Drug-Induced Perturbations of the Bile Acid Pool, Cholestasis, and Hepatotoxicity: Mechanistic Considerations Beyond the Direct Inhibition of the Bile Salt Export Pump

A. David Rodrigues, Yurong Lai, Mary Ellen Cvijic, Lisa L. Elkin, Tatyana Zvyaga and Matthew G. Soars

Pharmaceutical Candidate Optimization, Bristol-Myers Squibb, Princeton, New Jersey (A.D.R., Y.L.); Pharmaceutical Candidate Optimization, Bristol-Myers Squibb, Wallingford, Connecticut (M.S.); Leads Discovery and Optimization, Bristol-Myers Squibb, Princeton, New Jersey (M.E.C.); and Leads Discovery and Optimization, Bristol-Myers Squibb, Wallingford, Connecticut (L.E., T.Z.)
Running title: Mechanisms leading to drug-induced cholestasis

Correspondence Address:

A. David Rodrigues, PhD
Pharmaceutical Candidate Optimization
Bristol-Myers Squibb, Mail Stop F12-04
P.O. Box 4000, Princeton, New Jersey 08543
U.S.A
TEL: (609) 252-7813
FAX: (609) 252-6802
david.rodrigues@bms.com

Number of text pages: 38
Number of tables: 2
Number of figures: 3
Number of references: 73
Number of words in Abstract: 249
Number of words in Introduction: 733
Total number of words: 8,744
**Abbreviations:** Bile acid, BA; SULT2A1, sulfotransferase 2A1; FXR, farnesoid X receptor; PXR, pregnane X receptor; BSEP, bile salt export pump; MRP, multidrug resistance-associated protein; OATP, organic anion transporting peptide; NTCP, sodium-taurocholate co-transporting polypeptide; ASBT, apical sodium-dependent bile acid transporter; OST, organic solute transporter; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PKC, protein kinase C; NHR, nuclear hormone receptors; EHR, enterohepatic recirculation; DILI, drug-induced liver injury; DIC, drug-induced cholestasis; BACS, bile acid-CoA ligase (bile acid-CoA synthetase); BAAT, bile acid-CoA: amino acid (glycine/taurine) N-acetyltransferase; Akt, protein kinase B; OAT, organic anion transporter; CAR, constitutive androstane receptor; MDR3, multidrug resistant protein 3; PK-ADME-TOX, pharmacokinetic-absorption-distribution-metabolism-excretion-toxicity; LCA, lithocholic acid; G-LCA, glycolithocholic acid; T-LCA, taurolithocholic acid; G-UDCA, glycoursodeoxycholic acid; T-UDCA, taouroursodeoxycholic acid; UDCA, ursodeoxycholic acid; DCA, deoxycholic acid; G-DCA, glycodyoxycholic acid; T-DCA, taurodeoxycholic acid; CDCA, chenodeoxycholic acid; G-CDC, glycochenodeoxycholic acid; T-CDC, taurochenodeoxycholic acid; CA, cholic acid; G-CA, glycocholic acid; T-CA, taurocholic acid; CAT, chloramphenicol acetyltransferase; ATP8B1, ATPase-aminophospholipid transporter.
Abstract

The bile salt export pump (BSEP) is located on the canalicular plasma membrane of hepatocytes and plays an important role in the biliary clearance of bile acids (BAs). Therefore, any drug or new chemical entity that inhibits BSEP has the potential to cause cholestasis and possibly liver injury. In reality, however, one has to consider the complexity of the BA pool, BA enterohepatic recirculation (EHR), extrahepatic (renal) BA clearance, and the interplay of multiple participant transporters and enzymes (e.g., sulfotransferase 2A1, multidrug resistance-associated protein 2, 3 and 4). Moreover, BAs undergo extensive enzyme-catalyzed amidation and are subjected to metabolism by enterobacteria during EHR. Importantly, the expression of the various enzymes and transporters described above is governed by nuclear hormone receptors (NHRs) that mount an adaptive response when intracellular levels of BAs are increased. The intracellular trafficking of transporters, and their ability to mediate the vectorial transport of BAs, is governed by specific kinases also. Finally, bile flow, micelle formation, canalicular membrane integrity, and BA clearance can be influenced by the inhibition of MDR3 (multidrug resistant protein 3)- or ATP8B1 (ATPase-aminophospholipid transporter)-mediated phospholipid flux. Consequently, when screening compounds in a discovery setting, or conducting mechanistic studies to address clinical findings, one has to consider the direct (inhibitory) effect of parent drug and metabolites on multiple BA transporters, as well as inhibition of BA sulfation and amidation, and NHR function. Vectorial BA transport, in addition to BA EHR and homoeostasis, could also be impacted by drug-dependent modulation of kinases and enterobacteria.
Introduction

Interest in the liver canalicular bile salt export pump (BSEP) has grown in recent years, because it plays an important role in the biliary clearance of numerous bile acids (BAs). Specifically, focus has shifted to BSEP inhibition as a cause of drug-induced cholestasis (DIC) and possibly drug-induced liver injury (DILI) (Yang et al., 2013; Kubitz et al., 2012; Bjornsson and Jonasson, 2013; Stepan et al., 2011). Interest in BSEP (ABC11) has been fueled also by reports that subjects carrying certain loss-of-function/expression ABC11 alleles are predisposed to DILI (Ulzurrun et al., 2013). As a result, a considerable amount of effort has been made to set up high-throughput in vitro inhibition screens in an attempt to mitigate drug interactions involving BSEP inhibition and DIC (Morgan et al., 2010; Dawson et al., 2012; Warner et al., 2012; Pedersen et al., 2013). Some groups have gone on to attempt to relate BSEP inhibitory potency in vitro to cholestasis and DILI. Unfortunately, such exercises have rendered mixed results even after consideration of drug exposure (Morgan et al., 2010; Dawson et al., 2012).

