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Current insights in the complexities underlying drug-induced cholestasis

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Abstract

Drug-induced cholestasis (DIC) poses a major challenge to the pharmaceutical industry and regulatory agencies. It causes both drug attrition and post-approval withdrawal of drugs. DIC represents itself as an impaired secretion and flow of bile, leading to the pathological hepatic and/or systemic accumulation of bile acids (BAs) and their conjugate bile salts. Due to the high number of mechanisms underlying DIC, predicting a compound's cholestatic potential during early stages of drug development remains elusive. A profound understanding of the different molecular mechanisms of DIC is, therefore, of utmost importance. Although many knowledge gaps and caveats still exist, it is generally accepted that alterations of certain hepatobiliary membrane transporters and changes in hepatocellular morphology may cause DIC. Consequently, liver models, which represent most of these mechanisms, are valuable tools to predict human DIC. Some of these models, such as membrane-based *in vitro* models, are exceptionally well-suited to investigate specific mechanisms (*i.e.,* transporter inhibition) of DIC, while others, such as liver slices, encompass all relevant biological processes and therefore offer a better representation of the *in vivo* situation. In the current review, we highlight the principal molecular mechanisms associated with DIC and offer an overview and critical appraisal of the different liver models that are currently being used to predict the cholestatic potential of drugs.

Keywords: drug-induced cholestasis, bile acids, hepatocytes, *in vitro* models, bile acid homeostasis, drug transporters, drug-induced liver injury

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1 Introduction

Cholestasis is characterized by the impairment of bile secretion and flow, leading to the pathological hepatic and/or systemic accumulation of bile acids (BAs) and their conjugate bile salts. Cholestasis is classified as either intrahepatic or extrahepatic. Intrahepatic cholestasis is predominantly associated with the bile canaliculi and the intrahepatic bile ducts, while the common hepatic or bile duct and the extrahepatic bile ducts are related to extrahepatic cholestasis (Nguyen et al. 2014). From a clinical point of view cholestasis leads to acute hepatotoxicity, increases in the number of bile ducts and fibrosis progressing to cirrhosis, ultimately triggering liver failure and necessitating liver transplantation (Wagner et al. 2009). The possible causes of cholestasis are divergent, ranging from inherited mutations in canalicular transporter genes to cholestasis caused by external agents (*e.g.,* drugs) (Pauli-Magnus et al. 2006). The latter is of great interest to the pharmaceutical industry, as it is one of the principal causes of drug attrition and post-marketing drug withdrawals (Tonder et al. 2013). Indeed, since 1964 a total of 63 drugs were withdrawn from various markets, due to hepatotoxic adverse reactions. Approximately 30% of the withdrawn drugs were reported to induce cholestatic liver injury (Figure 1) [Figure 1 near here]. Yet, due to the idiosyncratic nature of cholestasis as well as the many known issues regarding *in vitro-in vivo* extrapolation (IVIVE) and interspecies differences in toxic response, the cholestatic potential of drugs remains difficult to predict during the early phases of drug development (de Lima Toccafondo Vieira et al. 2014). Currently, hepatic safety testing is mainly achieved by *in vivo* screening of drug candidates (Tonder et al. 2013). However, a study of the predictive value of human drug toxicity by animal studies showed that only 72% of human toxicities were found to be associated with toxicity in animals (Olson et al. 2000).

Although animal studies are inevitable during pre-clinical and clinical drug development, their poor predictive power and low-throughput makes them less favorable for the assessment of liver toxicity, and in particular drug-induced cholestasis (DIC). Instead, a multitude of experimental and theoretical methodologies need to be explored and data combined in order to predict human cholestatic liver injury. In order to achieve this, a profound understanding of the different underlying mechanisms of DIC is of utmost importance. In the last decade, considerable resources have been invested by both academia and industry to unravel the molecular mechanisms of DIC, not only leading to the development of new predictive tools but also impacting patient care by identifying novel drug targets for the treatment of DIC (Wagner et al. 2009; M. Chen et al. 2014).

The aim of the present review is to give an overview of the principal molecular mechanisms associated with DIC as well as the different liver models that are currently used to predict the cholestatic potential of drugs.

2 Physiological mechanisms of bile flow

Bile is formed *via* an energy-dependent process during which osmotically active substances are transported from blood into the bile canalicular lumen, effectively creating an osmotic gradient that serves as the driving force for passive movement of water and small solutes into biliary networks (Boyer et al. 2013). The latter is facilitated by water channels aquaporin (AQP) 8 and AQP9, which alter water permeability across the basolateral and canalicular membranes in response to increasing concentrations of biliary solutes in the canaliculi (Masyuk et al. 2006).

BAs are the main organic solutes in bile and the primary determinants of BA-dependent flow (BADF) (Figure 2) [Figure 2 near here]. BAs are *de novo* synthesized from cholesterol *via* two major pathways: the 'neutral' pathway, which involves conversion of cholesterol to 7α-hydroxycholesterol *via* 7α-hydroxylase (CYP7A1), and the 'acidic' pathway, which is initiated by formation of 27-hydroxycholesterol (Axelson and Sjövall et al. 1990; Javitt et al. 1994). Both pathways generate primary BAs namely, chenodeoxycholic acid (CDCA) and cholic acid (CA), although the acidic pathway predominantly produces CDCA (Setchell et al. 1988). The final step of BA synthesis involves conjugation of primary BAs with either glycine or taurine, a process which takes place in peroxisomes and is mediated by two enzymes namely, bile acid:CoA synthase (BACS) and bile acid CoA:amino acid N-acyltransferase (BAAT) (Fuchs et al. 2003). Conjugated and unconjugated BAs can further undergo sulfation or glucuronidation inside hepatocytes, after which they are secreted into bile canaliculi *via* the bile salt export pump (BSEP/ABCB11) and the multidrug resistance-associated protein 2 (MRP2/ABCC2), located in the canalicular membrane of the hepatocyte (Pauli-Magnus et al. 2006).

Once secreted, BAs are partly reabsorbed into cholangiocytes, a process which is mediated by the apical sodium-dependent bile salt transporter (ASBT/SLC10A2) and the organic anion transporting polypeptide (OATP/SLCO) (Hagenbuch et al. 2004). Reabsorbed BAs are, subsequently, secreted into the peribiliary plexus by the organic solute transporter (OSTα-β/SLC51A and B), making them available for renewed secretion into bile (Ballatori et al. 2009). The circulation of BAs between hepatocytes and cholangiocytes is known as the cholehepatic shunt pathway. However, most of the primary BAs are transferred to the gallbladder and intestine, where they are dehydroxylated by intestinal bacteria, thereby forming secondary BAs, such as deoxycholic acid (DCA) and lithocholic acid (LCA). Approximately 90% of both conjugated and unconjugated BAs can then be reabsorbed in the distal intestine *via* passive diffusion or *via* ASBT and undergo an enterohepatic circulation, which preserves the BA pool (Boyer et al. 2013). Reuptake of BAs from the sinusoidal blood into hepatocytes is, subsequently, mediated by the basolateral carrier proteins, Na^+-taurocholate co-transporting polypeptide (NTCP/SLC10A1) and, 1B1 and 1B3 isoforms of the OATP family (Trauner et al. 2003).

Other key determinants of hepatic bile flow are bicarbonate (HCO_3^-) and glutathione, each driving up to 50% of the BA-independent flow (BAIF) (Figure 2) (Hardison et al. 1978). Glutathione, both in its thiol-reduced (GSH; approximately 80%) and in its disulfide-oxidized (GSSG) form, is actively transported across the canalicular membrane by MRP2, where it can reach concentrations up to 10 mM, representing a significant driving force for bile secretion (Ballatori et al. 1992). Upon secretion into the biliary lumen, glutathione and its conjugates are rapidly hydrolysed by the enzyme γ-glutamyl transpeptidase (γGT) and dipeptidases to release their amino acid constituents. Consequently, unlike BAs, reabsorption of glutathione only occurs to a limited extent (Dutczak et al. 1994).

Canalicular transport of bicarbonate is mediated by the chloride/bicarbonate (Cl^-/HCO_3^-) exchanger (AE2), which is localized at the apical membrane in human hepatocytes as well as at the luminal surface of cholangiocytes (Boyer et al. 2013). Although secretion of HCO_3^- into the canalicular lumen does not directly drive the osmotic gradient, some reports have demonstrated the importance of HCO_3^- in the maintenance of bile flow. For instance, omission of HCO_3^- decreases BAIF, while enhanced AE2 exchange activity, produces an immediate increase in bile flow (Hardison et al. 1978; Strazzabosco et al. 1996). Moreover, AE2 deficiency is thought to play an important role in primary biliary cirrhosis (PBC). Biliary HCO_3^- is known to be regulated by a plethora of local and neurohormonal factors, including BAs, secretin and glucagon (Concepcion et al. 2013). Indeed, numerous studies have shown that glucagon and specific BAs, such as taurocholic acid (TCA), stimulate bicarbonate secretion and bile flow (Lenzen et al. 1990; Benedetti et al. 1994; Alvaro et al. 1995; Alpini et al. 1999; Alpini et al. 2001).

Primary canalicular bile is further modified by a series of absorptive and secretory ductular processes carried out by cholangiocytes that alkalinize and dilute bile through secretion of fluid, HCO_3^-, Cl^-, whilst reabsorbing glucose, glutamate, conjugated bilirubin and, as mentioned previously, BAs (Strazzabosco et al. 1996; Esteller et al. 2008). These ductular processes generate a bicarbonate-rich watery fluid, which represents 30% and 10% of basal bile flow in humans and rats, respectively (Banales et al. 2006).

3 Perturbation of bile acid homeostasis

BA homeostasis encompasses a complex and dynamic interplay of various hepatobiliary transporters and enzymes. Any given drug that interferes with these intricate mechanisms could, theoretically, alter the composition of the BA pool, resulting in stagnation of BADF, hence leading to hepatocellular damage. Table 1 gives an overview of a number of drugs known to compromise BA homeostasis. The following section discusses different pathophysiological mechanisms involved in drug-induced perturbations of BA homeostasis (Table 1) [Table 1 near here].

3.1 Disturbance of bile acid synthesis and conjugation

BA synthesis is mainly located in the liver and serves as a predominant route for cholesterol elimination. As such, various congenital disorders related to primary BA deficiency are known to result in steatorrhea and growth retardation as well as inadequate gastro-intestinal absorption of fat-soluble vitamins and their *sequelae* (Vaz et al. 2017). Moreover, there is increasing evidence supporting the hypothesis that drugs are capable of disturbing these BA synthesis and conjugation pathways, eventually leading to hepatocellular damage. A recent *in vitro* study of Sharanek *et al.* demonstrated that both intracellular and extracellular total unconjugated BA content increased, when cells were treated with well-known cholestatic drugs, such as cyclosporin A, chlorpromazine or troglitazone (Sharanek et al. 2017). These findings were further corroborated by the finding that, besides inhibition of BA efflux, inhibition of BA amidation underlies the onset of troglitazone-induced cholestasis (Ogimura et al. 2017). In our group, the cholestatic drug bosentan was additionally observed to be able to alter both endogenous and exogenous BAs to a comparable extent *via* inhibition of BA conjugation and synthesis (Oorts et al., unpublished data).

In addition to disturbing BA synthesis, certain drugs may also increase BA synthesis, effectively leading to increased intracellular BA levels. Indeed, Terelius *et al.* have recently reported that non-nucleoside reverse transcriptase inhibitor nevirapine upregulates *CYP7A1*. The latter resulted in an increase in BA synthesis. Interestingly, the increment in BA formation was not accompanied with a compensatory upregulation of genes for BA efflux transporters (Terelius et al. 2016). Although it is evident that cholestatic drugs may cause aberrant states of BA homeostasis, by disturbing both BA synthesis and conjugation, the frequency by which this occurs is rather limited, indicating that other (primary) mechanisms are more likely to contribute to DIC (Figure 3) [Figure 3 hear here].

