Can Bile Salt Export Pump Inhibition Testing in Drug Discovery and Development Reduce Liver Injury Risk? An International Transporter Consortium Perspective

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Bile salt export pump (BSEP) inhibition has emerged as an important mechanism that may contribute to the initiation of human drug-induced liver injury (DILI). Proactive evaluation and understanding of BSEP inhibition is recommended in drug discovery and development to aid internal decision making on DILI risk. BSEP inhibition can be quantified using *in vitro* assays. When interpreting assay data, it is important to consider *in vivo* drug exposure. Currently, this can be undertaken most effectively by consideration of total plasma steady state drug concentrations ($C_{ss,plasma}$). However, because total drug concentrations are not predictive of pharmacological effect, the relationship between total exposure and BSEP inhibition is not causal. Various follow-up studies can aid interpretation of *in vitro* BSEP inhibition data and may be undertaken on a case-by-case basis. BSEP inhibition is one of several mechanisms by which drugs may cause DILI, therefore, it should be considered alongside other mechanisms when evaluating possible DILI risk.

BILE SALT EXPORT PUMP BACKGROUND

Role of bile salt export pump in bile flow

Bile is a complex biological fluid that is formed within the liver and excreted through the bile ducts into the intestine. The principal components of bile are bile acids, cholesterol, phospholipids, conjugated bile pigments, inorganic electrolytes, and water.[1] Primary bile acids are produced within hepatocytes via cytochrome P450 (CYP)-mediated oxidation of cholesterol and further metabolized to glycine or taurine conjugates, which are actively excreted into bile. In the intestine, bile acids may be dehydroxylated by bacterial enzymes to secondary bile acids, and undergo extensive reabsorption (enterohepatic circulation). Bile acids are amphipathic molecules that form micelles and thereby solubilize lipids and other compounds (e.g., bile pigments and vitamins), which otherwise exhibit poor solubility. They also exert hormone-like biological activities, especially via activation of nuclear hormone receptors (most notably the farnesoid X receptor (FXR)) and G-protein coupled receptors (such as the membrane-bound receptor for bile acids, TGR5).[2] Bile salt export pump (BSEP) (*ABCB11*; abbreviated Bsep in animal species) is an ATP-dependent membrane transport protein present in the apical (canalicular) domain of hepatocytes and constitutes the rate-determining step for bile acid secretion from hepatocytes[3] (**Figure 1**). Thus, BSEP is essential for normal bile flow and healthy liver function (see genetic diseases and drug-induced liver injury (DILI) in the "Other Hepatobiliary Transporters, Their Roles in DILI and Interdependencies with BSEP" section).[3]

Genetic evidence that defective BSEP expression causes liver injury in humans

Cholestasis is a reduction or interruption in bile flow.[1] Cholestasis that arises via inhibition of biliary transporter activities results in

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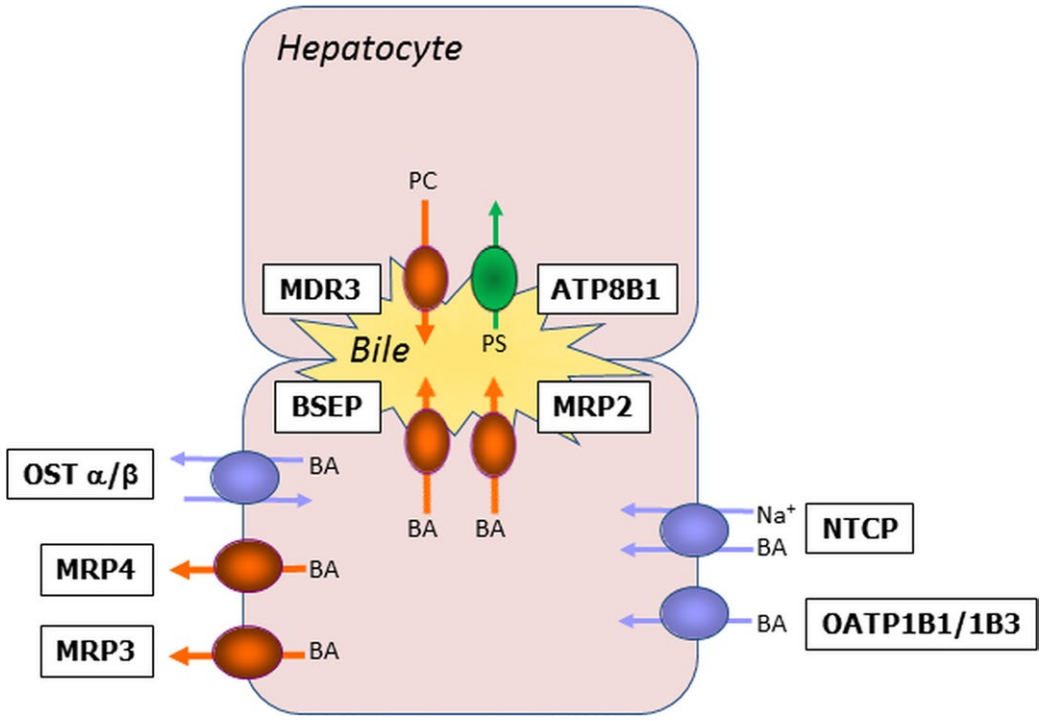


Figure 1 Localization of hepatic bile acid and lipid transporters. ATP8B1, ATPase aminophospholipid transporter 8B1; BA, bile acids; BSEP, bile salt export pump; MDR3, multidrug resistance protein 3; MRP, multidrug resistance protein; NTCP, Na^+-taurocholate co-transporting polypeptide; OATP, organic anion transporting polypeptide; OST, organic solute transporter; PC, phosphatidylcholine; PS, phosphatidylserine. Red, ATP binding cassette (ABC) transporters; green, P-type ATPase; purple, solute carrier (SLC) transporters.

increased intracellular accumulation of bile components within hepatocytes.[1] In humans, genetic defects in *ABCB11* result in reduced BSEP expression/activity, reduced bile acid excretion, and liver injury.[3,4] The most severe genetic defect is progressive familial intrahepatic cholestasis type 2 (PFIC2), which is characterized by a complete loss of BSEP function. Patients with PFIC2 present with cholestasis on average within ~3 months after birth and the disease can progress rapidly, leading to cirrhosis during infancy, or may progress relatively slowly with minimal scarring well into adolescence. Left untreated, most patients die before the age of 30.[3–5] A similar pattern of progressive cholestatic liver damage has been observed in homozygous $Bsep^{-/-}$ knockout mice[6] (see the "Other Hepatobiliary Transporters, Their Roles in DILI andInterdependencies With BSEP" section for more details). Functionally less severe human *ABCB11* gene polymorphisms lead to expression of BSEP variants that retain some activity and result in benign recurrent intrahepatic cholestasis type 2 (BRIC2) or intrahepatic cholestasis of pregnancy, which are characterized by cholestasis but not severe liver injury.[7] Historically, it was assumed that the hepatic injury due to BSEP dysfunction (e.g., genetic or drug-mediated) was a result of the detergent-like properties and "high" intracellular concentrations of bile acids. However, recent work has suggested that bile acid accumulation following BSEP inhibition by drugs causes hepatocyte injury by multiple mechanisms, which include mitochondrial toxicity and initiation of an inflammatory response.[8,9]

A final reflection on the translatability of the *ABCB11* pharmacogenetic data in humans to drug discovery risk assessment is that the level of sustained *in vivo* BSEP inhibition caused by typical drug molecule competitive inhibitors is poorly understood, and could be less than the complete BSEP deficiency that occurs in PFIC2. The severity of liver injury that occurs during chronic administration of a drug that does not completely inhibit BSEP *in vivo* might be more similar to the relatively mild cholestatic liver injury observed in BRIC2.