There is no doubt that BSEP plays an important role in the biliary clearance of BAs. However, is it the major player in DIC and DILI? When one considers the complexity of the BA pool (Fig. 1), the differential properties of BAs (hydrophobicity, toxicity, etc), and the complex and dynamic interplay of multiple participant transporters and enzymes (Fig. 2A), is it too simplistic to think that a drug (at a threshold exposure) only needs to directly inhibit BSEP to bring about cholestasis and DILI? Is it possible that the direct inhibition of BSEP is simply the “trigger” event that leads to elevations in BA levels and perturbation of the BA pool, followed by an adaptive response and shunting of BAs to alternative (salvage) pathways? Such a concept is well established and is consistent with the observed increases in serum BAs (~50-fold) in cholestatic
versus non-cholestatic subjects (Xie et al., 2001; Stahl et al., 2008; Humbert et al., 2012).

From the standpoint of the pharmaceutical industry, however, what has not received as much attention is the concept that a drug (or new chemical entity) can trigger BA pool perturbations by alternative mechanisms, or combinations of mechanisms, in addition to (or instead of) the direct inhibition of BSEP. Furthermore, a drug could not only act as a “trigger” but also interfere with the adaptive response mounted by the different organs participating in the enterohepatic recycling (EHR) of BAs. Could the latter be the important step that leads to DILI? It is worth noting that increases in circulating BAs are not always associated with DILI, and concerns only arise when additional “liver signals” are evident (Ozer et al., 2008). Careful review and interpretation of clinical data is warranted (see Supplemental Fig. 1), because hepatotoxicity could lead to hepatocellular damage and reduced bile flow. In such a scenario, cholestasis would be a secondary phenomenon. On the other hand, the direct impact of a drug on BA homeostasis, by whatever mechanism(s), could lead to an increase in the intracellular concentrations of toxic BAs. In this second scenario, cholestasis would be reflective of the primary event leading to hepatotoxicity. Given such complexity, and the known species differences in enzyme and transporter expression, activity and inhibition, as well as the composition of the BA pool, it is not surprising that animal models often fail to predict DIC/DILI in human subjects. Despite the known species differences, however, rodent data (e.g., Bsep knockout mouse) do support the concept of a stunted adaptive response leading to severe intra-hepatic cholestasis (Wang et al., 2009).

In short, when it comes to drug-induced perturbations of the BA pool, DIC and DILI, it is likely that one has to consider additional factors beyond the direct inhibition of hepatic BSEP. In reality, the PK-ADME-TOX (pharmacokinetic-absorption-distribution-
metabolism-excretion-toxicity) profiles of individual BAs are complex and have to be viewed in light of their EHR (Ballatori et al., 2009; Gonzalez, 2012), renal clearance, vectorial flux by multiple transporters, transport out of cells back into blood, 3-O-sulfation, glucuronidation, amidation, and the nuclear hormone receptor (NHR)-mediated “adaptive” responses of the liver, kidney and intestine (Fig. 2B; liver shown). A given drug can inhibit (or modulate) any one or more of these important processes and alter the composition of the BA pool, the balance of BAs in the pool, and increase intracellular concentrations of toxic BAs.

Major Considerations

Complex and Dynamic BA Pool

Primary BAs (cholic acid [CA] and chenodeoxycholic acid [CDCA], Fig. 1) are synthesized from cholesterol in the liver via two multi-step biosynthetic pathways (initiated by cholesterol 7- and 27-hydroxylation) involving various cytochromes P450 (Gonzalez, 2012). Once formed, they can undergo extensive enzyme-catalyzed taurine and glycine conjugation to form “amidated” BAs (taurocholic acid [T-CA]; glycocholic acid [G-CA]; glycochenodeoxycholic acid [G-CDCA]; and taurochenodeoxycholic acid [T-CDCA]). In turn, the mixture can be actively transported out of the liver and into the bile (Table 1). Under normal conditions, such transport renders a very concentrated BA pool in the gallbladder (~100 mM total BAs) when compared to liver tissue (~20 µM), small intestine lumen (2-10 mM), serum (~2 µM) and urine (~1 µM) (Humbert et al., 2012; Garcia-Canaveras et al., 2012; Takikawa et al., 1984; Northfield and McColl, 1973). Via the bile ducts, BAs travel to the gallbladder and are released into the upper small intestine (duodenum). Along the small intestine, BAs can be absorbed by passive
diffusion and, upon reaching the ileum, are subjected to active uptake via apical sodium-dependent bile acid transporter [ABST] and basolateral organic solute transporter [OST]. While in the gut, CA (to deoxycholic acid [DCA]) and CDCA (to lithocholic acid [LCA] and ursodeoxycholic acid [UDCA]) are metabolized by bacteria to form secondary BAs (Fig. 1) (Ridlon et al., 2006; Ballatori et al., 2009; Gonzalez, 2012; Trauner and Boyer, 2003). Any taurine- or glycine-conjugated BAs in the intestine are also subjected to de-amidation by enterobacteria, especially in the mid to lower ileum and large intestine (Northfield and McColl, 1973; Ridlon et al., 2006). As shown in Table 1, the BA pool in the caecum (versus liver tissue and gallbladder bile) is dominated by non-amidated LCA (17.5%), DCA (29.5%), CDCA (20.1%), CA (14.8%) and UDCA (3.5%).