3.2 Inhibition of bile acid detoxification pathways

BA detoxification depends on complex metabolic activities, and it is reasonable to expect that drugs that interfere with these intricate mechanisms are capable of inducing a cholestatic phenotype. LCA, a rare toxic endobiotic, is formed by bacterial 7-dehydroxylation from the primary BA CDCA or the secondary BA ursodeoxycholic acid (UDCA), and is efficiently detoxified by mono-sulfation at the 3-OH position (Hofmann et al. 2004). The latter is catalysed by enzyme sulfotransferase-2A1 (SULT2A1), also known as hydroxysteroid sulfotransferase (HST) (Alnouti et al. 2009). Being a prominent BA detoxification pathway, it is conceivable that any drug or metabolite that is able to inhibit SULT2A1 in the liver alters the hydrophobic-hydrophilic balance, and thus induces toxic effects. A recent *in vitro* study of Sharanek *et al.* provided the first direct evidence that cholestatic drugs are able to inhibit LCA sulfation, leading to its intracellular accumulation (Sharanek et al. 2017). However, further investigation is required to substantiate these findings. Besides sulfation, detoxification of BAs also occurs *via* cytochrome P450 (CYP) 3A4-mediated hydroxylation, which increases the hydrophilicity of BAs and effectively decreases their toxicity (J. Chen et al. 2014).

Several studies have indicated the importance of CYP3A4 in maintaining a healthy BA homeostasis. For example, Stedman *et al.* showed that upregulation of CYP3A4 played part in an *in vivo* adaptive response to cholestasis, while Bremmelgaard *et al.* observed extensive urinary excretion of 6α-hydroxylated BAs in cholestasis patients (Bremmelgaard et al. 1980; Stedman et al. 2004). In addition to sulfation and hydroxylation, BAs can also be detoxified through glucuronidation, catalysed by UDP-glucuronosyltransferases UGT2B4, UGT2B7 and UGT1A4 (Takikawa et al. 1983; Barbier et al. 2003; Trottier et al. 2006). This step renders BAs more water soluble and facilitates their renal excretion. However, in most cases, glucuronidation can only take place when BAs are already hydroxylated. In this context, UGT2B4 is associated with glucuronidation of 6α-hydroxylated BAs, whereas UGT2B7 mediates glucuronidation of both 3α-hydroxylated BAs and 6α-hydroxylated BAs (Li et al. 2015). Upregulation of UGT2B4, UGT2B7 and UGT1A4 is a well-documented response to cholestasis, effectively protecting the hepatocyte against toxic effects of supra-physiological intracellular BA concentrations (Fröhling et al. 1976). Although it is unlikely that a given drug induces cholestasis solely through inhibition of hydroxylation or glucuronidation enzymes, disturbance of CYP-mediated and UGT-mediated detoxification pathways during cholestasis could lead to more severe hepatic injury.

3.3 Alterations of hepatobiliary transporters

Hepatobiliary transporters play an essential role in maintaining BA homeostasis. They control hepatic exposure to BAs both in normal and cholestatic conditions and are therefore key determinants of intrahepatic and systemic BA levels. Numerous reports have suggested the involvement of drug-mediated alterations of transporter activity in the accumulation of potentially toxic BAs, thereby resulting in the development of DIC.

Indeed, approximately 75% of all drugs reported to cause cholestatic liver injury, either directly or indirectly, modify the function and/or expression of several hepatobiliary transporters (Figure 3). The following section discusses the various hepatocellular transporters capable of transporting BAs and their potential involvement in cholestatic liver injury.

3.3.1 Canalicular efflux transporters

3.3.1.1 BSEP

BAs are predominantly extruded to the canalicular lumen by BSEP, a member of the ATP-binding cassette (ABC) family of transporters. The importance of BSEP in BA homeostasis is illustrated by the fact that progressive intrahepatic familial cholestasis type 2 (PFIC2), a rare genetic disorder characterized by drastic decreases in BA secretion, results from mutations in the *ABCB11* gene (Wagner et al. 2009). Mutations and polymorphisms in BSEP have also been linked to intrahepatic cholestasis of pregnancy (ICP), an acquired form of cholestasis that evokes pruritus and elevated plasma concentrations of both BAs and transaminases (Pauli-Magnus et al. 2010). A multitude of different drugs that cause cholestatic liver injury also inhibit BSEP-mediated biliary secretion of BAs, resulting in excessive intracellular accumulation of BAs (Funk et al. 2001; Byrne et al. 2002; Kostrubsky et al. 2006). For example, bosentan is known to dose-dependently inhibit BSEP, an effect that has been shown to aggravate upon glibenclamide co-administration (Fattinger et al. 2001). Moreover, canalicular BA transport is also competitively inhibited by sulindac, while estradiol-17β-glucuronide ($E_2$17G) *trans*-inhibits Bsep (murine homolog of BSEP)-mediated BA transport and therefore exerts its cholestatic action only after it has been secreted to the canalicular lumen *via* Mrp2 (Bolder et al. 1999; Stieger et al. 2000). Taken together, inhibition of BSEP can be considered as a primary mechanism leading to DIC.

The European Medicines Agency (EMA) has therefore specified in its Guideline on the Investigation of Drug Interactions that drug-mediated inhibition of BSEP should be "preferably investigated". Additionally, EMA states: "If *in vitro* studies indicate BSEP inhibition adequate biochemical monitoring, including serum bile salts, is recommended during drug development" (EMA 2012). Furthermore, studies have shown a strong correlation between the potency of a drug to inhibit BSEP *in vitro* and its propensity to cause cholestatic liver injury in humans (Morgan et al. 2010; Dawson et al. 2012).

3.3.1.2 MRP2

Secretion of various conjugated organic anions into bile, including bilirubin, glutathione-S-conjugate and glucuronides is facilitated by MRP2, a member of the ATP binding cassette family of transporters (Nies et al. 2007). Although BSEP serves as the major route of canalicular BA secretion, certain BAs, such as sulfated taurolithocholate (TLCA) or glycolithocholate (GLCA), are preferably extruded *via* MRP2, suggesting that MRP2 could play a role in the pathogenesis of cholestasis (Akita et al. 2001). Indeed, patients with ICP display mutations in the *MRP2* gene, while cholestasis in rats has been related to impaired Mrp2 (murine homolog of MRP2) -mediated transport as well as down-regulation and altered localization of Mrp2 (Trauner et al. 1997; Bolder et al. 1999; Sookoian et al. 2008). Several cholestatic agents, such as estradiol, lipopolysaccharide, LCA and phalloidin, are known to alter Mrp2 activity by decreasing its insertion into the plasma membrane, while fasiglifam (TAK-875), a selective G-protein-coupled receptor 40 agonist, has been shown to potently inhibit MRP2/Mrp2, leading to hyperbilirubinemia and cholestatic liver injury (Kubitz et al. 1999; Beuers et al. 2001; Mottino et al. 2002a; Li et al. 2015; Rost et al. 1999).

In addition, Morgan *et al.* showed that the well-known cholestatic compounds tolcapone, troglitazone, rifampicin and cyclosporin A are able to inhibit both BSEP-mediated ^{3}H-TCA and MRP2-mediated ^{3}H-$E_2$17βG transport, suggesting that inhibition of MRP2 contributes to the development of DIC (Morgan et al. 2013).

3.3.1.3 MDR3

The multidrug resistance protein 3 (MDR3/ABCB4) is a canalicular phospholipid flippase that mediates translocation of phosphatidylcholine (PC) into the outer leaflet of the canalicular membrane (Nies et al. 1996). PC forms mixed micelles with BAs and cholesterol, thereby protecting cholangiocytes from damage, due to detergent effects of BAs present in bile (Fickert et al. 2004). Consequently, both BSEP and MDR3 need to act concomitantly for the formation of these micelles (Groen et al. 2011). As such, alterations in MDR3 activity might contribute to the manifestation of cholestasis and might impact biliary toxicity of BAs. A recent study of Aleo *et al.* has evaluated the role of MDR3 inhibition in the onset of hepatic liver injury. MDR3 inhibition was investigated across 125 compounds, of which more than 30% were able to both inhibit MDR3 and BSEP (Aleo et al. 2017). Zhao *et al.* have recently evaluated the potential of 8-methoxypsoralen (8-MOP), a photochemotherapeutic agent used to treat psoriasis and vitiligo, to induce cholestatic liver injury *via* secondary alterations of MDR3. Their results suggested 8-MOP administration to down-regulate both mRNA and protein expression of MDR3, resulting in disruption of MDR3-mediated PC efflux, and subsequent disturbance of BA homeostasis (Zhao et al. 2017). Furthermore, the triazole antifungal agent itraconazole is also known to decrease efflux of PC *via* MDR3 in MDR3-overexpressing cells. However, BSEP-mediated TCA transport remained unaltered, suggesting that inhibition of canalicular PC secretion mediated by MDR3 can be an additional risk factor for DIC even if BA secretion remains unaffected (Yoshikado et al. 2011).

3.3.1.4 FIC1 (ATP8B1)

The familial intrahepatic cholestasis 1 (FIC1) transporter represents a P-type ATPase that has been shown to facilitate transport of aminophospholipids from the outer to the inner leaflet of the canalicular membrane, thereby maintaining membrane asymmetry (Paulusma et al. 2008). Loss of lipid asymmetry, due to impaired FIC1 function, may result in dysfunction of BSEP (Davit-Spraul et al. 2010). Consequently, FIC1 deficiencies, either inherited or acquired, may cause intracellular accumulation of BAs. Multiple studies have indicated that mutations in the genes encoding FIC1 trigger cholestatic liver diseases, of which PFIC and benign recurrent intrahepatic cholestasis (BRIC) are two well-known examples (Bull et al. 1998; Klomp et al. 2004). Moreover, similar to BSEP and MRP2, mutations in the *FIC1* gene have also been associated with ICP (Müllenbach et al. 2005; Painter et al. 2005). No evidence of drug-induced alterations of this transporter has been found to date. However, due to its importance in maintaining BSEP activity, inhibition of FIC1 by exogenous substances might predispose hepatocytes to cholestatic liver injury and thus warrants further investigation.

3.3.1.5 BCRP

The breast cancer resistance protein (BCRP/ABCG2) is part of the ABC transporter family (Doyle et al. 1998). BCRP is implicated in transport of various drugs, endogenous substances and environmental agents (Polgar et al. 2008). Whether BCRP plays a significant role in BA homeostasis has not yet been cleared out. For instance, studies in Bcrp (murine homolog for BCRP) - expressing bacteria and transfected K562 cells demonstrated that primary BAs as well as TLCA and TLCA sulfate could be transported by Bcrp, while other *in vitro* studies failed to find such evidence (Imai et al. 2003; Suzuki et al. 2003; Janvilisri et al. 2005).

A more recent *in vivo* study of Mennone *et al.*, which investigated the role of Bcrp in cholestasis resulting from bile duct ligation (BDL) in mice, concluded that BDL does not cause more liver injury in Bcrp null mice, pleading against a hepatoprotective role of Bcrp during cholestasis (Mennone et al. 2010). Furthermore, a study in pregnant Bcrp knockout mice indicated that Bcrp may play a key role in BA transport in the placenta, but not in the liver (Blazquez et al. 2012). Altogether, data suggest that Bcrp is able to transport BAs, nonetheless liver Bcrp does not seem to be implicated in the adaptive response of cholestasis. Although the exact role of human BCRP during cholestasis remains to be elucidated, it does seem rather unlikely that secondary alterations (*i.e.*, repression of transcription, posttranslational modifications or direct inhibition) of BCRP alone will lead to cholestatic liver injury. However, due its broad substrate specificity, dysfunction of BCRP might contribute to the onset of DIC by altering canalicular secretion of known cholestatic drugs.