BSEP inhibition and DILI

Liver toxicity is a relatively frequent finding during preclinical safety testing in animals and is an important cause of compound attrition prior to clinical trials.[10] In addition, numerous drugs cause DILI in humans, but not in animals. In general, such "human-specific" DILI arises infrequently and unpredictably in susceptible individuals, and has been termed "idiosyncratic." Human idiosyncratic DILI (iDILI) is a leading cause of failed clinical drug development or cautionary labeling that restricts prescribing, with hundreds of licensed drugs having reports of iDILI.[11,12] Due to its low frequency, iDILI often is not evident until phase II/III clinical studies of the drug, or even post-marketing.[13,14] The most clinically concerning consequence of iDILI is acute liver failure, which has a high fatality rate unless treated by liver transplantation. However, acute liver injury arises infrequently in patients treated with drugs that cause iDILI. The mechanisms by which drugs cause iDILI are complex and include both drug-related processes and patient-related susceptibility factors.[15]

Many drugs that cause iDILI have been shown to inhibit BSEP activity *in vitro*[16,17] at concentrations proposed to be relevant

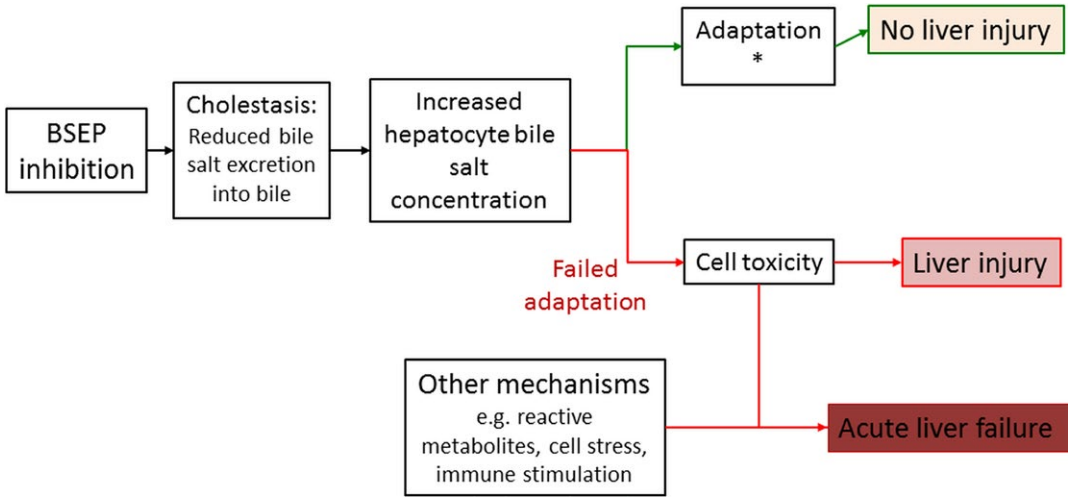


Figure 2 Proposed role of bile salt export pump (BSEP) inhibition in drug-induced liver injury. *Adaptation may arise via upregulation of BSEP expression and upregulation or downregulation of other hepatic plasma membrane efflux or uptake transporters, respectively, plus intracellular mechanisms that include farnesoid X receptor (FXR)-mediated downregulation of bile acid synthesis (see text for details).

to *in vivo* human total plasma steady state drug concentrations ($C_{ss,plasma}$).[18,19] In addition, drug exposure-based quantitative systems toxicology (QST) modeling of BSEP inhibition for the antidiabetic drug troglitazone and its sulfated metabolite, in conjunction with experimentally determined cytotoxicity potencies of bile acids, provided simulations that aligned well with the frequency and time of onset of iDILI observed in clinical trials.[20] QST modeling studies also have indicated that BSEP inhibition is a plausible explanation for iDILI due to tolvaptan treatment,[21] whereas lixivaptan treatment was correctly predicted to be less likely than tolvaptan to cause liver injury in clinical trials.[22]

To a toxicologist, liver injury due to altered bile acid homeostasis is termed "cholestatic." However, to a clinician, DILI is divided into "hepatocellular," "cholestatic," or "mixed" based on the ratio of serum alanine aminotransferase (indicating hepatocyte death) to serum alkaline phosphatase (reflecting reduced bile flow).[23,24] Because bile acids are toxic to the hepatocytes, inhibition of BSEP may present clinically as a hepatocellular and not cholestatic injury, as is the case for tolvaptan and troglitazone.

Even with the recent success in QST modeling, currently, it is not possible to predict whether BSEP inhibition in an individual patient will cause hepatocyte injury that may pose a risk of acute liver failure. This limitation reflects the complexity of DILI, and that development of acute liver failure in patients with iDILI often involves both innate and adaptive immune responses.[25] *In vitro* studies undertaken using mouse hepatocytes and hepatocyte-derived cell lines have shown that bile acid retention sensitizes hepatocytes to the lethal effects of Fas ligand or tumor necrosis factor (i.e., extrinsic cell death), and causes other adverse consequences that depend on the degree of bile acid retention and the overall health of the liver (e.g., capacity for cellular adaptive responses to remove bile acids or dampen the toxic stress imposed by bile acids, including oxidative, mitochondrial, and endoplasmic reticulum stress).[26] The adaptive responses are under complex environmental and genetic control,[26] which may help explain the marked human population variability in DILI caused by drugs that inhibit BSEP. It is also notable that some drugs that inhibit BSEP can affect these other processes via mitochondrial injury, reactive metabolite generation, and/or oxidative stress, which are important additional DILI risk factors.[27–29] The multiple steps between BSEP inhibition and DILI are illustrated schematically in **Figure 2** and discussed further in the sections "Data Interpretation: *In Vitro*/*In Vivo* Extrapolation, Quantitative Simulations" and "Recommendations on When and How to Generate and Interpret BSEP Inhibition Data." Thus, it is important to take into account the complexity of DILI when translating *in vitro* BSEP inhibition data to the design and selection of safe drugs.

Interspecies differences in BSEP inhibition

For several drugs, markedly (up to 10-fold) more potent inhibition of human BSEP, when compared with inhibition of Bsep from preclinical species, has been reported (e.g., troglitazone).[16,17] However, for most drugs the experimentally determined half maximal inhibitory concentration (IC_{50}) values for human and rat BSEP/Bsep inhibition have been found to be very similar. This suggests that species variability in inhibition of BSEP/Bsep is not a plausible explanation for why drugs that inhibit BSEP and cause human DILI do not cause liver injury when evaluated in animal safety studies. Humans have a significantly more hydrophobic bile acid pool than rats and dogs, as evidenced by higher levels of lithocholic acid and other monohydroxylated bile acids.[30] Furthermore, bile acid feeding experiments undertaken in rats revealed a good correlation between increased bile acid hydrophobicity and hepatotoxicity.[31–33] This raises the possibility that interspecies differences in bile acid pools might contribute to the poor predictive power of rodent safety studies for detection of human DILI caused by BSEP inhibition. Interestingly, rodents detoxify monohydroxylated bile acids through extensive hydroxylation,[1] and experiments performed using Cyp null mice revealed that the key enzymatic pathway that further oxidizes bile acids to

more hydrophilic alpha-muricholic or beta-muricholic acid species is catalyzed by members of the rodent Cyp2c family, which are not expressed in humans and other higher animal species.[34] QST modeling has provided additional evidence that the less toxic profile of bile acids in rats is a plausible hypothesis for why troglitazone did not exhibit liver toxicity when tested in animals, yet caused iDILI in humans.[35]

Much less is known about traditional large animal toxicity species, such as dogs and cynomolgus monkeys, in relation to their value for detection of liver injury caused by Bsep inhibition. However, notable differences in bile acid amidation and conjugation are known, which could lead to species differences in hepatotoxicity.[36]

OTHER HEPATOBILIARY TRANSPORTERS, THEIR ROLES IN DILI AND INTERDEPENDENCIES WITH BSEP

Multidrug resistance-associated protein 2 (MRP2; encoded by *ABC*C2) mediates ATP-dependent biliary excretion of glucuronide and sulfate conjugated bile acids, bilirubin glucuronides, glutathione conjugates, and conjugated drugs and other xenobiotics.[37] Inherited complete loss of human MRP2 function (Dubin–Johnson syndrome) is characterized by hyperbilirubinemia, although serum bile acid concentrations are unaffected and liver injury does not occur.[37] Hence, unlike BSEP, MRP2 inhibition is not in itself a plausible cause of DILI. However, there is indirect evidence of a relationship between MRP2 and liver injury. In a Korean population, an association was observed between *ABCC2* polymorphisms and 94 cases of toxic hepatitis, induced primarily by herbal remedies.[38] In UK patients with iDILI due to diclofenac, an increased frequency of the MRP2 C-24T allelic variant was observed, which was proposed to arise because of MRP2-mediated biliary excretion of glucuronide metabolites of the drug.[39] In addition, numerous drugs that caused DILI and inhibited BSEP also inhibited MRP2 activity in hepatocyte[40] and membrane vesicle[18,19] assays, although overall a poor concordance between inhibition of the two transporters by drugs was observed.[18,19]

In humans, multidrug resistance protein 3 (MDR3, encoded by *ABCB4*) mediates biliary excretion of phosphatidylcholine, which forms mixed micelles with cholesterol and bile acids and thereby protects the biliary epithelia from bile acid toxicity.[41] Polymorphisms in *ABCB4* that markedly reduce protein expression cause the cholestatic disorders PFIC3, BRIC3, and low phospholipid-associated cholelithiasis, and predispose to intrahepatic cholestasis of pregnancy.[41,42] Furthermore, mice lacking Mdr2 (the rodent orthologue of MDR3) exhibit impaired biliary phosphatidylcholine secretion and develop hepatobiliary disease.[43] Several antifungal drugs inhibit MDR3 activity *in vitro* in transfected LLC-PK1 cells.[44,45] In addition, > 40% of 125 tested drugs inhibited phosphatidylcholine efflux from isolated human hepatocytes, which was assumed (but not proven) to be mediated by MDR3.[46]