During the EHR process, ~90% of the BA pool in the gut is absorbed. A small fraction of the BA pool (~10%) is lost in feces and replaced by de novo synthesis in the liver (Ridlon et al., 2006; Ballatori et al., 2009; Gonzalez, 2012; Trauner and Boyer, 2003). Of the fraction absorbed, the majority of the hepatic portal BA pool (~90%) is extracted by the liver and the remainder circulates and is cleared via the kidneys (Ballatori et al., 2009; Van Berge Henegouwen et al., 1976; Humbert et al., 2012). Once in the liver, re-extracted BAs enter the BA pool therein and undergo amino acid conjugation and vectorial transport to bile; it has been reported that a given BA will undergo ~20 cycles of EHR prior to elimination (Gonzalez, 2012). Cholestasis can, therefore, be caused by “pre-hepatic” (e.g., inhibition of liver uptake), “intra-hepatic” (e.g., inhibition of biliary efflux) or “post-hepatic” (e.g., bile duct injury) events. Moreover, the kidneys serve as back-up clearance organs, when hepatic function is impaired, so factors impacting renal function could also perturb BA homeostasis.
In addition to conjugation with glycine and taurine, a number of BAs undergo sulfotransferase 2A1 (SULT2A1)-mediated 3-O-sulfation and UDP-glucuronosyltransferase-catalyzed glucuronidation. Consequently, it is not surprising that gallbladder bile, liver tissue, serum and urine samples render complex, and distinctive, BA signature profiles (Humbert et al., 2012; Garcia-Canaveras et al., 2012; Hamilton et al., 2007; Rossi et al., 1987; Takikawa et al., 1984; Trottier et al., 2013). Such “signatures” are reflective of the PK-ADME properties of individual BAs (Table 1), imparted by their unique transporter-enzyme-NHR profile (e.g., BSEP-NTCP [sodium-taurocholate co-transporting polypeptide]-OATP [organic anion transporting peptide]-MRP2 [multidrug resistance-associated protein 2]-FXR [farnesoid X receptor]-SULT2A1) (Heuman et al., 1989; Huang et al., 2010; Hayashi et al., 2005; Meier et al., 1997; Parks et al., 1999; Staudinger et al., 2001). In agreement, for individual BAs, one can find reports describing a wide range of uptake rates for human BSEP vesicles (~30-fold), sulfation rates for human SULT2A1 (>2000-fold), human NTCP- (~20-fold) and OATP- (~20-fold) mediated cell uptake rates, and human FXR- (7-fold) and pregnane X receptor (PXR; ~7-fold)-mediated reporter (chloramphenicol acetyltransferase [CAT] activity) induction in cells (Table 2).

Based on the available literature, it appears that multidrug resistance-associated protein 4 (MRP4)-mediated uptake rate is less varied amongst different BAs (~5-fold) (Table 2; Rius et al., 2006). Although additional data are needed, it implies that MRP4 may have a broader BA substrate selectivity; MRP4 serves as “salvage transporter” and is up-regulated in the liver during cholestasis (Gradhand et al., 2008). Unfortunately, even less is known regarding the selectivity of multidrug resistance-associated protein 3 (MRP3), which also likely plays a role in the efflux of certain BAs (Fig. 2A). Only one
report by Zeng et al (2000) describes G-CA as a human MPR3 substrate. Under the same assay conditions, however, no uptake of T-CA into MRP3 vesicles was detected. Does this mean that MRP3 is more selective (vs. MRP4) as a basolateral BA efflux transporter?

Importantly, one of the most toxic and hydrophobic BAs (LCA) is a poor human BSEP substrate in vitro, but is a relatively good SULT2A1 substrate (Table 2; Huang et al., 2010; Hayashi et al., 2005) and undergoes extensive conjugation with glycine and taurine in human subjects (Cowen et al., 1975). It is the amidated/3-O-sulfated form of LCA that serves as a BSEP substrate (Hayashi et al., 2005). So is the inhibition of BSEP irrelevant in the case of LCA itself? The same could be said for other hydrophobic BAs such as G-LCA, T-LCA, and DCA, although no BSEP data are available (Table 2). By comparison, T-DCA (taurodeoxycholic acid), G-CDCA, T-CDCA, G-CA and T-CA are better BSEP substrates, relatively less hydrophobic, poorer SULT2A1 substrates (Table 2), and together are major components of the BA pool in gallbladder bile (5.4%, 26%, 13%, 26%, 11% of total BA, respectively) (Table 1). Potent inhibition of BSEP in the liver would likely impact the biliary clearance of these 5 BAs.

In toto, when attempting to understand DIC mechanistically, it is evident that the PK-ADME, transporter, conjugation (amino acid and sulfation), and EHR properties of each individual BA have to be considered. This becomes even more critical in light of each BA’s physicochemical and cytotoxicity profile.

**Transporter-Enzyme Interplay**

As shown in Fig 2A, hepatic canalicular BSEP functions coordinately with sinusoidal NTCP to enable vectorial transport of circulating BAs into the bile. However,
this “NTCP-BSEP axis” should only be viewed as one possible mechanism by which BAs are transported. BAs such as LCA, G-LCA (glycolithocholic acid) and T-LCA (taurolithocholic acid) can also undergo SULT2A1-catalyzed sulfation to form a 3-O-sulfate conjugate that can serve as a substrate of the “OATP-MRP2 axis.” Therefore, SULT2A1 can be regarded as an important junction point between the two BA transport axes (Alnouti, 2009; Huang et al., 2010). Beyond NTCP, BSEP, OATP and MRP2, liver OST and additional multidrug resistance-associated proteins (MRP3 and MRP4) also play an important role in BA transport (Fig. 2A). Such transporters mediate the transport of BAs into the blood (from the liver) and enable “hopping” between individual hepatocytes, and more efficient extraction, as the blood flows along the sinusoids. Such a concept has been introduced by van de Steeg et al (2012).

As described above, SULT2A1 can play an important role in the clearance and disposition of certain BAs. The enzyme is expressed in liver (~1500 ng/mg cytosol protein), small intestine (~400 ng/mg cytosol protein), and kidneys (~5 ng/mg cytosol protein), but as expected the rate of BA sulfation is highest in the liver and not detectable in kidneys (Riches et al., 2009; Loof and Wengle, 1979). SULT2A1 could, therefore, determine the composition of the BA pool in both the liver and intestine. In the absence of renal BA sulfation, it is noteworthy that BA 3-O-sulfates represent ~75% of the BA pool in urine (sulfated forms of G-UDCA, G-LCA, T-LCA, T-UDCA, DCA, UDCA, and CDCA represent 23.6%, 21.8%, 12.7%, 6.3%, 5.8%, 2.9%, and 2.3% of the BA pool in urine, respectively). By comparison, sulfated BAs represent only a small fraction (<5%) of the BA pool in the bile (Table 1). Under normal conditions, it is likely that 3-O-sulfate forms of BAs are substrates of MRP3 and MRP4, circulate, and are taken up by renal transporters. Unfortunately, the transporters involved in the renal clearance of sulfated
and non-sulfated BAs have not been reported. To date, there is only information for two non-sulfated BAs (CA and T-CA). Both are detectable in urine (Table 1) and have been shown to be substrates of organic anion transporter 3 (hOAT3) in vitro (Brandoni et al., 2012; Chen et al., 2008). Interestingly, it has been reported that OAT3 expression is elevated in kidneys of cholestatic subjects (Brandoni et al., 2012; Chen et al., 2008). It is accepted that OAT3 will likely function coordinately with other renal transporters, possibly apical organic anion transporter 4 and MRP2, to mediate the vectorial transport of BAs in kidneys.