3.3.1.6 MDR1 (P-gp)

The multidrug resistance protein 1 (MDR1/ABCB1), an adenosine triphosphate (ATP)-dependent transporter, protects the body from multiple xenobiotics and plays an important role in the development of multidrug resistance (Wolking et al. 2015). MDR1 transports several biliary constituents (*e.g.*, cholesterol and phospholipids), but its exact contribution to hepatic BA homeostasis, and its capability to transport BAs, remains unclear (Cuperus et al. 2014). MDR1 does seem to act as a canalicular overflow transporter under cholestatic conditions, as liver samples of patients with obstructive cholestasis and PBC showed an increase in MDR1 expression (Schrenk et al. 1993; Zollner et al. 2003). Furthermore, mouse studies using tetrahydroxylated BAs did indeed show Mdr1 (murine homolog of MDR1)-mediated transport, albeit with much lower affinity as compared to Mrp2, the main polyhydroxylated BA transporter (Megaraj et al. 2010).

In toto, these data suggest a compensatory role for MDR1 during cholestasis. Noteworthy, involvement of MDR1/Mdr1 in BA transport is probably more important in mice than in man, as only mice have been observed to produce tetrahydroxylated BAs (Perwaiz et al. 2003). Similar to BCRP, drug-induced deregulation of MDR1 alone will probably not lead to cholestasis in humans. However, due to its role in exporting toxins under cholestatic conditions, drug-drug competition for MDR1 binding sites might be involved in DIC pathogenesis. For instance, co-administration of cyclosporin A and verapamil, two known MDR1 inhibitors, could theoretically lead to increased intracellular concentrations of cyclosporin A and thus may trigger cholestatic liver injury (Wessler et al. 2013).

3.3.2 Basolateral uptake transporters

3.3.2.1 OATPs

OATPs are membrane uptake transporters that regulate cellular uptake of various organic compounds, such as conjugated and unconjugated BAs as well as bilirubin (Slijepcevic et al. 2017). In humans, two liver specific OATP isoforms, namely OATP1B1 and OATP1B3, are known to transport BAs (Geier et al. 2007). The exact role of OATPs in the pathogenesis of cholestasis remains elusive. However, it has been speculated that enhanced OATP activity might increase intracellular hepatic concentrations of certain drugs and BAs, thus predisposing hepatocytes to liver injury. This hypothesis is corroborated by a study that demonstrated a decrease in plasma exposure of atorvastatin, a known substrate of OATP1B1/Oatp2, in diabetic rats, due to increased expression and activity of both hepatic Cyp3a (murine homolog of CYP3A) and Oatp2 (Shu et al. 2016). Moreover, our group has previously tested over 2000 compounds as potential modulators of the OATP1B subfamily (De Bruyn et al. 2013).

Next to inhibitors, several activators of both OATP1B1 and OATP1B3 were identified that could theoretically increase intracellular BA levels, eventually leading to hepatotoxicity.

3.3.2.2 NTCP

NTCP is a member of the solute carrier 10 family and facilitates transport from the space of Disse into hepatocytes (Boyer et al. 1994). Its substrate specificity is virtually limited to conjugated BAs and certain steroid sulfates. Consequently, NTCP mediates a large portion of total BA uptake. Hence, intact NTCP function is considered critical for the maintenance of enterohepatic circulation and hepatocyte BA homeostasis (Hagenbuch et al. 2004; Ho et al. 2004). Both sinusoidal NTCP and canalicular BSEP need to work in concert to ensure vectorial transport of circulating BAs into bile canaliculi. Accordingly, inhibition in either process affects total BA clearance. A study of Mita *et al.* suggested that a wide variety of cholestatic drugs, such as rifampicin, gliblenclamide and cyclosporin A, disturb BA transport. The disturbance can take place both in hepatocytes and at canalicular membranes, resulting in elevated serum BA concentrations and causing prehepatic cholestasis (Mita et al. 2006).

3.3.3 Basolateral efflux transporters

3.3.3.1 MRP3 and MRP4

Pathological accumulation of biliary constituents in cholestasis is postulated to be counteracted by induction of alternative basolateral export pumps, namely MRP3 (ABCC3) and MRP4 (ABCC4), resulting in subsequent renal elimination of BAs (Soroka et al. 2001; Denk et al. 2004). Indeed, studies in cholate fed and BDL rodents showed induction of Mrp3 (murine homolog of MRP3) and Mrp4 (murine homolog of MRP4) expression.

Similarly, human patients with obstructive cholestasis and PFIC are also associated with an increase in hepatic MRP4 expression (Wagner et al. 2003; Keitel et al. 2005; Teng et al. 2007). Consequently, loss of MRP3 and/or MRP4 function, due to drugs, may result in drastic increases in intracellular BA concentrations. The loss in transporter function of MRP3 and/or MRP4 may serve, in addition to inhibition of BSEP, as a confounding risk factor for the onset of cholestatic liver injury (Köck et al. 2014; Welch et al. 2015). This is exemplified by troglitazone sulfate, a major metabolite of troglitazone and a known BSEP inhibitor, which has been shown to inhibit MRP4-mediated dehydroepiandrosterone (DHEAS) transport, potentially making hepatocytes more susceptible to toxicity (Yang et al. 2013). These results were supported by a study of Morgan *et al.*, who investigated whether additional information on the propensity of a BSEP inhibitor to inhibit MRP3 and/or MRP4 would improve the correlation with (cholestatic) liver injury. The authors concluded that it is recommended to assess MRP inhibition, next to BSEP inhibition and that several well-known cholestatic agents, such as rifampicin, troglitazone and bosentan, affect both BSEP and MRP3/MRP4, leading to an increased risk of cholestatic liver injury (Morgan et al. 2013).

3.3.3.2 OSTα-OSTβ

OSTα-OSTβ is a heterodimeric efflux transporter that functions to extrude BAs, other organic solutes and steroids, from the intracellular to the extracellular compartment (Ballatori et al. 2009). OSTα-OSTβ is known to transport estrone 3-sulfate, DHEAS 3-sulfate, digoxin and prostaglandin E2 as well as a variety of different BAs (Wang et al. 2001; Ballatori et al. 2009). Liver specimens of patients with PBC, BDL rats and mice as well as farnesoid X receptor *(Fxr)* knockout mice showed increased expression of OSTα-OSTβ, indicating that OSTα-OSTβ plays a protective role during cholestasis (Boyer et al. 2006).

Furthermore, given its central role in BA homeostasis, OSTα-OSTβ is believed to be associated with certain diseases that are related to BA malabsorption, cholestasis and cholelithiasis (Ballatori et al. 2009). A recent report identified a single nucleotide mutation in the gene encoding OSTβ, resulting in significantly reduced TCA uptake activity and reduced expression of its partner protein OSTα. Clinical manifestations of the mutation included chronic diarrhea with features of cholestatic liver injury (Sultan et al. 2017). Currently, involvement of OSTα-OSTβ in DIC remains undocumented. However, being an important compensatory pathway during cholestatic disease states, one could speculate that any drug, which might affect OSTα-OSTβ function, could theoretically predispose hepatocytes to liver injury.

3.4 Disturbance of nuclear hormone receptor activity

The aforementioned detoxification and secretion pathways are tightly regulated by at least four nuclear hormone receptors (NHRs), namely FXR, pregnane X receptor (PXR), vitamin D receptor (VDR) and constitutive androstane receptor (CAR) (Zollner et al. 2010; Rodrigues et al. 2014). NHRs exert their transcriptional activity by regulating the recruitment of coregulators and chromatin-modifying machineries, effectively orchestrating the expression of specific target genes (Mangelsdorf et al. 1995). Biliary constituents, lipid products, hormones and xenobiotics activate NHRs and thus co-ordinately regulate induction and inhibition of genes that encode detoxification and secretion proteins (Wagner et al. 2005; Zollner et al. 2006). PXR and CAR are classical drug receptors. These nuclear receptors can be activated *via* ligand-binding of xenobiotics, such as rifampicin, statins, phenobarbital or dexamethasone (Urquhart et al. 2007). Hydrophobic BAs are additional ligands of PXR and, CAR can be activated by bilirubin, thereby indicating that there is an overlap in regulation between both drug and BA metabolism.

FXR is strongly activated by BAs, such as CDCA, CA, DCA and LCA and, VDR is activated by LCA in addition to its natural ligand 1α,25-dihydroxyvitamin D_3 (Zollner et al. 2010). The importance of NHRs in the defence against BAs is underscored by the observations that LCA-fed Pxr-deficient mice showed increased liver injury, due to absence of Cyp3a11 induction, while Car transgenic mice displayed increased Sult2a1 expression resulting in resistance against LCA-induced toxicity (Staudinger et al. 2001; Xie et al. 2001; Saini et al. 2004). NHRs can also prevent BA accumulation within hepatocytes by controlling the expression of different secretion proteins. For instance, FXR regulates expression of BSEP/Bsep in response to BAs, whereas Mrp2 induction is lacking in PXR-deficient mice (Zollner et al. 2006). Moreover, expression of the basolateral efflux pumps MRP3/Mrp3 and MRP4/Mrp4 is mediated by PXR/Pxr and CAR/Car, respectively (Cherrington et al. 2002; Wagner et al. 2005; Teng et al. 2007). Due to their central role in maintaining BA homeostasis, drugs that disturb NHR activity might aggravate toxic effects of accumulating BAs. Indeed, both lopinavir and troglitazone have been reported to significantly reduce FXR activity, resulting in repressed BSEP transcription, thus causing excessive hepatic accumulation of BAs (Garzel et al. 2014). Results of a recent study of Guo and co-workers also suggested that cholestatic liver injury induced by pyrazinamide, a first-line drug for the treatment of tuberculosis, was related to FXR inhibition (Guo et al. 2016). Moreover, a study of Lim *et al.* indicated that PXR activation is disrupted by treatment with ketoconazole. This disturbance may lead to down-regulation of CYP3A4 gene expression and potentially affects BA homeostasis (Lim et al. 2009).

4 Disturbances of hepatocellular morphology and polarity

Establishment and maintenance of adequate hepatocyte polarity and bile canalicular networks require organized interplay of tight junctional, cytoskeletal and intracellular trafficking components (Fu et al. 2010). Several inherited cholestatic liver diseases are known to originate from disturbances in either of these processes, emphasizing their importance in bile secretory function (Gissen et al. 2015). Moreover, recent evidence also suggests that approximately 20% of all reported cholestatic drugs interact with one of these trafficking pathways (Figure 3). Different mechanisms by which drugs are known to disturb hepatocellular morphology and polarity are discussed here.

4.1 Altered membrane fluidity

The term fluidity refers to the motional freedom of membrane components and is affected by a number of factors (Schachter et al. 1984). A key determinant of membrane fluidity is the exact composition of the lipid bilayer, which primarily consists of phospholipids and cholesterol (Ray et al. 1969). The latter acts as a bidirectional regulator, effectively stabilizing the membrane at high and low temperatures. Phospholipids, on the other hand, regulate membrane fluidity through alterations in both length and degree of saturation of their acyl side chains (Smith et al. 1987). Changes in membrane fluidity are known to alter activity of certain liver membrane enzymes. For instance, activity of Na^+-K^+-ATPase and glucagon-stimulated adenylate cyclase correlates with fluidity of their respective lipid environment. Another example is impaired Bsep activity as a result of reduced canalicular membranes' cholesterol content (Whetton et al. 1983; Paulusma et al. 2009). Moreover, it has been speculated that low cortisol blood levels, in patients with PBC, may change fluidity of the canalicular membrane, resulting in impaired biliary secretion and aggravation of intrahepatic cholestasis (Reshetnyak et al. 2006).