The intestinal apical sodium-bile acid transporter (ASBT, encoded by *SLC10A2*) mediates efficient (95%) reabsorption of bile acids from the intestinal lumen.[47] Human sodium Na^+-taurocholate cotransporting polypeptide (NTCP; encoded by *SLC10A1*) is responsible for the highly efficient hepatic first-pass extraction of conjugated bile acids, which represent the bulk of the portal vein bile acid pool.[47] A small fraction of the bile acid pool may be transported by other basolateral transporters, such as the organic anion transporting polypeptides (OATPs).[47] Some drugs that inhibit BSEP also inhibit NTCP or OATPs.[48,49] Inhibition of hepatic bile acid uptake may result in increased systemic concentrations of bile acids which, in parallel with decreased intracellular bile acid concentrations, could serve as a protective mechanism when canalicular bile acid efflux is compromised.[49]

Additional protection against bile acid-mediated hepatotoxicity is provided by the basolateral plasma membrane domain efflux transporters MRP3 (encoded by *ABCC3*) and MRP4 (encoded by *ABCC4*).[50] In rats, these transporters mediate vectorial bile acid efflux into the systemic circulation, although human MRP3 is a very poor transporter of bile acids. MRP3 protein expression is similar to or slightly lower than BSEP expression under normal physiological conditions, whereas MRP4 protein expression is relatively low.[51,52] Studies undertaken in knockout mice have indicated that Mrp4 expression, but not Mrp3 expression, provides a compensatory mechanism that regulates intracellular bile acid concentrations following bile duct ligation.[53,54] Furthermore, human MRP4 is upregulated in patients with hereditary BSEP deficiencies.[55] Inhibition of MRP3 and/or MRP4 activities *in vitro* by some drugs that cause DILI and inhibit BSEP have been observed.[18,19,56] The functional significance of this observation is unclear, because it seems that the majority of drugs that inhibit BSEP and cause DILI do not inhibit MRP3.[18,19]

Organic solute transporter (OST)α/β is a bidirectional heterodimeric transporter expressed in the basolateral membrane of human hepatocytes and cholangiocytes, which is comprised of subunits encoded by *SLC51A* and *SLC51B*.[57] OSTα/β substrates include bile acids, sulfate conjugates of steroid hormones, and some drugs.[57,58] OSTα/β functions by facilitated diffusion[58] and the direction of transport depends on the electrochemical gradient. Hepatic OSTα and OSTβ expression is regulated through FXR (encoded by *NR1H4*)[57] and is increased significantly in obstructive cholestasis[59] and primary biliary cirrhosis.[60] In human sandwich-cultured hepatocytes (SCHs), it seems that this adaptive response functions as a "safety-valve" to protect cells from elevated concentrations of bile acids that arise under cholestatic conditions.[61] Among 22 drugs tested, troglitazone sulfate significantly inhibited bile acid transport at the single concentration tested in human cells that overexpressed OSTα/β,[62] which raises the possibility that hepatoprotection mediated by OSTα/β may be impaired following administration of troglitazone.

Studies undertaken in homozygous *Bsep*$^{-/-}$ knockout mice and rats have provided further insight into interdependencies between hepatobiliary transporters and their role in liver injury. In the initial studies of *Bsep*$^{-/-}$ mice, little evidence of liver injury was observed unless the animals were fed a diet enriched in bile acids.[63] This was because the animals exhibited adaptive upregulation of hepatic enzymes that metabolize bile acids and other hepatobiliary efflux transporters, notably P-glycoprotein, Mdr2, and Mrp2.[63] Subsequently, another strain of *Bsep*$^{-/-}$ mice was developed by backcrossing the original strain with C57BL/6J mice for 10 generations. These animals did not exhibit the pronounced adaptive alterations present in the original mouse strain, developed

progressive cholestatic liver injury when fed a normal diet, and also exhibited plasma chemistry and liver histopathology abnormalities similar to human PFIC2.[6]

IN VITRO AND *IN VIVO* METHODOLOGIES TO ASSESS BSEP INHIBITION

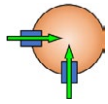
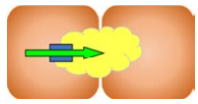
A survey sponsored by the International Transporter Consortium (ITC) was undertaken to assess the use by companies of BSEP inhibition methodologies. Seven companies provided information, all of whom reported the use of membrane vesicles as an initial tool to investigate BSEP inhibition, which often is evaluated in drug discovery. In four of the seven companies, inhibition observed in the vesicle assay could trigger additional studies, including use of SCHs and/or *in vivo* animal studies, in which plasma bile acids and clinical chemistry markers of cholestatic liver injury were measured. The survey results are in good agreement with the methodologies reviewed below.

In vitro methodologies: BSEP containing membrane vesicles

Membrane vesicles containing BSEP are available commercially and are most commonly used to assess BSEP inhibition potential. The vesicles are often produced from baculovirus infected *Spodoptera Frugiperda* 9 or 21 (sf9 or sf21) insect cell lines, which allow large-scale production. Mammalian cell lines, such as CHO, Hela, or HEK293, also have been utilized. Insect and mammalian membranes differ in cholesterol content, although this does not seem to influence BSEP substrate affinity (K_m) or inhibitory potencies (IC_{50}).[64] Preparation procedures and quality control assays have been published previously and will not be further discussed here.[16–18,65] The membranes obtained using all isolation procedures comprise a mixture of right-side out and inside-out vesicles. However, because only inside-out vesicles will have the ATP binding site facing the external environment, purifying inside-out vesicles has been considered unnecessary; instead, studies with AMP have been used to account for non-ATP dependent translocation and/or nonspecific binding to membranes.

Numerous groups have published methodologies for the assessment of BSEP inhibition in membrane vesicles.[16–19,56] Briefly, ATP-dependent vesicular uptake of a probe bile acid is examined in the presence or absence of test compound and compared to uptake in control vesicles that do not express BSEP. The most common substrate used is taurocholate (TCA). However, other bile acids, such as glycocholate, taurochenodeoxycholate, and glycochenodeoxycholate, also have been utilized. Probe substrate transport can be monitored by radiolabel, fluorescence, or liquid chromatography tandem mass spectrometry measurements. The inhibition assay should be performed within the linear range of probe substrate uptake and preferably well below the K_m of the probe substrate, where the IC_{50} approaches the K_i value.[65] Typical assay conditions are summarized in **Table 1**. Use of known BSEP inhibitors (e.g., troglitazone, pioglitazone, ritonavir, or cyclosporine)[16,17] as positive controls in each run is highly recommended to provide confidence that the assay data can be translated for clinical risk assessment. When BSEP inhibition is observed, the IC_{50} value should be determined.

Table 1 Typical *in vitro* BSEP inhibition assay conditions

	Membrane vesicles	SCHs
		
Example probe substrate	Taurocholic acid	Taurocholic acid
Km (μM)	11 ± 7	n/a
Substrate (μM)	0.5–2 μM	1 μM
Temperature (°C)	37	37
Pre-incubation time	n/a	10 minutes ± Ca^+
Incubation time (minute)	5	10

Different probe substrates, probe concentrations, and assay temperatures have been published. Assay conditions should be optimized prior to evaluation of BSEP inhibition potential. Maximum concentration of test compound can vary depending on solubility limits or estimated target exposures needed in the clinic for efficacy.
BSEP, bile salt export pump; SCHs, sandwich-cultured hepatocytes.

Typically, BSEP inhibition by drugs is evaluated only at a single substrate concentration and, hence, competitive inhibition is assumed. Noncompetitive BSEP inhibition has been observed for some drugs and metabolites, which was not evident until K_i determinations were undertaken, and retrospective QST simulations indicated that this markedly influenced the hepatotoxic potential.[20,21] Therefore, it may be preferable to investigate the mode of BSEP inhibition in a more definitive K_i study when a more complete DILI risk assessment is required. However, these investigations are resource intensive and are not possible for "routine" compound screening. In addition, currently, it is unclear whether routine BSEP inhibition K_i determinations would substantially enhance DILI risk assessment if they were to be undertaken proactively and routinely, prior to clinical progression of new drug candidates.

Although membrane vesicles can serve as a low cost and high throughput tool for direct measurement of BSEP inhibition, they lack the ability to assess metabolites and/or interplay between BSEP and other bile acid transporters in hepatocytes. Hence, this assay can result both in false-positive and false-negative data[16–19] (**Table 2**), and further evaluation in a hepatocyte-based model may be warranted to improve data interpretation.