From the standpoint of BA clearance and disposition, therefore, one has to consider the inhibition of multiple transporters in liver and kidney. For example, the combined inhibition of liver BSEP, OST, MRP2, MRP3 and/or MRP4 could trigger intra-hepatic cholestasis, inhibition of liver OATP and/or NTCP could lead to pre-hepatic cholestasis, and the inhibition of renal BA transporters could give rise to extra-hepatic cholestasis. Interestingly, during the preparation of the present manuscript, Morgan et al (2013) reported that integration of exposure data, and knowledge of drug effect to not only BSEP, but also one or more of the MRPs, is a useful tool for informing the potential for DILI due to altered bile acid transport. Therefore, investigators are moving beyond BSEP data alone and starting to integrate inhibition data for additional BA transporters like MRP2, MRP3 and MRP4. In terms of BA sulfation, LCA, G-LCA and T-LCA are relatively hydrophobic and serve as good SULT2A1 substrates (Table 2). Consequently, any drug or metabolite that inhibits SULT2A1 in the liver (or small intestine) could alter the composition of the BA pool and impact the levels of hydrophobic BAs during EHR. While a theoretical consideration, the inhibition of SULT2A1 by known cholestatic compounds warrants investigation.
Importantly, as described below, NHRs mount an adaptive response when intracellular BA levels rise. Such a response leads to increases in transporter (e.g., OST and MRPs) and SULT2A1 expression. So any combination of BSEP, OST, MRP, and SULT2A1 inhibition could not only trigger DIC, but also stunt any NHR-mediated adaptive response and exacerbate cholestatic liver injury.

The Role of NHRs

As discussed previously, the liver is able to respond to increases in BA levels and BA pool perturbations. This is possible via the coordinated interplay of at least 3 NHRs; FXR, PXR and CAR (constitutive androstane receptor) (Fig. 2B). Numerous BAs have been shown to be agonists of these receptors (Li and Chiang, 2013; Stahl et al., 2008; Guo et al., 2003). For example, CDCA, DCA, and UDCA are relatively good FXR agonists, whereas LCA behaves as a good FXR and PXR agonist (Parks et al., 1999; Staudinger et al., 2001). FXR agonism increases BSEP, MRP2, and OST expression and represses NTCP and OATP expression in hepatocytes. CAR/PXR agonism increases OATP, SULT2A1, MRP2, MRP3 and MRP4 expression (Halilbasic et al., 2013; Xie et al., 2001; Stahl et al., 2008). The net effect of this NHR interplay, and its modulation by members of the BA pool, is increased efflux of BAs out of the hepatocyte, as well as shunting of BAs towards MRPs, especially after sulfation to 3-O-sulfates that are excreted in the urine (Keppler, 2011; Van Berge Henegouwen et al., 1976; Humbert et al., 2012). This is reflected in the serum BA profile of cholestatic versus healthy subjects; elevations in primary BAs (amidated with or without sulfation) accompanied by decreases in secondary-hydrophobic/nonamidated/non-sulfated-BAs. Importantly, the NHRs “compete” with each other and can elicit differential effects on transporter
expression. Such a phenomenon is exemplified by the opposing effect of CAR (up-regulation) and FXR (down-regulation) on MRP4 expression (Renga et al., 2011).

If a given drug disrupts NHR function (e.g., by interfering with transcription factor, and/or co-activator function, etc), then the adaptive ability of the liver will be muted and the toxic effects of BAs can be exacerbated. Ketoconazole is one example of a drug that has been shown to inhibit the activation of PXR (Huang et al., 2007). Similarly, the sulfate conjugates of certain hormones (e.g., progesterone) are known to inhibit FXR (Abu-Hayyeh et al., 2013). So it is possible that one has to consider BSEP inhibition in concert with NHR inhibition. As in the case of SULT2A1, inhibition of NHRs (e.g., PXR and FXR) by known cholestatic drugs warrants further study.

**Inhibition of BA Conjugation**

All BAs undergo extensive conjugation with glycine and taurine. In fact, the amidated forms of DCA, CDCA, and CA dominate the BA pool (> 90%) in liver and bile (Table 1). Such conjugation is catalyzed (step-wise) by two enzymes that are highly expressed in the liver (bile acid-CoA ligase or bile acid-CoA synthetase [BACS] and bile acid-CoA: amino acid (glycine/taurine) N-acetyltransferase [BAAT]) (Falany et al., 1994; Solaas et al., 2000; O’Bryne et al., 2003). Therefore, inhibition of one or both enzymes can perturb the BA pool and the balance of toxic versus less toxic BAs. For example, cyclosporine (which is cholestatic) has been shown to inhibit BA conjugation in vitro (Vessey and Kelley, 1995) and lack of BA conjugation (related to BAAT genotype) has been associated with cholestasis (Hadzic et al., 2012). Importantly, hydrophobic BAs like LCA and DCA are extensively conjugated with glycine and taurine, so one can imagine the consequences of inhibiting BACS and/or BAAT.
Additional Considerations (Involving the Direct Inhibition of BA Transporters)

To date, as in the case of BSEP, investigators have largely focused on transporter (direct) inhibition screening in vitro (Morgan et al., 2010; Dawson et al., 2012; Warner et al., 2012; Pedersen et al., 2013). Typically, this requires a fully- or semi-automated high-throughput (multi-well plate-based) assay employing a well-characterized transporter substrate. However, based on current literature, it is apparent that one has to evaluate metabolites as transporter inhibitors also. Moreover, the possibility of “trans” inhibition, especially in the case of canalicular transporters like BSEP, has to be considered.