Several cholestatic drugs are known to alter membrane fluidity. Administration of ethinylestradiol and chlorpromazine to rats decreases bile flow through modification of the lipid composition of the basolateral membrane, while cyclosporin A reduces bile secretion by increasing canalicular membrane fluidity (Davis et al. 1978; Keeffe et al. 1980; Schachter et al 1984; Yasumiba et al. 2001). Furthermore, phalloidin has been shown to uncouple and decrease secretion of cholesterol and phospholipids, causing a rearrangement of fatty acyl chain species among the phospholipids present in the canalicular membrane. Consequently, canalicular membrane fluidity can be effectively altered (Hyogo et al. 2000).

4.2 Changes in bile canalicular dynamics

Unidirectional flow of bile towards bile ducts is facilitated by spontaneous rhythmic contractions of the biliary lumen. These contractions, also termed bile canalicular dynamics, are controlled by a complex network of actin and myosin microfilaments as well as other contractile proteins (Oshio et al. 1981). Myosin ATPase activity, which catalyses recurrent interactions of actin with myosin *via* hydrolysis of ATP. The latter is an important step in the development of acto-myosin-based contractile forces (Burbank et al. 2016). These contractions are preceded by activation of the RhoA/Rho-kinase (ROCK) pathway, which results in inhibition of myosin light chain phosphatase (MLCP) and subsequent enhanced phosphorylation of myosin light chains (MLCs) (Figure 4) [Figure 4 near here] (Mills et al. 1998). Contractile activity is equally stimulated through phosphorylation of MLCs by Ca^{2+}/calmodulin (CaM)-dependent myosin light-chain kinase (MLCK) (Isotani et al. 2004). RhoA/ROCK is known to regulate intrahepatic vascular tone in human cirrhotic liver and in BDL rats, whilst also playing a crucial role in the assembly of tight junctions (Zhou et al. 2006; González-Mariscal et al. 2008).

Moreover, a recent study of Sharanek and co-workers demonstrated the direct contribution of the ROCK/MLCK pathway in developing a bile canalicular disorder. Their results showed that cholestatic agents, chlorpromazine, cyclosporin A, alpha-naphthyl isothiocyanate (ANIT) and bosentan were able to cause constriction or dilatation of the canalicular lumen. Cholestatic drugs can undergo an interaction with the ROCK/MLCK pathway and its associated enzymes, resulting in loss of bile canalicular contractions and impaired secretion of BAs (Sharanek, Burban, Burbank, Rémy Le Guevel, et al. 2016). These findings were reinforced in a follow-up study that assessed the propensity of 12 cholestatic and 6 non-cholestatic compounds to alter bile canalicular dynamics *via* disruption of the ROCK/MLCK pathway. All tested cholestatic drugs were able to either constrict or dilate bile canaliculi. None of the non-cholestatic compounds caused bile canalicular deformations, further validating the role of the ROCK/MLCK signalling pathway in association with bile canalicular dynamics in drug-induced intrahepatic cholestasis (Burbank et al. 2016).

4.3 Increased paracellular permeability

Tight junctions form a physical barrier between the bile canalicular lumen and the remainder of the intercellular space. They are comprised of various proteins, such as claudins, occludin and junction adhesion molecules (JAMs), which are anchored to actin-based cytoskeleton (Chen et al. 2009). The cytoplasmic tails of occludin and claudins interact with several peripheral membrane proteins, such as zonula occludens (ZO)-1, -2, -3, multi-PDZ domain protein-1 (MUPP1) and membrane-associated guanylate kinase with inverted orientation (MAGI)-1, -2, -3. These membrane proteins are in turn linked to the underlying cytoskeleton to constitute functional protein complexes (Takano et al. 2014). Altered integrity of hepatocyte tight junctions has been associated with inherited human liver diseases.

For instance, mutations in tight junction protein 2 (TJP2) and claudin-1 (CLDN1), which encode integral tight junctional proteins, cause familial hypercholanemia (FHC), neonatal ichtyosis-sclerosing cholangitis syndrome (NISCH) and PFIC-4 (Carlton et al. 2003; Hadj-Rabia et al. 2004; Sambrotta et al. 2014). Several studies in experimental models of cholestasis provide additional evidence that disturbances in junctional complexes can influence bile secretion. For example, BDL rats show significant alterations in the structure and function of tight junctions. Ethinylestradiol administration to male and female rats produces cholestasis, while increasing the canalicular permeability to sucrose (Metz et al. 1977; Elias et al. 1983). Furthermore, a plethora of studies have indicated that ANIT induces cholestasis in rodents through disruption of tight junctional complexes, leading to dilation of bile ducts and loss of bile components into liver interstitial tissue (Connolly et al. 1988; Yang et al. 2017). Rifampicin has also been shown to both alter and internalize hepatocyte tight junctions in mice, further emphasizing the role of leaky tight junctions in the onset of DIC (Chen et al. 2009).

4.4 Disturbed localization of hepatic transporters

Intracellular trafficking of hepatic transport systems is a tightly regulated process that involves delivery of *de novo* synthesized carriers to either the basolateral or canalicular membrane. Depending on the physiological demand to secrete bile or detoxify endobiotics and xenobiotics, hepatocellular transporters undergo recycling from and to the endosomal compartment, ensuring that a sufficient amount of transporters is present at the membrane (Roma et al. 2008). This dynamic interplay between exocytic insertion and endocytic retrieval is modulated by a number of signalling pathways. For instance, activation of protein kinase C (PKC) isoforms aPKCζ and nPKCδ by cyclic adenosine monophosphate (cAMP) induces insertion of NTCP and MRP2 into the basolateral and canalicular membrane, respectively.

Activation of cPKC by phorbol myristate acetate (PMA) leads to translocation of NTCP from the basolateral membrane to intracellular structures (Anwer et al. 2014). Conversely, exocytic insertion of Bsep is enhanced by means of pericanalicular Ca^{2+} signalling mediated *via* inositol 1,4,5 trisphosphate receptor type II (InsP3R2) (Kruglov et al. 2011). Hence, it is evident that vesicle-mediated internalization/insertion of hepatic transporters plays a considerable role in bile formation. But, besides their role in biliary physiology, accumulating evidence suggests that also an altered endocytic retrieval of hepatocellular transporters to pericanalicular or intracellular domains contributes to cholestasis. Indeed, Kojima and co-workers found that in patients suffering from PBC, localization of MRP2 partially shifts from the canalicular membrane to cytoplasmic structures, while Chai *et al.* reported significant MRP2 internalization and subsequent degradation during human obstructive cholestasis (Kojima et al. 2003; Chai et al. 2015). Endocytic retrieval of both Mrp2 and Bsep has also been reported to occur in experimental models of both obstructive and hepatocellular cholestasis. For example, rats undergoing 48 hours of BDL showed decreased immunofluorescent detection of Mrp2, accompanied by intracellular colocalization of Bsep in pericanalicular vesicular structures, while administration of cholestatic compounds, such as phalloidin, $E_2$17G and cyclosporin A, to rats also induces endocytic internalization of Mrp2 and Bsep from the canalicular membrane (Paulusma et al. 2000; Mottino et al. 2002b; Rost et al. 1999). Redistribution of canalicular transporters induced by certain drugs has also been shown to occur in acute intrahepatic cholestasis (AIC). For instance, an immunohistochemical study, performed on liver biopsies from patients treated with tiopronin, chlorpromazine or diclofenac, showed a remarkable redistribution of MRP2 from canalicular membrane into intracellular structures and basolateral membranes (Watanabe et al. 2007).

The same phenomenon has also been reported by Milkiewicz *et al.*, where liver biopsies of two patients with antidepressant-induced cholestasis showed noticeable relocalization of canalicular MRP2 into basolateral membrane (Milkiewicz et al. 2003).

5 Liver models to predict human drug-induced cholestasis

During the last decade, a considerable amount of research by both academia and industry has been devoted to developing new tools for predictive assessment of human DIC. Numerous liver models, which will be discussed in the following section, have been successfully developed and are now being used to predict and evaluate DIC as well as to elucidate its underlying mechanisms (Table 2) [Table 2 near here].

5.1 Membrane-based in vitro models

Although the translation of an *in vitro* inhibitory capacity of a small molecule on a specific transporter to human cholestasis most likely dependents on various factors, early high-throughput screening of drug candidates using membrane-based *in vitro* models has been proven to be useful to prioritize compounds with the lowest likelihood to induce cholestatic liver injury (Morgan et al. 2010; Dawson et al. 2012). Two of the most commonly used membrane-based *in vitro* systems to investigate drug-transporter interactions are isolated canalicular liver plasma membrane (cLPM) vesicles and membrane vesicles originating from BSEP-overexpressing cells.

5.1.1 Isolated canalicular liver plasma membrane vesicles

Preparation of cLPM vesicles from liver tissue was first described by Meier *et al.*, who was able to separate basolateral from canalicular membranes using a discontinuous sucrose gradient (Meier et al. 1984).

Since then, cLPM vesicles have been widely applied in drug-transporter interaction studies. For example, Funk *et al.* used cLPM vesicles isolated from wild type and Mrp2-deficient rats to show that both troglitazone and troglitazone sulfate inhibit Bsep in a *cis*-dependent manner (Funk et al. 2001). Furthermore, using the same *in vitro* system, Bode *et al.* found that antibiotic fusidate inhibits transport of Mrp2 substrates 17 beta-glucuronosyl estradiol and leukotriene C4 and Bsep substrate cholyltaurine (Bode et al. 2002). Despite their routine application in earlier transporter studies, cLPM have now been largely replaced. Membrane vesicles originating from BSEP-overexpressing cells circumvent the major disadvantages of cLPM, including complexities involved in their preparation and absence of BSEP specificity (Kis et al. 2012). Nevertheless, BSEP-overexpressing cells are limited to only investigate BSEP, while cLPM still offer the important advantage that any relevant influence of other canalicular transporters is taken into account.

5.1.2 Membrane vesicles originating from BSEP-overexpressing cells

Membrane vesicles are predominantly prepared from baculovirus-infected insect cells (*Spodoptera frugiperda;* Sf9) or mammalian cell lines (*e.g.*, Chinese hamster ovary (CHO) cells), transfected with BSEP cDNA (Kis et al. 2012; Noe et al. 2001). BSEP-transfected insect cells are more convenient to produce large amounts of transporter protein. It should be kept into account that the lipid composition of insect membrane can differ significantly from mammalian membranes (Gimpl et al. 1995). The latter considerably influences both ATPase and transport activity of BSEP. Kis *et al.* showed that insect membrane vesicles loaded with 1 mM cholesterol have significantly increased V_{max} values. However, K_m values remained unaltered, indicating that differences in cholesterol membrane composition do not seem to change the affinity for the transporter (Kis et al. 2009).

Plasma membranes containing overexpressed human BSEP, have been used extensively to screen for drugs' inhibitory capacity. For instance, both Morgan *et al.* and Dawson *et al.* used membrane vesicles, originating from BSEP-overexpressing cells, to study the potential correlation between the propensity of a compound to inhibit BSEP and human cholestatic liver injury (Morgan et al. 2010; Dawson et al. 2012). Interference with BSEP was quantified using the membrane vesicle transport (VT) assay, which measures the intravesicular accumulation of a reporter compound (*e.g.*, TCA) by utilizing inside-out oriented vesicles. Both studies concluded that inhibition of BSEP correlates with a molecule's ability to cause cholestatic liver injury. Early high-throughput screening of drug candidates for *in vitro* BSEP inhibition could, as such, be useful in categorizing compounds based on their likelihood to induce BSEP-mediated liver injury.

5.2 Cell-based in vitro models

Unlike membrane-based *in vitro* models, cell-based systems are able to recapitulate most of the liver functions and mechanisms that play an important role in drug-mediated hepatotoxicity. Both 2D and 3D cellular models are used extensively in drug development processes, since they offer a simple, fast and cost-effective alternative to large-scale animal testing.