In vitro methodologies: hepatocyte-based models

SCHs are used most commonly to evaluate inhibition of BSEP-mediated biliary excretion. Detailed methodologies have been reviewed elsewhere,[65,66] and typical assay conditions are outlined in **Table 1**. SCHs are pre-incubated in buffer with or without calcium to modulate cellular tight junctions and distinguish between cellular and bile canalicular compartments, and then incubated with a probe substrate in the absence and presence of test drugs. This enables calculation of the biliary excretion index (BEI) and quantification of the *in vitro* biliary clearance of the probe substrate (e.g., bile acid) from the medium ($CL_{biliary}$) and from the intracellular compartment ($CL_{bile,int}$).[8,65,66] The *in vitro* $CL_{biliary}$

Table 2 Advantages and disadvantages of common methodologies to assess BSEP inhibition potential

Method	Advantages	Disadvantages
In vitro		
Membrane vesicles	Commercially available Affordable High throughput Easy to use Direct BSEP interaction Cytotoxic compounds can be assessed Supports more facile K_i generation and differentiation of competitive vs. noncompetitive inhibition Can be run as part of assay suite (along with other transporter and nontransporter DILI-related assays)	Lack metabolism Lack physiological relevance Captures only one step in bile acid excretion False-positives and false-negatives reported
SCHs	More physiologically relevant *in vitro* model Express metabolic enzymes Intact regulatory machinery Express multiple transporters Determine biliary clearance and basolateral clearance Determine intracellular concentrations Commercially available	Time consuming Sometimes difficult to distinguish between effects on different transporters Complex and require experience
In vivo		
Plasma bile acid measurements	Provides indirect measurement of potential inhibition of bile acid clearance Noninvasive Suitable for preclinical and clinical studies	No standardized assay available Nonspecific biomarker Several confounding factors complicate data interpretation Uncertainties regarding preclinical to human translation

BSEP, bile salt export pump; DILI, drug-induced liver injury; SCHs, sandwich-cultured hepatocytes.

represents uptake of the probe across the sinusoidal membrane into the hepatocyte and its excretion into the bile canaliculi, whereas $CL_{bile,int}$ represents the canalicular excretion only. The BEI represents the fraction of accumulated substrate that resides in the bile canaliculi. Inhibition by a test compound of *in vitro* $CL_{biliary}$, with no change in BEI or $CL_{bile,int}$, indicates that the uptake of the probe is inhibited, whereas inhibition of both *in vitro* $CL_{biliary}$ and BEI or $CL_{bile,int}$ indicates that both hepatocyte uptake and canalicular efflux pathways are inhibited. SCHs are lower throughput and may be more costly compared to BSEP membrane vesicles, depending on the experimental design. However, they provide more holistic data integrating extracellular and intracellular protein binding, and take into account the multiple transporters involved in bile acid uptake and efflux (**Table 2**).

SCHs also can be used in investigatory studies to explore potential transporter-mediated regulatory mechanisms and the possible role of metabolism. Several drugs, such as troglitazone[67] and tolvaptan,[68] produce metabolites that are markedly more potent BSEP inhibitors than the parent drug. For these compounds, valuable additional information is provided by data obtained using SCHs. It is always important to consider the possibility that drug metabolites may inhibit BSEP. Micropatterned hepatocyte-stromal cell cocultures[69] and 3D spheroid, organoid, and microfluidic liver models[70] have been shown to be metabolically active and may provide future utility in this area. However, these models require validation of transporter function.

In vivo methodologies

Direct measurement of the effects of drugs on BSEP function *in vivo* is needed to enhance our understanding of *in vitro/in vivo* translation and how *in vivo* data in animal species translate to humans. Currently, there are no well-characterized exogenous probe substrates that are transported specifically by BSEP and are suitable for use in either preclinical or clinical studies. Endogenous bile acids are useful biomarkers and can be quantified in plasma and other biofluids using liquid chromatography mass spectrometry methods that are markedly more sensitive and specific than traditional clinical chemistry techniques.[71,72]

In a previous ITC publication, Hillgren *et al.*[73] recommended measuring total plasma bile acid concentrations and serum transaminases in preclinical species in a multiple dose study conducted over several weeks at doses leading to at least the expected clinical systemic concentration. Such studies may provide useful insight into whether a compound that inhibits Bsep *in vitro* will also affect Bsep function *in vivo*. Furthermore, single and repeat dosing can be used to assess transient increases in bile acid concentrations due to transporter inhibition and/or effects due to compensatory mechanisms. For example, notable increases in plasma bile acid concentrations were observed in patients administered the BSEP inhibitor bosentan, either alone or in combination with glyburide.[74] However, numerous factors complicate the interpretation of the bile acid data, such as the potential for drugs to inhibit NTCP/OATP-mediated bile acid uptake, affect bile acid synthesis/composition, or activation/antagonism of nuclear receptors, such as FXR (see the "Data Interpretation: *In Vitro/In Vivo* Extrapolation, Quantitative Simulations" section).

In addition, careful consideration of study design is important when undertaking bile acid biomarker studies. Considerable intra-individual variability in endogenous human bile acid concentrations has been observed in some studies,[75,76] but not in others in which patients were fasted.[77] In view of this possibility, it is advantageous to conduct longitudinal sampling from baseline or pretreatment

with drug. Marked effects of food on the bile acid pool can also confound the interpretation of drug effects, in particular for taurine-conjugated bile acids.[76] There is also a diurnal rhythm of bile acids; therefore, optimizing sampling times is required. Urinary bile acid profiling is less affected by the aforementioned variables than plasma, so this could be a possible alternative.[75,78] Profiling individual bile acids also may be advantageous because BSEP inhibition has been reported to increase serum concentrations of some selected bile acids. For example, tauromuricholic acid and taurocholic acid in rat serum were markedly more sensitive to troglitazone-mediated Bsep inhibition than total serum bile acids.[79]

BSEP INHIBITION COMPUTATIONAL MODELING

Several computational approaches have been used to model BSEP inhibitors (**Table 3**). In principle, computational models represent a useful approach for prediction of compounds in drug discovery with reduced BSEP inhibition risk prior to compound synthesis and *in vitro* testing. Quantitative structure-activity relationship (QSAR) analyses have correlated molecular descriptors, and sometimes molecular fragments, with BSEP inhibition, in order to identify molecular properties that influence BSEP inhibition. Nonlinear, random forest models have been described that can delineate compounds in a series of closely related structural analogues that have high BSEP inhibition potency.[80,81] Furthermore, structure-based probabilistic Bayesian modeling, which uses molecular descriptors to analyze the frequency of structural features associated with BSEP inhibition by statistical discriminant analysis, can extract important substructures and thereby identify favorable and unfavorable structural fragments for BSEP inhibition.[82]

A high-resolution crystal structure of the BSEP protein is not available. Jain *et al.*[83] used mouse P-glycoprotein, which is the most structurally related template protein, to create a protein homology model that allowed docking-based classification of BSEP inhibitors and noninhibitors. Pharmacophore models have been developed that used low energy 3D molecular conformations of BSEP ligands to predict important binding pocket domains, without prior knowledge of the protein crystal structure.[81] These ligand-based pharmacophore models are restricted to predictions within the chemical space on which they are built and often have lower predictive power than QSAR and Bayesian models.

The overall performance of the BSEP inhibition computational models published to date is insufficient to enable a general rational design of compounds that will not exhibit BSEP inhibition. A major problem is posed by the great structural diversity of compounds that inhibit BSEP and other hepatic efflux transporters, which makes the identification of common and "BSEP-specific" structural features difficult. Additionally, variances in reported inhibition potency based on different *in vitro* systems, assay types, and laboratories make data interpretation challenging. However, important chemical features and properties of BSEP inhibitors have been identified within certain scaffolds. For example, an ester or thioester directly attached to a heterocyclic carbon, high lipophilicity, the number of halogen atoms, and a carbocyclic system containing at least one aromatic ring, have been positively correlated with BSEP inhibition.[80,84,85] These are useful findings, which can aid compound design.

Computational models that can be used to predict the inhibition of other hepatic bile acid efflux transporters potentially involved in DILI also are sparse, as are models that incorporate BSEP-inhibitory metabolites in addition to parent compounds. An integrated computational modeling approach that accurately predicts inhibitors of all relevant hepatic bile acid efflux transporters, including BSEP, could be especially useful.

DATA INTERPRETATION: *IN VITRO/IN VIVO* EXTRAPOLATION, QUANTITATIVE SIMULATIONS

In vitro BSEP data

Quantification of IC_{50} values in BSEP vesicle assays has been reported to distinguish between human DILI-positive or DILI-negative drugs with moderately high specificity (70–80%), but only modest sensitivity (~50%).[16–19] However, there are large discrepancies between the BSEP IC_{50} cutoff values claimed by different investigators to identify "concerning levels" of BSEP inhibition. For example, Morgan *et al.*[17] proposed a value of 25 μM, whereas Dawson *et al.*[16] proposed 300 μM, and Yucha *et al.*[19] proposed 50 μM. The different BSEP IC_{50} cutoff values are likely due to a combination of differences in the drugs tested by different investigators, the annotation of test drugs as DILI positive or negative, and/or different experimental methodologies that yielded variable values when the same drugs were tested in different laboratories. In the future, it will be important to standardize the methods used to generate data, the reference drugs used to calibrate assays, and the DILI annotation process. Widespread use of the DILI classification developed by the US Food and Drug Administration (Liver Toxicity Knowledgebase)[86] is recommended as a starting place for classification to enable more consistent drug annotation. Verification of the classification should be ensured by comparison to the drug label.

When interpreting *in vitro* BSEP inhibition data, it is critical to consider both potency of inhibition and *in vivo* drug exposure. BSEP inhibition that occurs only at drug or drug metabolite concentrations that are not achieved *in vivo* should not be considered clinically relevant. Conversely, BSEP inhibition that exhibits low potency (i.e., high IC_{50}) may be biologically significant if *in vivo* drug exposure is sufficiently high.