Metabolites as Transporter Inhibitors

While current screening efforts focus on parent molecules, there is a growing appreciation that some metabolites may inhibit transporters. For example, progesterone metabolites have been shown to inhibit NTCP (Abu-Hayyeh et al., 2010), whereas the sulfate conjugate of troglitazone is a more potent inhibitor (10-fold) of rat bsep than parent troglitazone (Funk et al., 2001). Given the high levels of some metabolites in the liver and bile, it is likely that many could behave as transporter co-substrates (inhibitors) and impact BA clearance also. Therefore, careful metabolic profiling of human bile would be warranted in such cases and the major metabolites screened as transporter inhibitors. Because human ADME data are not available in early discovery, there is some risk when advancing parent molecules that have been shown to be weak inhibitors of human BA transporters in vitro.

Cis- Versus Trans-Inhibition
Classically, drugs are thought to inhibit BSEP activity by impacting ATP-dependent transport. This is known as “cis-inhibition” and involves binding to the (intracellular) ATP-binding site or substrate binding site. Typically, this renders competitive inhibition. “Trans-inhibition”, on the other hand, involves the interplay of BSEP with at least one additional canalicular transporter. For example, certain glucuronides (and other metabolites) are transported into the bile via a second transporter (e.g., MRP2), are present at high concentrations and inhibit BSEP once in the bile canaliculus (Pauli-Magnus and Meier, 2006). In such a scenario, a drug (or metabolite) would inhibit BSEP only after its biliary secretion by a second transporter.

**Additional Considerations (Involving Indirect Effects Leading to DIC)**

Although direct inhibition of BA transporters and BA-metabolizing enzymes by drugs is important, one has to consider the possibility that DIC, and possibly DILI, may be caused by indirect effects. In this instance, a given parent drug (or a metabolite) does not have to bind to, or interact directly with, a BA transporter. Three possibilities are described below.

**Modulation of Kinases**

Numerous publications have described the regulation of transporter (intracellular) trafficking by various kinases (Crocenzi et al., 2012; Roma et al., 2008; Boaglio et al., 2010). “Translocation” of OATP and NTCP from the basolateral membrane of hepatocytes to endosomes is stimulated by activation of Ca^{2+}-dependent protein kinase C (PKC). Likewise, the trafficking of MRP2 and BSEP away from the canalicular membrane is also mediated by PKC. Trafficking from endosomes can be disrupted also
by activation of the PI3K-Akt (phosphoinositide 3-kinase/protein kinase B) pathway. Conversely, trafficking of transporters to the basolateral and canalicular membranes is mediated by protein kinase A (PKA). Because the activity of these various kinases is linked to the levels of oxidative stress in hepatocytes, any pro-oxidant drug (or metabolite) can cause cholestasis by elevating Ca\(^{2+}\) levels and decreasing the concentration of reduced glutathione; transporters such as BSEP and MRP2 are known to be internalized under conditions of oxidative stress (Perez et al., 2006; Sekine et al., 2011). Therefore, a given drug or metabolite could decrease the vectorial transport of BAs by modulating the balance of PKA, PKC, PI3K activity without direct inhibition of transporters such as BSEP and MRP2. The internalization of key BA transporters would negate any effort by the liver to mount a NHR-mediated adaptive response.

**Impact on Phospholipid Flux**

At least two transporters function coordinately to maintain membrane integrity and mediate phospholipid flux across the canalicular plasma membrane of hepatocytes (Groen et al., 2011). Importantly, loss of function phenotype in either case has been associated with intra-hepatic cholestasis. Therefore, inhibition of either transporter could give rise to DIC.

The first is MDR3 (multidrug resistant protein 3; ABCB4), also known as a “phosphatidylcholine translocase” or “floppase”, which is the locus of progressive familial intra-hepatic cholestasis type 3 (“PFIC-3”) (Dzagania et al., 2012; Harris et al., 2005; Groen et al., 2011). It plays a major role in the secretion of phospholipids out of the liver and into bile. Such phospholipids are important components of BA-containing biliary micelles. So it is likely that MDR3 and BSEP function together to form mixed
Therefore, inhibition of MDR3 could bring about cholestasis and impact the biliary clearance of BAs. Compared to BSEP, however, inhibition of MDR3 has received relatively little attention to date and there is only one report describing the inhibition of MDR3 by a drug (itraconazole) (Yoshikado et al., 2011).

The second transporter, ATP8B1 (ATPase-aminophospholipid transporter), is associated with progressive familial intra-hepatic cholestasis type 1 (“PFIC-1”) (Groen et al., 2011; Harris et al., 2005). Unlike MDR3, canalicular ATP8B1 acts as a “flippase” and mediates the translocation of phosphatidylserine from the outer to the inner leaflet of the canalicular membrane. The continued inward flux of phosphatidylserine is thought to be essential for the maintenance of membrane integrity in the presence of high concentrations of detergent BAs (Groen et al., 2011). To date, there are no reports of any drug inhibiting ATP8B1.

**Impact on Gut Bacteria**

As described earlier, gut bacteria are involved in the metabolism of BAs (amino acid and sulfate deconjugation, as well as 7α/β-dehydroxylation) during EHR (Ridlon et al., 2006; Robben et al., 1989). This is reflected in the BA profiles of stool and caecal contents (LCA, DCA and CDCA represent >70% of the BA pool, low levels of amidated and sulfated BAs detected; Table 1). Moreover, there is evidence that the expression of host BA transporters (e.g., MRP2 and ABST) in the intestine can be modulated by enterobacteria (Mercado-Lubo et al., 2010; Miyata et al., 2011). Therefore, any orally dosed drug that perturbs gut bacteria could impact BA homeostasis, alter the composition of the BA pool (primary vs. secondary BAs, amidated vs non-amidated BAs; sulfated vs.
non-sulfated BAs), by altering BA metabolism and/or transport, and give rise to cholestasis.

**Conclusions (Going Beyond “BSEP Bias”)**

The growing awareness of the complex and dynamic interplay between transporters, enzymes, and NHRs in multiple organs, and its likely impact on BA EHR and disposition, will compel industry researchers and regulators to garner a more “systems view” of DIC. As described herein, DIC could be triggered not only by direct inhibition of BSEP, but by any combination of direct and indirect mechanisms involving enterobacteria, BA-metabolizing enzymes, kinases and other participating BA transporters (Fig. 3, liver shown). From the standpoint of DILI, the impact of drug or new chemical entity on any subsequent NHR-mediated (adaptive) responses could be very important and should be considered also. Therefore, it is likely that efforts will be made to expand inhibition screening in vitro beyond BSEP and include assays for additional transporters, NHRs, kinases, and Phase II (conjugation) enzymes.