5.2.1 2D models

5.2.1.1 Sandwich-cultured hepatocytes (SCH)

One of the most valuable cell-based *in vitro* tools for the assessment of DIC is the SCH model. SCH consist of primary animal hepatocytes or human hepatocytes cultured between two layers of an extracellular matrix (ECM). Dunn *et al.* were the first to provide direct evidence that hepatocytes cultured in sandwich configuration preserve both their phenotype and liver specific functions for extended periods of time (Dunn et al. 1989).

Most importantly, SCH have relevant expression levels of basolateral and canalicular transporters as well as enzymes involved in BA homeostasis. Additionally, SCH develop large canalicular tubular structures, making them specifically suited for the assessment of DIC (De Bruyn et al. 2013). The potential BSEP inhibitory effect of different drug candidates is typically determined by measuring the effect on BSEP-mediated efflux of TCA (Kostrubsky et al. 2003; Ansede et al. 2010). TCA is secreted into the bile canaliculi, thus a decrease of its biliary efflux, in presence of a test compound indicates that the compound is likely an inhibitor. Alterations in biliary efflux can be evaluated using the B-Clear® method. With this method, the biliary excretion index (BEI) is determined through changes in Ca^{2+}/Mg^{2+} levels in the incubation buffer, thereby inducing opening (absence of Ca^{2+}/Mg^{2+}) of bile compartments. Subsequently, the biliary efflux ratio can be determined using the following equation:

$$\text{BEI (\%)} = \frac{\text{Accumulation}_{\text{standard buffer}} - \text{Accumulation}_{Ca^{2+}/Mg^{2+}\text{ - free buffer}}}{\text{Accumalation}_{\text{standard buffer}}} \text{ x } 100 \qquad (1)$$

By comparing the BEI of a reporter substrate in both absence and presence of a test compound, the potential inhibitory effect of the test compound can be determined. Although determination of the BEI is a well-known and widely used method for the evaluation of drug-transporter interactions, it possesses several drawbacks, which complicate its use. The method has a number of steps, including extraction of culture homogenate and quantification of the probe substrate, making it rather labor-intensive and requiring specifically trained staff (Miszczuk et al. 2015). An alternative method has been proved successful for measuring BSEP-mediated transport processes. This method is based on changes in fluorescence intensity in bile canaliculi, as a result of the presence or absence of a transporter inhibitor. The fluorescence intensity is measured in SCH *via* quantitative time-lapse imaging (QTLI) analysis.

The imaging technique combines automated image analysis with real-time fluorescence microscopy, enabling rapid processing of large datasets (Muzzey et al. 2009; De Bruyn et al. 2014). In addition to assessment of biliary efflux, SCH also allow evaluation of hepatic uptake and basolateral efflux, rendering them particularly useful for determining alterations in BA disposition. Using SCH, Kemp *et al.* observed that while troglitazone and bosentan both decreased the BEI of TCA, intracellular accumulation of TCA remained unaltered (Kemp et al. 2005). The consistent intracellular TCA concentration, even though BEI was decreased, could be attributed to changes in NTCP-mediated uptake. Collectively, these results indicate that drugs, known to inhibit canalicular efflux, may simultaneously inhibit hepatic uptake, thereby dissipating elevations of intracellular BA concentrations (Swift et al. 2010). An additional SCH-based *in vitro* assay is developed in our group to flag compounds with a cholestatic liability based on their potential in modulating BA disposition. The assay relies on determination of drug-induced cholestasis index (DICI) values, which reflect the ratio in urea production of SCH co-exposed to a compound and BAs and SCH solely exposed to a compound. The assay was able to adequately distinguish cholestatic compound from hepatotoxic non-cholestatic and non-hepatotoxic compounds. This SCH-based *in vitro* assay, thus, offers a unique tool to reliably detect cholestatic drug candidates (Chatterjee et al. 2014; Oorts et al. 2016; Deferm et al. 2019). In conclusion, the possibility to directly determine alterations in intracellular concentrations of BAs is one of the greatest assets of the SCH model, as it may provide useful mechanistic information on compounds that cause cholestasis (Chatterjee et al. 2014). However, SCH suffer from a time-dependent downregulation of hepatic transporters and enzymes, making them less suited for the assessment of long-term toxicity (De Bruyn et al. 2013).

5.2.1.2 Immortalized cell lines

5.2.1.2.1 HepaRG

Another commonly used *in vitro* tool to study DIC is the hepatic hepatoma cell line HepaRG. HepaRG cultures are composed of two cell population, one of which expresses features of mature hepatocytes, including bile canaliculi, while a second cell population resembles cholangiocytes. HepaRG cells stably express both phase I and II enzymes as well as hepatic transporters and nuclear receptors for several weeks in culture (Kanebratt et al. 2008). However, both human BA uptake and efflux transporters display lower mRNA expression levels compared to SCH, resulting in a lower prediction accuracy for cholestatic DILI risk. This has recently been shown in a study where toxicity responses of HepaRG cells were compared to those of sandwich-cultured human hepatocytes (SCHH) (Susukida et al. 2016). Canalicular BA efflux, BA-dependent toxicity as well as mRNA expression levels of relevant BA transporters were first determined in each cell type. Next, *in vitro* toxicity data was gathered for a wide selection of different drugs. The biliary efflux ratio of [^{3}H]taurocholic acid, which was assessed by the B-Clear® method, did not significantly decrease when HepaRG cells were exposed to 10 µM cyclosporin A. Furthermore, HepaRG cells showed lower mRNA expression levels of the tested BA transporters as compared to SCHH. Despite the aforementioned discrepancies between HepaRG cells and SCHH, BA-dependent drug toxicities towards each cell type did show a strong correlation between HepaRG cells and SCHH. Hence, HepaRG cells might serve as an alternative cell-based assay system for drug screening.

5.2.1.2.2 HepG2

The human liver cancer cell line HepG2 is another commonly used *in vitro* system for the assessment of DIC. In contrast to HepaRG cells, the HepG2 line does not natively express NTCP and must be transfected in order to express the protein (Woolbright et al. 2015).

In several studies, NTCP-transfected HepG2 cells were used to evaluate the effect of BAs (Denk et al. 2012). In general, these cells recapitulate responses seen in rat hepatocytes, but not in human hepatocytes, making them not suited as a surrogate for PHHs. Also, similar to the HepaRG cell line, HepG2 cells originate from a single donor, thereby limiting their predictive value for the human population and its associated variability.

5.2.1.3 Transfected cell lines

Human embryonic kidney 293 (HEK293) and CHO cells are being used extensively by both academia and industry. These cells are commonly transfected with specific uptake transporter proteins (*e.g.*, NTCP and OATP), and are therefore utilized to study drug-transporter interactions *in vitro* (Molina et al. 2008; Chen et al. 2016). As discussed earlier, certain cholestatic drugs may modulate their uptake and subsequent intracellular exposure, effectively altering their cholestatic liability. Transfected HEK293 and CHO cells are, at their very nature, particularly well-suited to identify these specific molecules. Other cell lines include polarized pig kidney proximal tubule (LLC-PK_1) and Madin-Darby canine kidney type II (MDCKII) cells, which can be co-transfected with NTCP/Ntcp, BSEP/Bsep and/or MDR3/Mdr3 (Mita et al. 2005). These multiple-expressing systems can be used to study changes in basal-to-apical transport of BAs under cholestatic conditions. For instance, Mita *et al.* showed that rifampicin, rifamycin SV, glibenclamide and cyclosporin A reduced the vectorial transport of TCA across NTCP- and BSEP- co-expressing LLC-PK_1 cells. Mahdi *et al.* successfully reproduced biliary lipid secretion *in vitro* by stably transfecting LLC-PK_1 cells with NTCP, BSEP, MDR3 and ABCG5/8. This allowed efficient testing of drugs that interact with biliary lipid and BA secretion (Mita et al. 2006; Mahdi et al. 2016).

Overall, although transfected cell lines are a valuable tool to assess whether specific compounds disrupt BA transport mechanisms, one still has to consider the lack of metabolic enzymes, NHRs and the differences in expression levels of the transporters present, when comparing data obtained in these *in vitro* systems to the *in vivo* situation.

5.2.2 3D models

5.2.2.1 Spheroids

Spheroids have been shown to better maintain the mature hepatocyte phenotype, due to formation of homotypic cell-cell interactions, preservation of cell polarity and production of ECMs (Landry et al. 1985). They closely mimic the *in vivo* liver tissue in terms of protein expression and CYP activity levels. In addition, spheroids typically preserve their morphology, viability, and hepatocyte-specific functions (*i.e.*, formation of bile canaliculi) for multiple weeks in culture (Bell et al. 2016). The ability of a spheroid *in vitro* model to maintain hepatic cells in a differentiated state makes them especially suited for long-term toxicity testing. A recent study, conducted by six independent laboratories, compared the robustness and sensitivity of PHHs, either cultivated as 2D monolayers or 3D spheroids (Bell et al. 2018). Upon prolonged exposure, spheroids were found to be more sensitive to investigated hepatotoxic agents, emphasizing their applicability in long-term hepatotoxicity studies. Spheroids have also been extensively evaluated for their ability to detect human DIC. For instance, Hendriks *et al.* tested two hepatic spheroid systems, one originating from PHHs and one from HepaRG cells (Hendriks et al. 2016). Both spheroid types were repeatedly exposed to various compounds known to cause cholestatic liver injury for 14 days. The two spheroid systems were able to correctly identify cholestatic risk in almost all compounds, with the exception of ticlopidine, which was classified as having a low cholestatic risk in the PHH spheroid system.

Furthermore, a recent study conducted by Parmentier and co-workers highlighted the importance of long-term exposure studies in order to identify compounds that may cause cholestasis (Parmentier et al. 2018). The cholestatic toxicity of chlorpromazine and troglitazone was detected during long-term repeated exposures (14 days) in spheroids, while they were not classified as being cholestatic during short-term exposures (48-72 h). Taken together, spheroids are especially valuable when assessing chronic drug toxicity events as they closely resemble the *in vivo* liver tissue and preserve a mature phenotype for several weeks in culture.

5.3 Microfluidic systems

Conventional static *in vitro* systems, such as SCH, do not adequately resemble the *in vivo* microenvironment, where hepatocytes are in close proximity with the microvascular network, which continuously supplies cells with oxygen, nutrients and hormones. Microfluidic perfusion systems, on the other hand, more closely mimic the *in vivo* situation, as they enable researchers to precisely adjust both supply and removal of soluble factors, effectively creating a strictly organised environment (Kim et al. 2007). Various studies have indicated positive effects of dynamic culturing conditions on hepatocyte longevity. PHHs cultured in a dynamic flow system reproducibly recapitulated *in vivo*-like structures, while maintaining liver-specific functions, such as albumin secretion and CYP3A4 activity, for up to 7 weeks (Choi et al. 2014). The same observation has been made by Hegde *et al.*, who demonstrated that hepatocytes cultured under flow not only secrete higher levels of albumin and urea, but also of collagen, which might in part explain the beneficial effects of dynamic culturing conditions (Hegde et al. 2014). Additionally, several groups have postulated that flow-induced shear stress can lead to increased hepatic functions. For instance, Vinci *et al.* showed that genes involved in human drug metabolism, including CYP2B6, 2C9, 1A1, 1A2 and 3A4, were positively regulated by shear stress, while Rashidi *et al.* indicated that fluid

shear stress improved Cyp1A2 activity of hepatocyte-like cells (Vinci et al. 2011; Rashidi et al. 2016). Although perfusion systems have shown to greatly improve both hepatocyte function and longevity, their routine application in biomedical fields has not yet been achieved, due to their complexity. Moreover, microfluidic systems are inherently low-throughput, therefore impeding their use in a high-throughput screening setting. To overcome this issue, current research focuses on integrating many sensitive micro-assays into microfluidic perfusion systems, thus providing a more rapid assessment of drug effects. For example, Choucha-Snouber *et al.* developed a liver microfluidic biochip to investigate metabolic responses to anticancer drug flutamide. Cell culture media were analysed using both nuclear magnetic resonance and mass spectrometry, resulting in the identification of specific *in vitro* toxicity markers that showed to be consistent with previous literature reports (Choucha-Snouber et al. 2013). Furthermore, Bavli *et al.* established a liver-on-chip device, which was able to maintain HepG2/3A cells for over 28 days. Oxygen and glucose uptake rate as well as lactate production were monitored in real-time, thus permitting detection of minute shifts from oxidative phosphorylation to glycolysis or glutaminolysis, both of which are indicative of mitochondrial damage (Bavli et al. 2016). Taken together, microfluidic systems offer a broad range of advantages over conventional static *in vitro* cultures. Once routinely applied, microfluidic technologies may potentially define the way drug toxicity-related problems are being solved.