The key *in vivo* exposure parameter is the unbound drug (or inhibitory metabolite) concentration in hepatocytes at the site of interaction with BSEP in patients. In practice, this concentration is not known and is challenging to determine experimentally. Drug and metabolite concentrations present in venous blood or plasma are routinely determined, enabling unbound venous plasma drug concentrations to be estimated. However, there is debate about whether unbound or total concentrations of the perpetrator should be used when interpreting *in vitro* BSEP inhibition values as part of the clinical hazard assessment. This is because unbound maximum plasma drug concentrations at steady state are much lower than the *in vitro* BSEP IC_{50} values for the majority of tested drugs.[18,19]

Currently, unbound plasma drug concentrations are considered unsuitable for exposure adjustment of BSEP IC_{50} data. A plausible explanation for this apparent incongruity is that drug concentrations in plasma do not accurately reflect drug concentrations

Table 3 BSEP computational models

Ref no.	Type of model	*In vitro* system	Substrate	Inhibitor cutoff	No. of compounds	Outcome and model characteristics	MCC
83	QSAR and Ligand Docking (random forest, REPTree, LibSVM and Naive Bayes)	Membrane vesicles (Sf9, Sf21)	[^{3}H]-TCA	$IC_{50} \leq 10$ μM	Training Set: 408 (113 inhibitors; 295 noninhibitors) (Sf21)[80]; External Test sets: 166 (44 inhibitors; 122 noninhibitors) (Sf9)[85]; and 638 (248 inhibitors; 390 noninhibitors) (Sf21)[80]	Developed a BSEP homology model and chemical structure-based classification models. Ligand-based model resulted in the most balanced predictions with an MCC value of 0.69. Ligand-based models combined with structure-based models resulted in decreased level of FPs but increased level of FNs	0.69
81	QSAR and Chemical Structure-based (random forest and pharmacophore)	Membrane vesicles (Sf9, Sf21)	[^{3}H]-TCA	$IC_{50} \leq 300$ μM	Training Set: 618 (324 inhibitors; 294 noninhibitors) (Sf21)[80]; External Test set: 55 (38 inhibitors; 17 non-inhibitors)[16]; (Sf21) (Sf9),[17] (Sf9)[85]	Developed chemical structure-based classification models with the aim to decrease the number of FPs that had been reported previously. Final random forest model resulted in MCC values of 0.7, 0.76, and 0.92 for the training set, internal test set, and external test set, respectively. The best pharmacophore model contained two hydrophobic features and two H-bond acceptor lipids	0.92
82	QSAR and Chemical Structure-based (Bayesian and pharmacophore)	Membrane vesicles (Sf9, Sf21)	[^{3}H]-TCA	$IC_{50} \leq 135$ μM	Training Set: 171 (43 inhibitors; 128 noninhibitors); External Test set: 86 (22 inhibitors; 64 non-inhibitors). Both datasets combined from (Sf21),[16] (Sf9)[18]	Developed structure-based models to identify common features within BSEP and MRP4 inhibitors. Final Bayesian model resulted in MCC values of 0.93 and 0.58 for the training set and test set, respectively. The final pharmacophore model contained two hydrophobic features and one H-bond acceptor	0.58
109	QSAR (Bayesian, k-nearest neighbor, J48, and random forest)	Membrane vesicles (Sf9, Sf21)	[^{3}H]-TCA	Inhibitors: $IC_{50} \leq 10$ μM Non-Inhibitors: $IC_{50} \geq 50$ μM	Training Set: 670 (220 inhibitors; 450 noninhibitors) (Sf21); External Test sets: 168 (55 inhibitors; 113 non-inhibitors) (Sf21) and 156 (39 inhibitors; 117 noninhibitors); combined from (Sf9)[18,84]	Developed linear and nonlinear structure-based models to investigate classification predictions for a large number of compounds. The best performing model was a random forest; hydrophobicity, aromaticity, and H-bond donor characteristics were identified to be important for BSEP inhibition	0.69
110	Chemical Structure-based (pharmacophore)	Membrane vesicles (HEK293)	[^{3}H]-TCA	50% inhibition at 50 μM	Training Set: 32 (5 inhibitors; 27 noninhibitors); External Test set: 59 (12 inhibitors; 47 noninhibitors)	Developed a 3D pharmacophore model to compliment previously reported 2D models. The final model contained two H-bond acceptors and four hydrophobic or aromatic features. MCC values were 0.43 and 0.52 for the training set and test set, respectively	0.52
85	QSAR (OPLS-DA)	Membrane vesicles (Sf9)	[^{3}H]-TCA	50% inhibition at 50 μM	Training Set: 163 (55 inhibitors; 108 noninhibitors); External Test set: 86 (31 inhibitors; 55 noninhibitors)	Developed a chemical structure-based model to aid in early identification of potential BSEP inhibitors. The final model based on molecular descriptors describing charge, lipophilicity, hydrophobicity, and size correctly classified 84% and 91% of the inhibitors and the noninhibitors in the external test set	0.73

(*Continues*)

Ref no.	Type of model	*In vitro* system	Substrate	Inhibitor cutoff	No. of compounds	Outcome and model characteristics	MCC
80	QSAR (recursive partitioning, PLS, random forest, and support vector machine)	Membrane vesicles (Sf21)	[^{3}H]-TCA	$IC_{50} \leq 300$ μM	Training Set: 437 (231 inhibitors; 206 noninhibitors); External Test set: 187 (94 inhibitors; 93 noninhibitors)	Developed chemical structure-based models to provide a cost-effective computational method to screen for BSEP inhibition. Out of the five models, the best model developed was based on support vector machine. It resulted in 85% and 90% probability for correct classifications of BSEP inhibitors and noninhibitors, respectively	0.74
84	QSAR (MLR)	Membrane vesicles (Sf9)	[^{14}C]-TCA	Percent inhibition at 100 μM	Training Set: 42 compounds	Developed the first computational model designed to describe BSEP inhibitors. The final model suggested that an ester on a heterocyclic ring and aromatic carbocyclic systems were important for BSEP interaction	—

BSEP, bile salt export pump; FN, false negative; FP, false positive; H-bond, hydrogen bond; IC_{50}, half maximal inhibitory concentration; MCC, Matthew's correlation coefficient; MRP, multidrug resistance protein; QSAR, quantitative structure-activity relationship; TCA, taurocholic acid.

within hepatocytes *in vivo*,[87] which for many drugs are many fold higher. It is also possible that the reported BSEP IC_{50} values may be inaccurate, because the nominal drug concentration used in the assay may not reflect the unbound drug concentration. The actual unbound concentration is rarely determined experimentally in these assays. BSEP inhibition assay buffers typically do not contain physiologically relevant concentrations of plasma proteins so that drug binding to plasma proteins can be discounted. However, binding of drugs to the membrane vesicles, or to the wells of the assay plates, might occur.

In contrast to the findings with unbound drug concentrations, good correlations have been observed in several studies between maximum total (i.e., protein-bound plus protein-unbound) plasma concentrations and *in vitro* BSEP IC_{50} values for drugs that caused DILI, but not for drugs that inhibited BSEP *in vitro* but did not cause DILI. A common observation was that drugs that exhibited total $C_{ss,plasma}$/BSEP $IC_{50} \geq 0.1$, and were administered systemically for prolonged durations, caused human DILI concern.[18,19,27] These drugs included both troglitazone and bosentan. Calculation of this ratio reduced the number of false-positive results that were observed when BSEP IC_{50} cutoff values were used to try to discriminate between drugs that did and did not cause DILI; however, this ratio still yields a high false-negative rate.[18,19] It should be noted, however, that because total drug concentrations are not predictive of pharmacological effect, the relationship between total drug plasma exposure and BSEP inhibition cannot be considered causal. This highlights that there are important gaps in our current understanding of bile acid mediated toxicity due to BSEP inhibition by drugs.

The modest (< 50%) DILI sensitivity of BSEP inhibition data is not surprising because DILI also can be caused by numerous other mechanisms.[27,28] Interestingly, compounds that are dual inhibitors of BSEP and mitochondrial function have been associated with increased risk of acute liver failure,[29] as have compounds that also form reactive metabolites and/or exhibit other DILI liabilities.[27,28]

MRP-mediated drug transport is an important compensatory mechanism of bile acid disposition (see the "Other Hepatobiliary Transporters, Their Roles in DILI and Interdependencies With BSEP" section) and associations between MRP2 inhibition by drugs and cholestatic DILI have been reported.[16–19] However, recent work assessing > 200 drugs revealed that, when compared with exposure adjusted *in vitro* BSEP inhibition data, assessment of MRP2, MRP3, or MRP4 inhibition provided no additional DILI predictive benefit, whereas high molecular weight (> 600 Da) and high cLogP (> 3) markedly enhanced DILI predictivity.[19]

This physiochemical property observation is consistent with a recent re-analysis of previously published data showing that most BSEP inhibitors are Biopharmaceutics Drug Disposition Classification System class 2 drugs (highly metabolized and poorly soluble).[88] Further, these authors provide a position that although BSEP inhibition is a source of liver toxicity, *in vitro* BSEP IC_{50} values are not in themselves useful predictors of DILI. Their analysis correctly highlights several limitations of the *in vitro* assay, which are also discussed in this white paper. In particular, there is strong agreement that considering only the IC_{50} for inhibition of BSEP results in both false-positive and false-negative predictions. Further

research is warranted to place *in vitro* measures of BSEP inhibition into context by also considering *in vivo* drug exposure, mechanism of inhibition, and additional mechanisms associated with DILI.