Ultimately, progress will be enabled by the development and wide use of validated and more sophisticated in vitro models, the availability of complete in vitro data sets for greater numbers of individual BAs (Table 2), and improved modeling and simulation tools that support in vitro-in vivo extrapolations and provide mechanistic insight. Integrated strategies will also require the development of robust analytical methods that facilitate the more routine profiling of individual BAs and the generation of BA “signatures” in human subjects. Ideally, individual subject BA profiles would be interpreted in light of genotype data (see Supplemental Fig. 2).
As evidenced by the recent reports of Thompson et al (2012), Chang et al (2013), and Morgan et al (2013), researchers are already migrating to assay panel-based (integrative) strategies to enable high-throughput compound screening, support the building of structure-activity relationships and facilitate risk assessment in a discovery setting. Therefore, the widespread and rapid transition from “BSEP inhibition” screens to “cholestasis assay panels” and “assay batteries” is envisioned. Concomitantly, there will also be a push for “physiologically relevant” primary cell-based screens, coupled to multiplexed readouts (e.g., human hepatocyte co-cultures, sandwich cultures, and 3-dimensional cultures), and more sophisticated data integration strategies (Godoy et al., 2013; Vinken et al., 2013; Pedersen et al., 2013).

Despite the obvious challenges, it is hoped that systematic approaches that enable investigation of the potential causes of DIC and DILI (beyond direct BSEP inhibition) will support the discovery and development of new chemical entities that are less cholestatic and hepatotoxic.
Authorship Contributions

Participated in research design: Rodrigues, Soars, Lai

Conducted experiments: Not applicable

Contributed to new reagents or analytic tools: Not applicable

Wrote or contributed to the writing of the manuscript: Rodrigues, Soars, Lai, Zvyaga, Elkin, Cvijic
References


Funk C, Pantze M, Jehle L, Ponelle C, Scheuermann G, Lazendic M, and Gasser R (2001) Troglitazone-induced intrahepatic cholestasis by an interference with the hepatobiliary...
export of bile acids in male and female rats. Correlation with the gender difference in
troglitazone sulfate formation and the inhibition of the canalicular bile salt export pump
(Bsep) by troglitazone and troglitazone sulfate. *Toxicology* **167**:83-98.

García-Cañaveras JC, Donato MT, Castell JV, and Lahoz A (2012) Targeted profiling of
circulating and hepatic bile acids in human, mouse, and rat using a UPLC-MRM-MS-

J, Borner C, Böttger J, Braeuning A, Budinsky RA, Burkhardt B, Cameron NR, Camussi
G, Cho CS, Choi YJ, Craig Rowlands J, Dahmen U, Damm G, Dirsch O, Donato MT,
Dong J, Dooley S, Drasdo D, Eakins R, Ferreira KS, Fonsato V, Fraczek J, Gebhardt R,
L, Guyot C, Hallifax D, Hammad S, Hayward A, Häussinger D, Hellerbrand C, Hewitt P,
Hoehme S, Holzhütter HG, Houston JB, Hrach J, Ito K, Jaeschke H, Keitel V, Kelm JM,
Naisbitt DJ, Nussler AK, Olinga P, Pampaloni F, Pi J, Pluta L, Przyborski SA,
Ramachandran A, Rogiers V, Rowe C, Schelcher C, Schmich K, Schwarz M, Singh B,
Stelzer EH, Stieger B, Stöber R, Sugiyama Y, Tetta C, Thasler WE, Vanhaecke T, Vinken
Recent advances in 2D and 3D in vitro systems using primary hepatocytes, alternative
hepatocyte sources and non-parenchymal liver cells and their use in investigating
mechanisms of hepatotoxicity, cell signaling and ADME. *Arch Toxicol* **87**:1315-1530.

**2**:2811-2828.


Stepan AF, Walker DP, Bauman J, Price DA, Baillie TA, Kalgutkar AS, and Aleo MD (2011) Structural alert/reactive metabolite concept as applied in medicinal chemistry to


**Figure Legends:**

**Figure 1. Structures of representative BAs**

**Figure 2. Liver BA transporters and their regulation**

Hepatic transporters involved in the vectorial transport of BAs (A) and their regulation by NHRs (B). OST, MRP3/4, OATP1B1, NTCP, BSEP, and MRP2 represent organic solute transporter, multidrug resistance-associated protein 3 and 4, organic anion transporting peptide 1B1, sodium-taurocholate co-transporting polypeptide, bile salt export pump, and multidrug resistance-associated protein 2, respectively. SULT2A1, FXR, PXR, and CAR represent sulfotransferase 2A1, farnesoid X receptor, pregnane X receptor and constitutive androstane receptor, respectively.

**Figure 3. Summary of the potential mechanism(s) by which a drug or metabolite can impact the hepatobiliary disposition of BAs**

ATP8B1, PKC, PI3K/Akt, MDR3, BACS and BAAT represent ATPase-aminophospholipid transporter, protein kinase C, phosphoinositide 3-kinase, protein kinase B, multidrug resistant protein 3, bile acid-CoA ligase (bile acid-CoA synthetase) and bile acid-CoA: amino acid (glycine/taurine) N-acetyltransferase, respectively. See Figure 2 for additional abbreviations.
Table 1
Summary quantitation of different BAs in human serum, urine, liver tissue, caecum, bile and feces