5.4 Liver slices

As opposed to many cell-based assay systems, precision-cut liver slices (PCLS) contain all hepatic cell types within their natural architecture (Olinga et al. 2013). Moreover, PCLS have been shown to express relevant levels of metabolizing and detoxification enzymes as well as transporters, while also producing BAs (Jung et al. 2007; Elferink et al. 2011; Starokozhko et al. 2017). However,

analogous to cell-based *in vitro* models, PCLS display a rapid loss in metabolic enzyme activity in function of culture time. Their use in studies that demand prolonged drug exposure is, therefore, limited. Indeed, rat PCLS (rPCLS) and human PCLS (hPCLS) have demonstrated a progressive decrease in CYP enzymes. Metabolism studies are, thus, best conducted with freshly-cut instead of cultured liver slices (Hashemi et al. 2000; Renwick et al. 2000; Martin et al. 2003). Improvements in culturing conditions have managed to partially surmount these issues. For instance, Starokozhko *et al.* optimized the incubation medium resulting in stable activity of both drug metabolizing enzymes and transporters during 5 days of culture. Moreover, Midwoud *et al.* allowed the production of phase I drug metabolites after 72 h of culture by embedding PCLS in Matrigel® and culturing them in a microfluidic system (van Midwoud et al. 2011; Starokozhko et al. 2017). Although these optimizations have improved longevity of PCLS, they still do not display high levels of functions for more than a few days, effectively limiting their use in chronic drug toxicity studies. Nonetheless, PCLS have been employed extensively for studies of xenobiotic-induced hepatotoxicity. For example, Vatakuti *et al.* performed a comparative analysis of gene expression profiles induced by acetaminophen (APAP) and CCl_4 using rPCLS. They found that observed changes in gene expression profiles reflected the characteristic difference between different compounds in their capacity to induce liver fibrosis. Hence, transcriptomic analysis of PCLS was suggested as a useful tool to identify potential fibrotic compounds (Vatakuti et al. 2015). Furthermore, Szalowska *et al.* assessed whether mouse PCLS (mPCLS) could be used to identify mechanisms leading to cholestatic liver injury (Szalowska et al. 2013). Accordingly, mPCLS were treated for 24 h with cyclosporin A and chlorpromazine, after which they were subjected to DNA microarray analysis. Their results showed that both compounds significantly down-regulated Fxr signalling, leading to changes in BA and lipid homeostasis.

Interestingly, several similarities were found when gene expression patterns of treated mPCLS were compared to expression data obtained from livers of cholestatic patients, indicating that some mechanisms of human cholestasis are reflected in the mPLCS *ex vivo* model. Recently, Vatakuti *et al.* investigated whether hPCLS could be used as a predictive model for DIC (Vatakuti et al. 2017). hPCLS were co-incubated with a non-toxic BA mixture and chemicals known to induce cholestasis. In the presence of BAs, all tested hepatotoxic agents induced regulation of genes and pathways associated with human cholestasis. In addition, the gene expression patterns were clearly distinct of those observed when PCLS of the same human donor were incubated with necrotic compounds. This suggests that observed regulation pathways are considerably related to cholestatic properties of the tested compounds. In conclusion, due to their multicellular composition and close resemblance to the *in vivo* liver architecture, PCLS are a suitable *ex vivo* model to study human DIC in a limited timeframe.

6 Conclusions and future perspectives

DIC poses a major hurdle for the pharmaceutical industry and regulatory authorities, since it is a leading cause of both attrition and post-approval withdrawal of drugs (Tonder and Gulumian 2013). Indeed, approximately 30% of all drugs withdrawals, due to hepatotoxic adverse events, were reported to induce cholestatic liver injury (Figure 1). Examples include benoxaprofen and troglitazone. Benoxaprofen was withdrawn in 1982, the same year it was marketed, while troglitazone was withdrawn in 1999, only two years after it reached the market (Onakpoya et al. 2016). Troglitazone accrued approximately $ 700 million per year in the US, stressing the fact that drug withdrawals lead to huge financial losses for the pharmaceutical industry (Tonder et al. 2013).

For this reason, as well as the accompanied concern for the safety of the patient, both academia and

industry have invested a considerable amount of resources in the development of safer, non-cholestatic, drugs. By doing so, multiple primary targets for DIC have been successfully identified. For instance, the potency of a compound to interfere with BSEP appears to be correlated with its likelihood to induce cholestatic liver injury in humans (Morgan et al. 2010). The correlation becomes even stronger when activities of the compound on other hepatobiliary transporters, such as MRP2, MRP3, MRP4 and MDR3 are integrated (Morgan et al. 2013; Aleo et al. 2017). One might obtain even more accurate predictions if alterations of other BA transporters, such as NTCP, OATP1B1 and OATP1B3 are included. The exact role of FIC1, BCRP, MDR1 and OSTα-β in BA homeostasis has not yet been unequivocally elucidated. Inhibition and/or activation of these transporters could significantly contribute to a compound's cholestatic potential, and thus warrants further investigation. Although predominant, disturbances of basolateral and canalicular transporters are not the sole mechanisms, by which a compound can induce cholestatic liver injury. For example, 17α ethinylestradiol and chlorpromazine not only inhibit specific hepatic transporters but, also showed to induce cholestasis by affecting canalicular membrane fluidity (Table 1). Other drugs conjure cholestatic liver injury without disturbing hepatobiliary transporters. This is the case for nevirapine, which upregulates CYP7A1, leading to intracellular accumulation of BAs (Table 1). Taken together, it is evident that a multitude of different mechanisms underlie DIC. However, certain molecular interactions may be implicated in cholestatic liver injury more frequently than others. For instance, approximately 75% of all drugs reported to cause cholestasis alter hepatobiliary transporters, while less than 2% act through disturbing NHR activity (Figure 3). In addition, other susceptibility factors also contribute to the overall cholestasis risk of a given drug. In this context, patients with certain genetic variants in BSEP are more at risk of developing cholestatic liver injury when treated with a BSEP-inhibiting drug.

Particular drug-drug interactions can lead to increased intracellular concentrations of a cholestatic drug, thereby aggravating the toxic response (Meier et al. 2006; Wessler et al. 2013). Taken together, it is evident that DIC encompasses a complex interplay of several underlying mechanisms and that multiple liability factors are considered when predicting the cholestatic potential of a given compound. Liver-based *in vitro* models have been developed and are used to predict and evaluate DIC along with elucidating its underlying mechanisms (Table 2). Some of these systems, like membrane vesicles originating from transporter-transfected cells, are exceptionally well-suited to investigate the interaction of a potentially cholestatic molecule with a single transporter, such as BSEP. In fact, several studies have been conducted to describe the inhibitory capacity of drugs on canalicular and basolateral efflux transporters, aiming to reveal their cholestatic behaviour (Stieger et al. 2000; Morgan et al. 2010). These systems are valuable tools to investigate the involvement of BSEP inhibition in DIC, but fail in accurately predicting human DIC, as they reduce the mechanistic complexity of DIC to merely the interaction of one transporter. Other models, like cell-based and biomimetic assay systems, serve as more holistic tools, since they encompass all relevant biological processes. Yet, most of these models are often too complex for high-throughput screening in an early drug development setting. The choice of the experimental system(s) is therefore primarily determined by the research question at hand. In addition, combined use of data obtained during high-throughput screening experiments and *in silico* modelling poses a promising alternative to the more traditional approaches. Using these mathematical models, data regarding perturbations of BA homeostasis, drug disposition and disturbances of hepatocellular morphology can be integrated to patient-specific factors, such as underlying disease states and genetic polymorphisms, resulting in improved predictions of human DIC during early stages of drug development.

This multifaceted modelling approach, which incorporates both drug disposition and physiological processes, is currently applied in DILIsym® initiative. DILIsym® utilizes quantitative systems pharmacology (QSP) to mathematically represent various forms of hepatic liver injury and, is thereby able to simulate the different scenarios of how a candidate drug interacts with known mechanisms of hepatotoxicity (*e.g.,* mitochondrial dysfunction, perturbation of BA disposition and formation of reactive oxygen species) (Shoda et al. 2014; Woodhead et al. 2017).

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8 Disclosure of interest

The current employment affiliation of the authors is as shown on the cover page. The authors have no financial or nonfinancial competing interests to declare and have sole responsibility for the writing and content of the review. No potential conflict of interest was reported by the authors.

9 Figures and tables

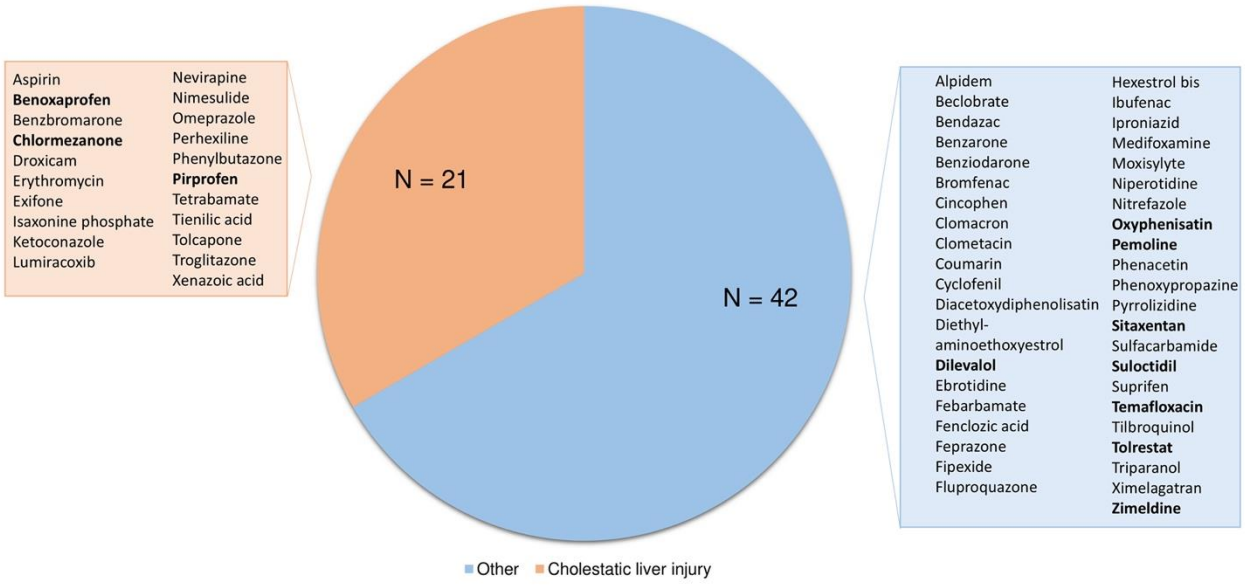

Figure 1. Overview of a number of drugs that were withdrawn from various markets due to hepatotoxic adverse reactions. Out of 63 withdrawn drugs, 21 were reported to induce cholestatic liver injury. Drugs marked in bold were withdrawn from the global market, while others were withdrawn in specific regions. Data are obtained from (Onakpoya et al. 2016).