Cellular models enable mechanistic investigation of the effects of test compounds on multiple uptake and efflux transporters, and of the possible contribution of drug metabolism,[89–91] and the FXR-mediated adaptive response. These additional data are valuable for risk assessment when investigating DILI mechanisms.

In vivo bile acid data interpretation and confounding factors

Plasma bile acid monitoring in animals has been proposed to provide a useful indirect biomarker of *in vivo* BSEP function[92] (also see section "Data Interpretation: *In Vitro/In Vivo* Extrapolation, Quantitative Simulations"). However, increased plasma bile acid concentrations may also arise due to extrahepatic biliary obstruction,[93] or as a secondary consequence of hepatocellular liver damage.[94] Other confounding factors can include: (i) inhibition of bile acid uptake across the sinusoidal membrane by NTCP and/or OATPs[95,96]; (ii) elevated plasma bile acid concentrations arising due to decreased gut microbial metabolism, which has been observed in patients treated with antibiotics that do not inhibit BSEP (e.g., clarithromycin)[94]; (iii) altered bile acid synthesis, bile acid pools, and/or transporter expression/localization; and (iv) impaired bile acid transport from hepatocytes to plasma due to nuclear receptor antagonism and/or inhibition of sinusoidal efflux transporters, such as MRP3/4 and OSTα/β that limit increases in plasma bile acid concentrations even though intrahepatocyte bile acid concentrations are elevated by BSEP inhibition.[97] When considering whether elevated plasma bile acid concentrations may be due to BSEP inhibition, these other possibilities should be contemplated and evaluated, if feasible.

Use of modeling and simulation to translate *in vitro/* preclinical data to humans

Physiologically based pharmacokinetic (PBPK) models combine drug characteristics with physiology to provide dynamic predictions of pharmacokinetic behaviors of drugs *in vivo*.[98,99] During early clinical drug development, PBPK modeling supports prediction of clinical exposure and aids interpretation of the human relevance of *in vitro* and preclinical data. PBPK models can also provide dynamic drug exposure predictions within hepatocytes, which is especially relevant for inhibition of efflux transporters, such as BSEP, and when compounds also inhibit hepatic uptake. PBPK modeling underpins QST, which integrates drug exposure, drug-specific mechanistic toxicity data, and known physiology and pathophysiology to predict the potential safety risk associated with a given compound.[100]

A particular advantage of QST is that it enables integration of multiple data from various sources. For example, one modeling approach simulated the cumulative effect of inhibiting multiple transporters (e.g., BSEP, NTCP, MRP3, and MRP4), plus the combined effects of multiple DILI mechanisms, including mitochondrial toxicity and oxidative stress. This QST model predicted liver exposure in a simulated population that encompasses variability related to genetic and nongenetic factors plus species variability.[101,102] Retrospective analyses undertaken using QST models that incorporate total plasma drug exposure have successfully reconstructed models of hepatotoxicity for numerous known hepatotoxic and nonhepatotoxic compounds in preclinical species and humans.[20,21,101–103] The predictivity of QST models depends on proper representation of physiology and reliable assay measurements (e.g., accurate and reproducible results validated using positive controls, and assays using parent compounds and metabolites). However, the complexity of the model, and the need for multiple data inputs, currently limits widespread and routine use of the approach in early drug discovery.

RECOMMENDATIONS ON WHEN AND HOW TO GENERATE AND INTERPRET BSEP INHIBITION DATA

Discovering that a promising new candidate drug exhibits unexpected human DILI signals during clinical trials is highly undesirable, because this may result in delayed progression, prolonged and larger clinical trials, and lead to either failed registration or cautionary and restrictive labeling. Therefore, it is preferable to use *in vitro* assays, such as BSEP inhibition or SCHs, along with other key DILI-related parameters (e.g., dose, reactive metabolites, etc.) to support selection of drug candidates with minimal possible DILI risk when their remains an opportunity to influence compound design and selection.

A potential guided workflow for generation and interpretation of BSEP inhibition data in drug discovery and early clinical development is shown in **Figure 3**. The workflow is based on current knowledge and is intended to aid internal decision making on potential BSEP liabilities within projects. The science has not evolved to a point where a standardized decision tree can be constructed and used by regulators, due to gaps in our current knowledge.

Computational models and *in vitro* studies

The primary focus of the workflow is on generation and interpretation of *in vitro* experimental data, because the currently available global computational models are unable to identify compounds that inhibit BSEP with high sensitivity or specificity. Nonetheless, computational approaches may aid compound design if a global model is refined by inclusion of BSEP inhibition data obtained with compounds from a specific chemical series.[78] This could be especially advantageous when seeking to eliminate or minimize BSEP inhibition caused by an otherwise promising lead compound or compound series.

The BSEP inhibition test method that is used most frequently for routine compound screening is the *in vitro* membrane vesicle assay described in section *In vitro* and *in vivo* methodologies to assess BSEP inhibition. As with other transporter assays, individual laboratories need to calibrate the assay and determine the appropriate decision criteria. Because the goal is to reduce possible human DILI risk, the assay should be undertaken using human BSEP. The workflow outlined in **Figure 3** references the large dataset published by Morgan *et al.*,[18] in which most drugs with *in vitro* BSEP IC_{50} < 25 μM were found to cause DILI. Therefore, a BSEP IC_{50} ≥ 25 μM can be expected to indicate "less BSEP concern," meaning that no formal additional BSEP evaluation may be necessary for that compound. This assumes that (i) systemic exposure