<table>
<thead>
<tr>
<th>BA$^c$</th>
<th>Mean % Total BA (n = number subjects)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum (n = 39)$^a$</td>
</tr>
<tr>
<td>Non-Sulfated$^f$</td>
<td></td>
</tr>
<tr>
<td>LCA</td>
<td>≤ 0.5</td>
</tr>
<tr>
<td>G-LCA</td>
<td>≤ 0.5</td>
</tr>
<tr>
<td>T-LCA</td>
<td>≤ 0.5</td>
</tr>
<tr>
<td>DCA</td>
<td>11.2</td>
</tr>
<tr>
<td>G-DCA</td>
<td>11.8</td>
</tr>
<tr>
<td>T-DCA</td>
<td>2.2</td>
</tr>
<tr>
<td>CDCA</td>
<td>8.2$^g$</td>
</tr>
<tr>
<td>G-CDC</td>
<td>32.6</td>
</tr>
<tr>
<td>T-CDC</td>
<td>3.9</td>
</tr>
<tr>
<td>CA</td>
<td>7.8$^g$</td>
</tr>
<tr>
<td>G-CA</td>
<td>5.2</td>
</tr>
<tr>
<td>T-CA</td>
<td>1.9</td>
</tr>
<tr>
<td>UDCA</td>
<td>2.7</td>
</tr>
<tr>
<td>G-UDCA</td>
<td>4.6</td>
</tr>
<tr>
<td>T-UDCA</td>
<td>≤ 0.5</td>
</tr>
<tr>
<td>Sulfated</td>
<td></td>
</tr>
<tr>
<td>LCA-3S</td>
<td>≤ 0.5</td>
</tr>
<tr>
<td>CA-3S</td>
<td>≤ 0.5</td>
</tr>
<tr>
<td>CDCA-3S</td>
<td>≤ 0.5</td>
</tr>
<tr>
<td>UDCA-3S</td>
<td>≤ 0.5</td>
</tr>
<tr>
<td>DCA-3S</td>
<td>≤ 0.5</td>
</tr>
<tr>
<td>T-UDCA-3S</td>
<td>≤ 0.5</td>
</tr>
<tr>
<td>T-LCA-3S</td>
<td>2.5</td>
</tr>
<tr>
<td>G-LCA-3S</td>
<td>3.5</td>
</tr>
<tr>
<td>G-UDCA-3S</td>
<td>1.8</td>
</tr>
<tr>
<td>Total</td>
<td>100.0</td>
</tr>
</tbody>
</table>
Table 1.
LCA, lithocholic acid; G-LCA, glycolithocholic acid; T-LCA, taurolithocholic acid; G-UDCA, glycoursodeoxycholic acid; T-UDCA, taouroursodeoxycholic acid; UDCA, ursodeoxycholic acid; DCA, deoxycholic acid; G-DCA, glycodeoxycholic acid; T-DCA, taurodeoxycholic acid; CDCA, chenodeoxycholic acid; G-CDCA, glycochenodeoxycholic acid; T-CDDA, taurochenodeoxycholic acid; CA, cholic acid; G-CA, glycocholic acid; T-CA, taurocholic acid; T-UDCA-3S, tauroursodeoxycholic acid 3-O-sulfate; G-UDCA-3S, glycoursodeoxycholic acid 3-O-sulfate; UDCA-3S, ursodeoxycholic acid 3-O-sulfate; T-LCA-3S, taurolithocholic acid 3-O-sulfate; CA-3S, cholic acid 3-O-sulfate; LCA-3S, lithocholic acid 3-O-sulfate; G-LCA-3S, glycolithocholic acid 3-O-sulfate; LCA-3S, lithocholic acid 3-O-sulfate; DCA-3S, deoxycholic acid 3-O-sulfate; CDCA-3S, chenodeoxycholic acid 3-O-sulfate.

aData reported by Humbert et al (2012) as nM, but calculated and presented as mean % of total BA. Low levels of amidated and sulfated BAs, as well as the high levels of LCA and DCA (93% of total BA), in the stool is the result of metabolism by enterobacteria. Approximately 75% and 8% of the BA pool in the urine and serum represents 3-O-sulfated BAs, respectively. It should be noted that Takikawa et al. (1984) have reported the presence of both BA glucuronide and sulfate conjugates in human serum and urine.

bData reported as fmol/mg tissue (Garcia-Canaveras et al., 2012), but calculated and presented as mean % of total BA in the table above. Note high levels of amino acid conjugated BAs in liver tissue (>99% of total BA).

cRossi et al., 1987 (gallbladder bile samples of healthy subjects). The authors do not report the levels of amidated or non-amidated CDCA, DCA and CA 3-O-sulfate or 3-O-
glucuronide conjugates. However, Takikawa et al. (1984) have reported that the levels of sulfated or glucuronidated BAs in human bile are low (<1% total BA).

*Hamilton et al., 2007 (authors reported % total BA, but only mean data are shown). It is assumed that the low level of amidated and sulfated BAs, as well as the high levels of LCA, DCA and CDCA (~70% of total BA), in caecum is the result of metabolism by enterobacteria.

*Non-sulfated BAs are ranked in terms of decreasing hydrophobicity index (see Table 2).

*Not reported

*Trottier et al (2013) report that 38% of serum CDCA is in the form of 3-O-glucuronide (amidated versus non-amidated not specified). The same authors report that about 14% of serum CA is in the form of an acyl glucuronide (non-amidated).
<table>
<thead>
<tr>
<th>BA</th>
<th>SULT2A1 $V_{\text{max}}/K_m$ (µL/min per mg)</th>
<th>BSEP Uptake (pmol/mg per 30sec)</th>
<th>MRP4 Uptake $V_{\text{max}}/K_m$ (µL/min per mg)</th>
<th>NTCP (OATP) Cell Uptake (fmol/min)</th>
<th>FXR (PXR) (CAT Activity Fold-Increase)</th>
<th>Hydrophobicity Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCA</td>
<td>23.3</td>
<td>&lt;45</td>
<td>-</td>
<td>-</td>
<td>35 (15)</td>
<td>-</td>
</tr>
<tr>
<td>G-LCA</td>
<td>22.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+1.05</td>
</tr>
<tr>
<td>T-LCA</td>
<td>16.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+1.0</td>
</tr>
<tr>
<td>DCA</td>
<td>0.63</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>40 (3)</td>
<td>+0.72</td>
</tr>
<tr>
<td>G-DCA</td>
<td>0.17</td>
<td>-</td>
<td>17</td>
<td>-</td>
<td>-</td>
<td>+0.65</td>
</tr>
<tr>
<td>T-DCA</td>
<td>0.14</td>
<td>600</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+0.59</td>
</tr>
<tr>
<td>CDCA</td>
<td>0.12</td>
<td>&lt;45</td>
<td>-</td>
<td>-</td>
<td>120 (3)</td>
<td>+0.59</td>
</tr>
<tr>
<td>G-CDCA</td>
<td>0.16</td>
<td>1000</td>
<td>16</td>
<td>-</td>
<td>-</td>
<td>+0.51</td>
</tr>
<tr>
<td>T-CDC A</td>
<td>0.01</td>
<td>1400</td>
<td>23</td>
<td>29 (0.9)</td>
<td>-</td>
<td>+0.46</td>
</tr>
<tr>
<td>CA</td>
<td>&lt;0.01</td>
<td>45</td>
<td>5</td>
<td>2 (0.3)</td>
<td>5 (2)</td>
<td>+0.13</td>
</tr>
<tr>
<td>G-CA</td>
<td>&lt;0.01</td>
<td>100</td>
<td>7</td>
<td>14 (0.1)</td>
<td>-</td>
<td>+0.07</td>
</tr>
<tr>
<td>T-CA</td>
<td>&lt;0.01</td>
<td>400</td>
<td>20</td>
<td>30 (0.1)</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>UDCA</td>
<td>0.91</td>
<td>&lt;45</td>
<td>-</td>
<td>10 (-)</td>
<td>0</td>
<td>-0.31</td>
</tr>
<tr>
<td>G-UDCA</td>
<td>1.02</td>
<td>300</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-0.43</td>
</tr>
<tr>
<td>T-UDCA</td>
<td>0.96</td>
<td>400</td>
<td>17</td>
<td>40 (1.8)</td>
<td>-</td>
<td>-0.47</td>
</tr>
</tbody>
</table>
Table 2:

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCA</td>
<td>lithocholic acid</td>
</tr>
<tr>
<td>G-LCA</td>
<td>glycolithocholic acid</td>
</tr>
<tr>
<td>T-LCA</td>
<td>taurolithocholic acid</td>
</tr>
<tr>
<td>G-UDCA</td>
<td>glycoursodeoxycholic acid</td>
</tr>
<tr>
<td>T-UDCA</td>
<td>taursodeoxycholic acid</td>
</tr>
<tr>
<td>UDCA</td>
<td>ursodeoxycholic acid</td>
</tr>
<tr>
<td>DCA</td>
<td>deoxycholic acid</td>
</tr>
<tr>
<td>G-DCA</td>
<td>glycodeoxycholic acid</td>
</tr>
<tr>
<td>T-DCA</td>
<td>taurodeoxycholic acid</td>
</tr>
<tr>
<td>CDCA</td>
<td>chenodeoxycholic acid</td>
</tr>
<tr>
<td>G-CDCA</td>
<td>glycochenodeoxycholic acid</td>
</tr>
<tr>
<td>T-CDCA</td>
<td>taurochenodeoxycholic acid</td>
</tr>
<tr>
<td>CA</td>
<td>cholic acid</td>
</tr>
<tr>
<td>G-Ca</td>
<td>glycocholic acid</td>
</tr>
<tr>
<td>T-Ca</td>
<td>taurocholic acid</td>
</tr>
<tr>
<td></td>
<td>aNo data reported.</td>
</tr>
<tr>
<td></td>
<td>bHuang et al., 2010 (authors reported kinetic parameters for the sulfation of each BA after incubation with recombinant human sulfotransferase 2A1, SULT2A1).</td>
</tr>
<tr>
<td></td>
<td>cHayashi et al., 2005 (authors reported BA uptake rate by human bile salt export pump [BSEP] vesicles and vector control vesicles; data represent difference between the two).</td>
</tr>
<tr>
<td></td>
<td>dRius et al., 2005 (authors reported kinetic parameters for BA uptake by human multidrug resistance-associated protein 4 [MRP4] vesicles).</td>
</tr>
<tr>
<td></td>
<td>eMeier et al., 1997 (authors reported BA uptake rate into oocytes containing human sodium-taurocholate co-transporting polypeptide [NTCP] or human organic anion transporting peptide [OATP]).</td>
</tr>
<tr>
<td></td>
<td>fParks et al., 1999; Staudinger et al., 2001 (human farnesoid X receptor (FXR)- and human pregnane X receptor [PXR]-mediated increase in chloramphenicol acetyltransferase [CAT] activity in CV-1 cells).</td>
</tr>
<tr>
<td></td>
<td>gHeuman et al., 1989.</td>
</tr>
<tr>
<td>Bile Acid</td>
<td>$R_1$ ($\alpha$)</td>
</tr>
<tr>
<td>------------------------------------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>Ursodeoxycholic acid (UDCA)</td>
<td>H</td>
</tr>
<tr>
<td>Cholic acid (CA)</td>
<td>H</td>
</tr>
<tr>
<td>Chenodeoxycholic acid (CDCA)</td>
<td>H</td>
</tr>
<tr>
<td>Deoxycholic acid (DCA)</td>
<td>H</td>
</tr>
<tr>
<td>Lithocholic acid (LCA)</td>
<td>H</td>
</tr>
<tr>
<td>Free Bile Acids</td>
<td></td>
</tr>
<tr>
<td>Glyco-Conjugated Bile Acids (G)</td>
<td></td>
</tr>
<tr>
<td>Tauro-Conjugated Bile Acids (T)</td>
<td></td>
</tr>
<tr>
<td>Non-Sulfated Bile Acids</td>
<td></td>
</tr>
<tr>
<td>Sulfated Bile Acids (S)</td>
<td></td>
</tr>
</tbody>
</table>

$R_5 = \text{OH}$

$R_5 = \text{NHCH}_2\text{COOH}$

$R_5 = \text{NHCH}_2\text{CH}_2\text{SO}_3\text{H}$

$R_6 = \text{OH}$

$R_6 = \text{OSO}_3\text{H}$
Figure 3

**Indirect Effects**

- Inhibition of BA hepatic uptake and efflux?

- Impact on NHR-mediated adaptive response to BA pool perturbations

- Impact on serum vs. liver BA levels

**Direct Inhibition**

- Inhibition of BA hepatic uptake and efflux?

- Impact on serum vs. liver BA levels

- Impact on intracellular BA levels

- Impact on BA biliary clearance?

- Impact on intracellular BA levels

Impact on BA amidation and/or sulfation?

Impact on BA pool composition

**Impact on NHRs?**

**Impact on NHR-mediated adaptive response to BA pool perturbations**