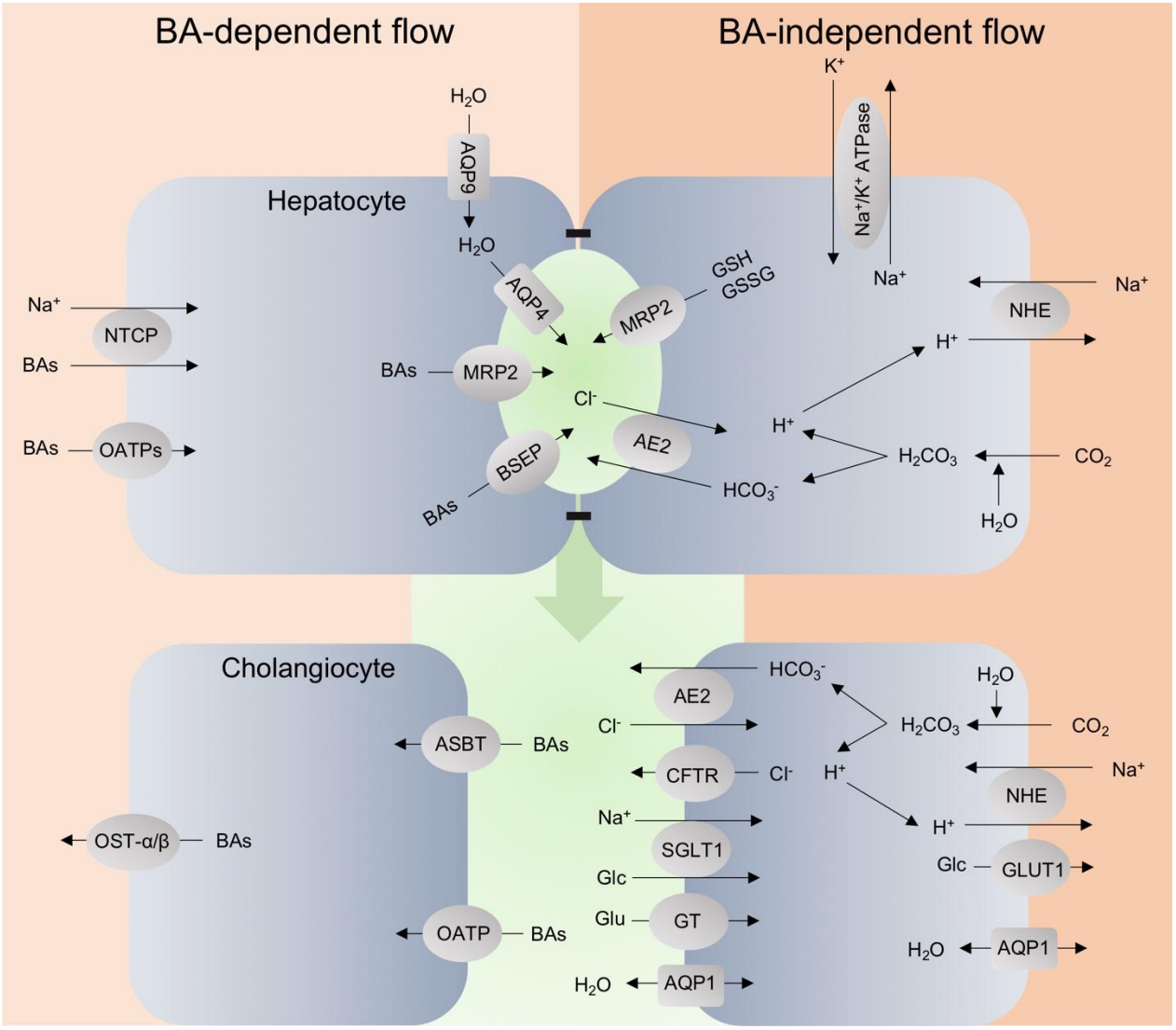

Figure 2. Schematic representation of BA-dependent and BA-independent bile flow. AE2; chloride/bicarbonate exchanger, AQP; aquaporin, ASBT; apical sodium-bile acid transporter, BA; bile acid, BSEP; bile salt export pump, CFTR; cystic fibrosis transmembrane conductance regulator, GLUT1; glucose transporter 1, GT; glutamate transporter, MRP; multidrug resistance-associated protein, NHE; sodium-proton exchanger, NTCP; sodium taurocholate cotransporting polypeptide, OATPs; organic anion transporting polypeptides, OST-α/β; organic solute and steroid transporter, SGLT1; sodium-dependent glucose cotransporter 1.

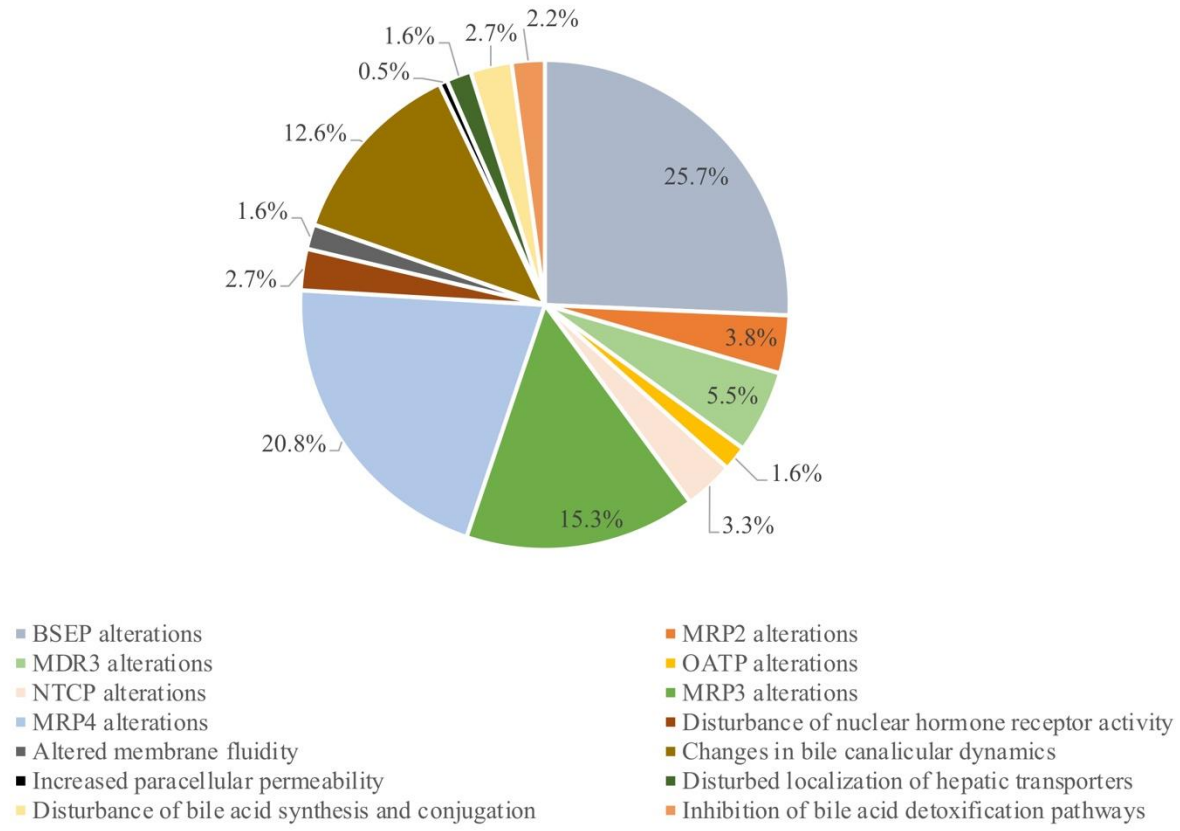

Figure 3. Relative frequencies (expressed as percentages) of molecular mechanisms involved in drug-induced cholestatic liver injury. Relative frequencies are calculated by dividing the times that a molecular mechanism is involved in drug-induced cholestatic liver injury by the sum of all reported mechanisms (as depicted in Table 1). BSEP; bile salt export pump, MDR; multidrug resistance protein, MRP; multidrug resistance-associated protein, NTCP; sodium taurocholate cotransporting polypeptide, OATP; organic anion transporting polypeptides.

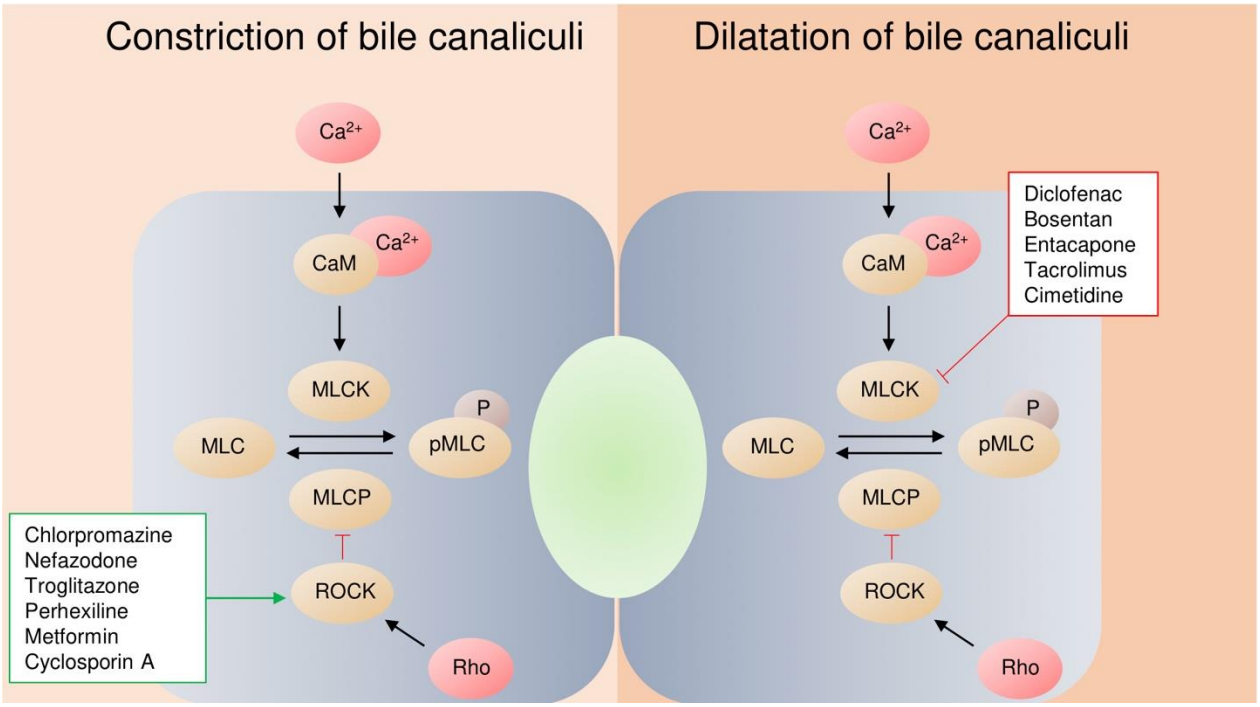

Figure 4. Schematic representation of molecular mechanisms involved in drug-induced bile canalicular constriction and dilatation. Chlorpromazine, nefazodone, troglitazone, perhexilline, metformin and cyclosporin A activate the ROCK pathway, resulting in inhibition of myosin light chain phosphatase (MLCP) and subsequent enhanced phosphorylation of myosin light chains (MLCs), thereby leading to constriction of bile canaliculi. Diclofenac, bosentan, entacapone, tacrolimus and cimetidine inhibit myosin light chain kinase (MLCK), thereby decreasing the phosphorylation of MLCs and causing dilatation of bile canaliculi. Ca^{2+}; calcium, CaM; calmodulin, MLC; myosin light chain, MLCK; myosin light chain kinase, MLCP; myosin light chain phosphatase, P; phosphate, ROCK; Rho-kinase (Modified from Sharanek et al. 2016).