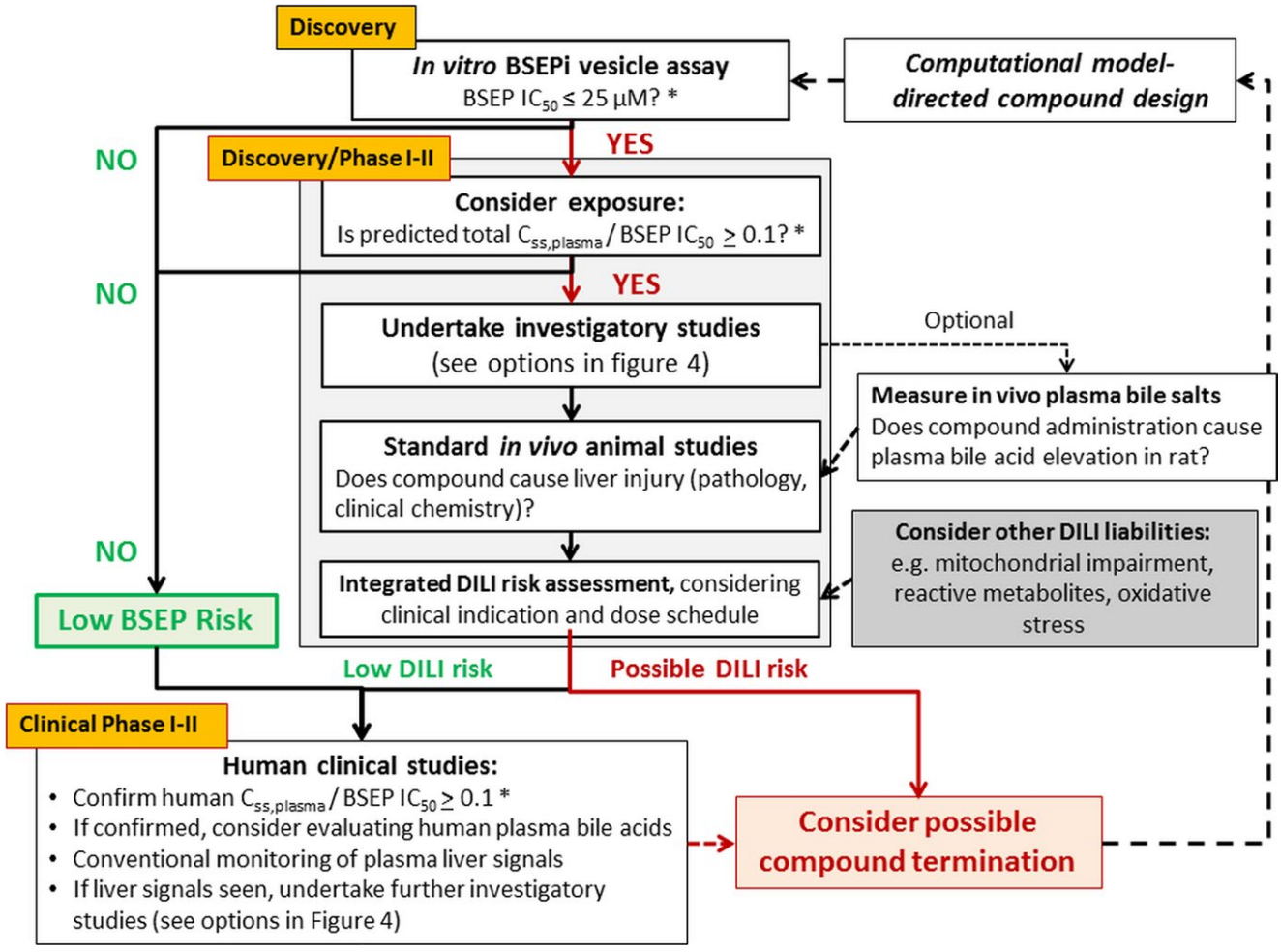


Figure 3 Potential guided workflow to interpret and mitigate bile salt export pump (BSEP) inhibition in drug discovery and/or early clinical development (phase I/II). The workflow is based on current knowledge and could be considered when making internal decisions on potential BSEP liabilities. The science has not evolved to a point where a standardized decision tree can be constructed and used by regulators due to gaps in our knowledge. (i) *The suggested cutoff values are based on limited published data and are intended to help focus additional discussion. Further research/justification is needed to reach final consensus on the feasibility of the suggested approaches. Typical assay conditions are summarized in **Table 1**. (ii) In the absence of clinical data, total concentration estimates may be from preclinical efficacy models or from other early predictions of human pharmacokinetics. Total plasma steady state drug concentrations ($C_{ss,plasma}$) correlation to BSEP concentration of half inhibition (IC_{50}) should be revisited when relevant clinical data are available. When total $C_{ss,plasma}$ is not known, estimated or determined total peak plasma concentration (C_{max}) data may be used instead. (iii) Higher likelihood of drug-induced liver injury (DILI) is expected if one or more DILI liabilities are flagged along with BSEP inhibition. (iv) Refer to **Figure 3** and the article for discussion follow-up studies and recommendations. (v) In clinical phase IIB/III studies, the strategy moves to considering metabolites and testing other transporters, in order to provide more comprehensive characterization of the drug prior to registration and labeling.

to the drug is low (estimated total $C_{ss,plasma}$ < 2.5 µM); (ii) inhibition is competitive in nature; (iii) there are no metabolites of the compound that might be more potent BSEP inhibitors; (iv) the compound does not inhibit BSEP in some indirect manner (e.g., trafficking to/from the canalicular membrane); and (v) BSEP inhibition IC_{50} values reported by Morgan *et al.*[18] can be reproduced in the relevant laboratory using selected test drugs.

As the molecule moves through lead optimization toward clinical dosing, new data need to be continuously integrated (e.g., toxicity and other hazard assessments). For compounds with an *in vitro* BSEP inhibition flag (IC_{50} ≤ 25 µM), a simple total $C_{ss,plasma}$/BSEP IC_{50} ratio is calculated. When total $C_{ss,plasma}$ has not yet been determined, the estimated or actual total plasma C_{max} value may be used in its place. If the ratio is < 0.1, this denotes low DILI concern.[18,19,27] Note that in this calculation, there is no correction for tissue accumulation (e.g., Kp) relative to plasma. If a Kp value for liver is known from *in vitro* hepatocyte or *in vivo* animal studies,[87] this could be used to refine the estimation. If a human total $C_{ss,plasma}$ is not available, an analysis across multiple $C_{ss,plasma}$ concentrations could provide an estimate of potential risk until the dose/exposure relationship is established. Another important consideration is that portal vein concentrations are higher than total C_{max} or C_{ss} concentrations in the systemic circulation after oral drug administration. As discussed in the section "Data Interpretation: *In Vitro*/*In Vivo* Extrapolation, Quantitative Simulations", the unbound drug concentration at the

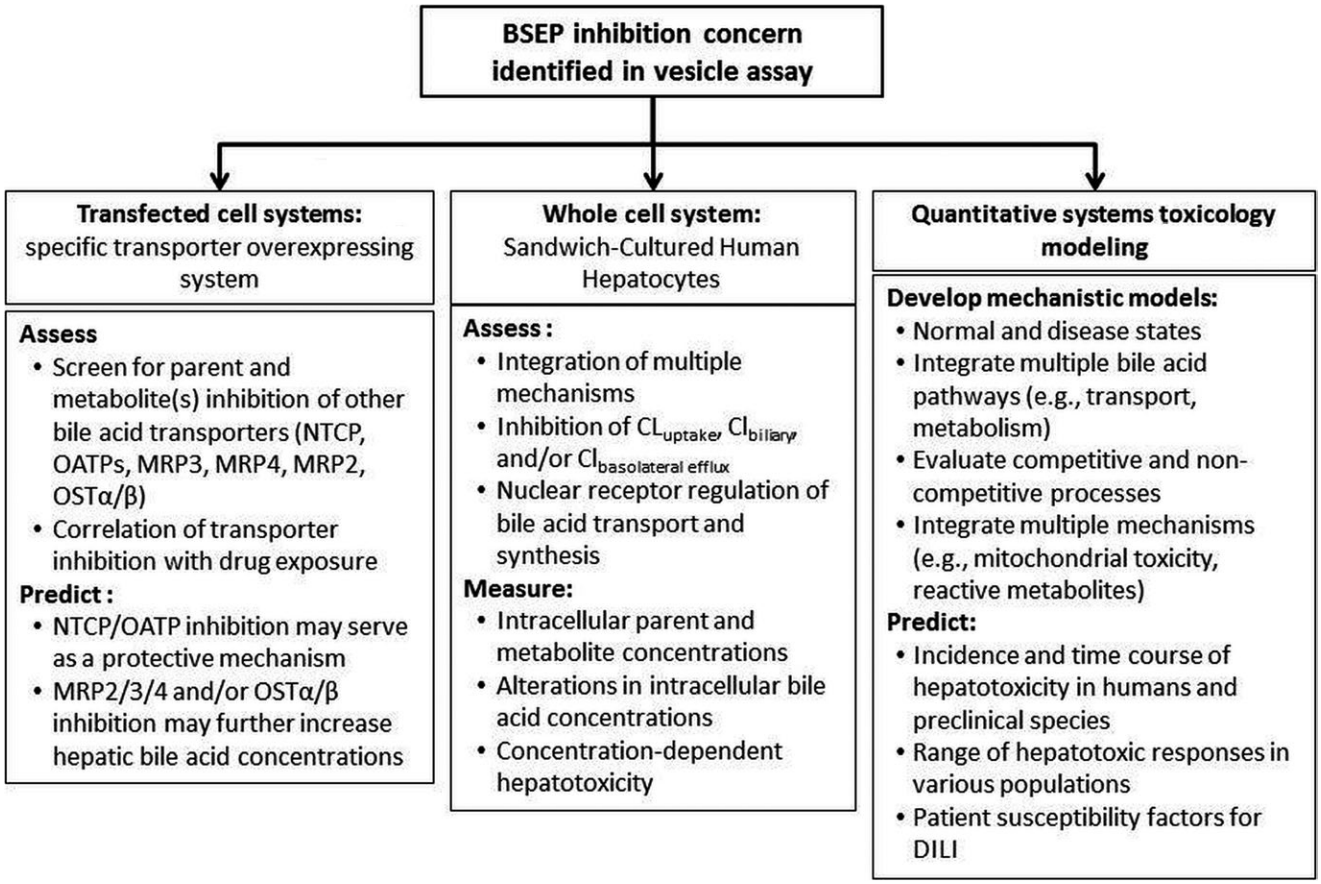


Figure 4 Follow-up studies that can be used to provide additional insight into the potential clinical relevance of bile salt export pump (BSEP) inhibition. (i) Refer to the article for discussion of follow-up studies and recommendations. (ii) To date, assays for organic solute transporter (OST)alpha and beta inhibition are not commercially available. CL, clearance; DILI, drug-induced liver injury; MRP, multidrug resistance-associated protein; NTCP, Na+-taurocholate co-transporting polypeptide; OATP, organic anion transporting polypeptide.

site of transport is the most relevant concentration, but is challenging to accurately determine. Unbound plasma drug concentrations have provided a poor correlation between *in vitro* BSEP IC_{50} and clinical DILI data.[16] Further work is needed to understand the unbound drug concentration in liver tissue and how best to incorporate this into the BSEP inhibition risk assessment.

It is important to note that the workflow and cutoffs values proposed in **Figure 3** and discussed above are preliminary guidances, which are based on the limited literature data available currently, and are intended to stimulate and focus discussions. Further research is needed to reach a final consensus on the applicability of the proposed approach. Individual laboratories may consider that alternative cutoff values are more appropriate for their internal use, providing these are justified following generation of suitable reference data.

If the total $C_{ss,plasma}$/BSEP IC_{50} ratio > 0.1, it is recommend to consider a series of investigatory studies (**Figure 4**). Effects on individual transporters can be assessed using appropriate membrane vesicle assays or other *in vitro* systems. More physiologically relevant primary cell systems, such as SCH, can provide additional and valuable insights into overall effects across multiple transporters, regulation changes due to BSEP inhibition and increased intracellular bile acid concentrations, and drug metabolite-mediated transporter interactions.[8,65,66] Finally, PBPK-based QST modeling can provide sophisticated insight into functional consequences that arise *in vivo*.[101–103] As outlined in **Figure 4**, the risk assessment for DILI moves from an early discovery *in vitro* BSEP inhibition "flag" to investigative and mechanistic *in vitro* studies. As BSEP inhibition is one of several mechanisms by which DILI can arise (**Figure 2**), the ITC recommends that BSEP screening should be undertaken in parallel with screening for other important DILI liabilities, especially inhibition of mitochondrial function and formation of chemically reactive metabolites (**Figure 3**). These additional mechanisms are outside the scope of this review but are discussed elsewhere, as are methods that can be used to integrate data on multiple DILI liabilities to assess overall DILI risk.[27–29]

The pragmatic test cascade shown in **Figure 3** has two inherent and important limitations that need to be considered. First, because vesicle-based BSEP inhibition assays lack metabolic capability, they may underestimate the DILI liability posed by a drug metabolite that is a more potent BSEP inhibitor than the parent compound (e.g., troglitazone sulfate). Drug metabolites that are chemically stable can be tested in vesicle assays, although data on human metabolites is often not available until clinical phase II trials. Use of metabolically competent cell-based BSEP inhibition assays (i.e., SCH) as primary BSEP screens, in place of vesicle assays,

would be preferable. Second, the commonly used BSEP vesicle assay conditions assume competitive BSEP inhibition and may not accurately estimate the effects of compounds that exhibit noncompetitive inhibition. It should be noted, however, that it is unclear currently whether differences in modes of inhibition have an effect on clinical translation of *in vitro* data or will add value in decision making if undertaken proactively during drug development.

When chemical choice has been exhausted and/or the anticipated clinical benefit of the compound is considered to outweigh BSEP inhibition risk, it may be desirable to progress a compound that exhibits *in vitro* BSEP inhibition. For example, the oncology drug ribociclib inhibited BSEP activity *in vitro*, and formed reactive metabolites that could be trapped using reduced glutathione and that bound covalently to human liver microsomal proteins.[104] Although quantitative comparisons between potencies of these *in vitro* effects and *in vivo* drug exposure have not been described, the anticipated clinical dose of the drug was high. Nonetheless, it was progressed into the clinic because the expected benefit in the intended clinical indication (patient survival in oncology) was considered to outweigh the possible DILI risk.[104] Ribociclib (Kisqali) was approved in 2017 for use as a first-line metastatic breast cancer treatment, at an oral starting dose of 600 mg q.d.[105] It remains to be seen whether DILI will occur in patients treated with this drug.

In vivo studies

Quantification of total serum bile acid concentrations in animals may provide useful, albeit indirect, information on whether biliary excretion of bile acids is perturbed by test compounds (see the "BSEP Inhibition Computational Modeling" section) and suitable assays are available commercially. This analysis can be undertaken alongside conventional animal safety studies, which assess effects of a range of compound doses on liver histopathology and serum or plasma clinical chemistry. However, there are many confounding factors that can hinder data interpretation and it remains unclear how data obtained in animals can relate to human risk. Due to these challenges, the value of serum bile acid profiling from preclinical species for human risk assessment is controversial. In contrast, serum bile acid profiles in individuals with PFIC2 aids the clinical evaluation of this genetic disease.[106] Therefore, plasma bile acid profiles in humans, measured alongside routine evaluation of other clinical chemistry parameters, may provide additional insight into the clinical risk of compounds that exhibit *in vitro* BSEP inhibition signals.

INFORMATION AND KNOWLEDGE GAPS AND OPPORTUNITIES

Prediction, assessment, and interpretation of BSEP inhibition by drugs

Improved computational models, which can accurately predict BSEP inhibition and thereby minimize the likelihood that tested compounds will exhibit this off-target risk, will be valuable. New and improved *in vitro* methods, which combine the advantages of SCH and other complex liver models (multiple hepatic transporter expression plus metabolite formation) with the low cost and high throughput afforded by membrane vesicle assays, also would be highly beneficial. *In vitro* approaches that help tease out the direct (or indirect) contribution of BSEP inhibition to clinical DILI also are needed. For example, co-incubation with a concentrated mixture of bile acids enhanced the hepatotoxicity of BSEP-inhibitory drugs in SCH and liver spheroids.[107] Data provided by organ-on-a-chip devices[70] could add additional value, once these have been suitably validated.

Accurate prediction of clinical exposure (systemic and within hepatocytes) to drugs and/or metabolites is required when translating *in vitro* and preclinical *in vivo* data to humans, and when undertaking QST simulations (see the "BSEP Inhibition Computational Modeling" section). This is challenging, especially during the early development phase; hence, improved drug exposure prediction methods are needed.

Another major gap is a lack of early specific and sensitive clinical biomarkers of functional BSEP inhibition by drugs, which are needed to underpin accurate *in vitro/in vivo* translation. Although plasma bile acid measurements are useful, these can change via mechanisms other than BSEP inhibition (e.g., NTCP inhibition), and concentrations in plasma may not correlate well with concentrations within hepatocytes (see the "BSEP Inhibition Computational Modeling" section). Recent advances in nuclear imaging technology show greater promise. Tracers for single photon emission computed tomography or positron emission tomography, such as [^{14}C]-rosuvastatin and [^{11}C]-labeled bile acids, have been used in preclinical and clinical studies to image drug-induced reduction of hepatobiliary transport and the accumulation of bile acids in the liver.[108]

Additional clinical issues

Elucidation of the role of BSEP inhibition and bile acid retention in the pathogenesis of idiosyncratic DILI that progresses to liver failure is the most clinically concerning open question, and cannot be directly addressed by consideration of BSEP inhibition alone. This requires further investigations, which should also address the multiple drug-related mechanisms by which DILI can arise, and the patient-related factors present in susceptible humans.

CONCLUSION

Proactive evaluation and understanding of BSEP inhibition is recommended in drug discovery and development to aid internal decision making on potential human DILI risk. When interpreting the data provided by *in vitro* BSEP inhibition assays, it is important to consider the *in vivo* drug exposure. Currently, this can be undertaken most effectively by consideration of total $C_{ss,plasma}$. It should be noted, however, that because total drug concentrations are not predictive of pharmacological effect, the relationship between total exposure and BSEP inhibition is not causal. This is an important gap in our current understanding of the relationship between BSEP inhibition by drugs and potential DILI risk. A variety of follow-up studies can aid interpretation of observed *in vitro* BSEP inhibition data and may be undertaken on a case-by-case basis. BSEP inhibition is one of several mechanisms by which drugs may cause DILI. Hence, it is important to consider BSEP inhibition alongside other important DILI initiating mechanisms when considering DILI risk, and especially the risk of possible acute drug-induced liver failure.

Key points from BSEP inhibition ITC paper

Inhibition of BSEP has emerged as an important mechanism that may contribute to human DILI. Bile acid accumulation following BSEP inhibition/deficiency causes hepatocyte injury by multiple mechanisms. The mechanisms by which drugs cause DILI are complex and include both drug-related processes and patient-related susceptibility factors. Therefore, it is important to consider the complexity of DILI when translating *in vitro* BSEP inhibition data to the design and selection of safe drugs.The ITC recommends that BSEP screening should be undertaken in parallel with screening for other important DILI liabilities, especially inhibition of mitochondrial function and formation of chemically reactive metabolites, along with the following considerations:

- An IC50 should be determined when BSEP inhibition is observed.
- The in vitro BSEP IC50 values should be compared to the maximum total plasma concentrations in calculation of an initial risk assessment.
- There remain important gaps in our current understanding of the relationship between BSEP inhibition by drugs and potential DILI risk, such as the lack of causality between total drug exposure and BSEP inhibition (e.g., effect of protein binding both intracellular and extracellular), importance of the mechanism of inhibition (e.g., noncompetitive) and the regulatory processes for bile acids.

SUPPORTING INFORMATION

Supplementary information accompanies this paper on the *Clinical Pharmacology & Therapeutics* website (www.cpt-journal.com).

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CONFLICTS OF INTEREST

J.G.K. is a drug safety consultant and a paid scientific adviser to Bioxydyn Ltd, which receives funding from the TRISTAN consortium (www.imi-tristan.eu). J.W.P. and K.S.T. are employees of GlaxoSmithKline and receive stock options as part of their employment. K.R.B. is an employee of BioIVT, and a co-inventor of the C-DILI assay used to predict cholestatic drug-induced liver injury. K.L.R.B. is co-inventor of the sandwich-cultured hepatocyte technology for quantification of biliary excretion (B-CLEAR) and related technologies, which have been licensed exclusively to Qualyst Transporter Solutions. K.Y. and C.B. are employees of DILIsym Services, a company that licenses the DILIsym software for commercial use and served as the coordinating member of the DILIsim Initiative. P.B.W. is a stock holder in Simulations Plus, which is the owner of DILIsym Services. Y.A.P. is an employee and a stock holder of Eli Lilly and Co. Y.L. is an employee of Gilead Sciences, Inc. and receives stocks as part of his employment. The other authors declared no conflict of interest.

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