Table 1. Overview of different drugs reported to cause drug-induced cholestasis and their postulated underlying mechanisms. Postulated primary mechanisms are marked in bold. BA; bile acids, BAAT; bile acid CoA:amino acid N-acyltransferase, BACS; bile acid:CoA synthase, BSEP; bile salt export pump, Fxr; farnesoid X receptor, DILI; drug-induced liver injury, MDR; multidrug resistance protein, MRP; multidrug resistance-associated protein, N.D.; not determined, NSAID; non-steroidal anti-inflammatory drugs, OATP; organic-anion-transporting polypeptide, SULT2A1; sulfotransferase-2A1. Data regarding DILI severity were obtained from

https://livertox.nlm.nih.gov/. The Cheng-Prusoff equation was used to calculate the K_i from IC_{50} values obtained in the different studies (Cheng and Prusoff et al. 1973).

Table 2. Comparison of several *in vitro* and *ex vivo* models used to investigate drug-induced cholestasis. BA; bile acid, DIC; drug-induced cholestasis.

Compound name	Therapeutic use	DILI severity	Postulated mechanism(s) of cholestatic liver injury	References
17α Ethinylestradiol	Contraceptive	Moderate to severe	**Alterations of hepatobiliary transporters:** • BSEP inhibition (K_i: 12.1 µM) • Repression of BSEP transcription Morphological disturbances: • Alterations of liver plasma membrane fluidity • Relocalisation of Mrp2	(Davis et al. 1978; Mottino et al. 2002b; Morgan et al. 2010; Morgan et al. 2013; Garzel et al. 2014)
Acitretin	Antipsoriatic	Moderate to severe	**Alterations of hepatobiliary transporters:** • BSEP inhibition (K_i: 17.2 µM) • MRP4 inhibition (K_i: 46.9 µM)	(Dawson et al. 2012; Morgan et al. 2013; Köck et al. 2014)
Aspirin	Anti-inflammatory, antipyretic	Mild	Unknown	(López-Morante et al. 1993)
Benoxaprofen	NSAID	Fatal	**Alterations of hepatobiliary transporters:** • BSEP inhibition (K_i: 166 µM)	(Dawson et al. 2012)

Benzbromarone	Uricosuric	Severe to fatal	**Alterations of hepatobiliary transporters:** • Repression of BSEP transcription	(Garzel et al. 2014)
Bosentan	Treatment of pulmonary arterial hypertension	Severe	Disturbance of bile acid synthesis: • Inhibition of BA conjugation • Inhibition of BA synthesis **Alterations of hepatobiliary transporters:** • BSEP inhibition (K_i: 36.0 µM) • Repression of BSEP transcription • MRP3 inhibition (K_i: 36.6 µM) • MRP4 inhibition (K_i: 21.1 µM) • NTCP inhibition (K_i: 18 µM) Morphological disturbances: • Dilatation of bile canaliculi	(Dawson et al. 2012; Morgan et al. 2013; Pedersen et al. 2013; Garzel et al. 2014; Burbank et al. 2016; Sharanek, Burban, Burbank, Rémy Le Guevel, et al. 2016)
Carbamazepine	Antidepressant	Moderate to severe	**Other:** • Hypersensitivity	(Dertinger et al. 1998)

Chloramphenicol	Antibiotic	Severe	**Alterations of hepatobiliary transporters:** • MRP3 inhibition (K_i: > 47.7 µM)	(Köck et al. 2014)
Chlormezanone	Anxiolytic	Fatal	Unknown	(Pomiersky et al. 1985)
Chlorpromazine	Antipsychotic	Moderate to severe	Disturbance of bile acid synthesis: • Inhibition of BAAT • Inhibition of BACS • Inhibition of CoA-BA translocation Inhibition of bile acid detoxification: • Inhibition of SULT2A1 **Alterations of hepatobiliary transporters:** • BSEP inhibition (K_i: 140 µM) • MRP3 inhibition (K_i: < 47.7 µM) • MRP4 inhibition (K_i: < 63.7 µM) • MDR3 inhibition (K_i: N.D.) • Repression of Bsep transcription • Repression of Ntcp transcription	(Boyer et al. 1978; Keeffe et al. 1980; Watanabe et al. 2007; Köck et al. 2014; He et al. 2015; Burbank et al. 2016; Donato et al. 2016; Sharanek et al. 2016; Sharanek et al. 2017)

			• Repression of Oatp1a1 transcription Morphological disturbances: • Alterations of liver plasma membrane fluidity • Constriction of bile canaliculi • Relocalisation of MRP2 Other: • Hypersensitivity	
Chlorpropamide	Antidiabetic	Severe to fatal	Unknown, but most likely hypersensitivity	(Rigberg et al. 1976)
Cimetidine	Anti-allergic	Mild to moderate	**Alterations of hepatobiliary transporters:** • BSEP inhibition (K_i: > 45.1 µM) Morphological disturbances: • Dilatation of bile canaliculi	(Pedersen et al. 2013; Burbank et al. 2016)
Cloxacillin	Antibiotic	Severe	Alterations of hepatobiliary transporters: • BSEP inhibition (K_i: 208 µM) **Other:** • Hypersensitivity	(Dawson et al. 2012)

Clozapine	Antipsychotic	Mild to moderate	Alterations of hepatobiliary transporters: • BSEP inhibition (K_i: 119 µM) • MRP3 inhibition (K_i: > 47.7 µM) • MRP4 inhibition (K_i: > 63.7 µM) **Other:** • Formation of toxic intermediate	(Dawson et al. 2012; Köck et al. 2014)
Cyclosporin A	Immunosuppressant	Mild	Disturbance of bile acid synthesis: • Inhibition of BAAT • Inhibition of BACS • Inhibition of CoA-BA translocation Inhibition of bile acid detoxification: • Inhibition of SULT2A1 **Alterations of hepatobiliary transporters:** • BSEP inhibition (K_i: 0.50 µM) • MRP2 inhibition (K_i: 7.84 µM) • MRP3 inhibition (K_i: 20.0 µM) • MRP4 inhibition (K_i: > 63.7 µM)	(Yasumiba et al. 2001; Mita et al. 2006; Dawson et al. 2012; Morgan et al. 2013; Köck et al. 2014; He et al. 2015; Burbank et al. 2016; Donato et al. 2016; Sharanek, Burban, Burbank, Rémy Le Guevel, et al. 2016; Sharanek et al. 2017)

			• MDR3 inhibition (K_i: N.D.) • NTCP inhibition (K_i: 0.27 µM) • Repression of Bsep transcription • Repression of Ntcp transcription • Repression of Oatp1a1 transcription Morphological disturbances: • Alterations of liver plasma membrane fluidity • Constriction of bile canaliculi	
Desipramine	Antidepressant	Mild	Alterations of hepatobiliary transporters: • MRP3 inhibition (K_i: > 47.7 µM) • MRP4 inhibition (K_i: > 63.7 µM) **Other:** • Hypersensitivity	(Köck et al. 2014)
Diclofenac	NSAID	Mild to fatal	**Alterations of hepatobiliary transporters:** • BSEP inhibition (K_i: > 45.0 µM) Morphological disturbances:	(Watanabe et al. 2007; Pedersen et al. 2013; Burbank et al. 2016)

			• Dilatation of bile canaliculi • Relocalisation of MRP2	
Dicloxacillin	Antibiotic	Moderate to severe	**Alterations of hepatobiliary transporters:** • BSEP inhibition (K_i: 66.0 µM) • MRP3 inhibition (K_i: < 47.7 µM) • MRP4 inhibition (K_i: > 63.7 µM)	(Dawson et al. 2012; Köck et al. 2014)
Droxicam	NSAID	Severe	Unknown	(Alvarez et al. 1993; Ferrer et al. 1994)
Entacapone	Antiparkinsonian	Mild	**Alterations of hepatobiliary transporters:** • BSEP inhibition (K_i: 47.9 µM) • MRP3 inhibition (K_i: 30.6 µM) • MRP4 inhibition (K_i: 6.51 µM) Morphological disturbances: • Dilatation of bile canaliculi	(Morgan et al. 2013; Burbank et al. 2016)
Erythromycin	Antibiotic	Mild to severe	Alterations of hepatobiliary transporters: • BSEP inhibition (K_i: 3.88 µM)	(Horn et al. 1999; Morgan et al. 2010; Dawson et al. 2012;

			• MRP4 inhibition (K_i: 65.3 µM) • Repression of BSEP transcription **Other:** • Hypersensitivity	Morgan et al. 2013; Pedersen et al. 2013; Garzel et al. 2014; Köck et al. 2014)
Exifone	Nootropic	Severe to fatal	Unknown	(Larrey et al. 1989)
Fasiglifam	Antidiabetic	Severe	**Alterations of hepatobiliary transporters:** • MRP2 inhibition (K_i: N.D.) • Ntcp inhibition • Oatp inhibition	(Li et al. 2015)
Fenofibrate	Antilipemic	Moderate to severe	**Alterations of hepatobiliary transporters:** • BSEP inhibition (K_i: 13.2 µM) • MRP4 inhibition (K_i: > 63.7 µM) • Repression of BSEP transcription	(Morgan et al. 2010; Pedersen et al. 2013; Garzel et al. 2014; Köck et al. 2014)
Flucloxacillin	Antibiotic	Moderate to severe	Alterations of hepatobiliary transporters:	(Dawson et al. 2012)

			• BSEP inhibition (K_i: 198 µM) **Other:** • Hypersensitivity	
Fluoxetin	Antidepressant	Moderate to severe	**Alterations of hepatobiliary transporters:** • BSEP inhibition (K_i: > 45.1 µM) • MRP3 inhibition (K_i: < 47.7 µM) • MRP4 inhibition (K_i: < 63.7 µM)	(Pedersen et al. 2013; Köck et al. 2014)
Flutamide	Antiandrogen	Moderate to fatal	**Alterations of hepatobiliary transporters:** • BSEP inhibition (K_i: 136 µM) Other: • Possible formation of a toxic intermediate	(Aizawa et al. 2003; Iwanaga et al. 2007; Dawson et al. 2012)
Fluvastatin	Antilipemic	Mild to moderate	**Alterations of hepatobiliary transporters:** • BSEP inhibition • MRP3 inhibition (K_i: < 47.7 µM)	(Morgan et al. 2010; Köck et al. 2014)

			• MRP4 inhibition (K_i: < 63.7 µM)	
Glimepiride	Antidiabetic	Severe to fatal	Alterations of hepatobiliary transporters: • BSEP inhibition (K_i: 13.5 µM) • MRP3 inhibition (K_i: 5.57 µM) • MRP4 inhibition (K_i: 61.2 µM) • Repression of BSEP transcription **Other:** • Hypersensitivity	(Morgan et al. 2010; Morgan et al. 2013; Pedersen et al. 2013; Garzel et al. 2014)
Glyburide	Antidiabetic	Severe to fatal	Alterations of hepatobiliary transporters: • BSEP inhibition (K_i: 5.02 µM) • MRP3 inhibition (K_i: 28.8 µM) • MRP4 inhibition (K_i: 10.1 µM) • Repression of BSEP transcription • Repression of Oatp1a1 transcription **Other:** • Hypersensitivity	(Byrne et al. 2002; Morgan et al. 2010; Dawson et al. 2012; Morgan et al. 2013; Pedersen et al. 2013; Garzel et al. 2014; Köck et al. 2014; Donato et al. 2016)
Haloperidol	Antipsychotic	Mild	**Alterations of hepatobiliary transporters:**	(Köck et al. 2014; He et al. 2015)

			• MRP3 inhibition (K_i: > 47.7 µM) • MRP4 inhibition (K_i: > 63.7 µM) • MDR3 inhibition (K_i: N.D.)	
Ibuprofen	NSAID	Severe	**Alterations of hepatobiliary transporters:** • BSEP inhibition (K_i: 567 µM) • MRP4 inhibition (K_i: > 63.7 µM) Other: • Hypersensitivity	(Nanau et al. 2010; Dawson et al. 2012; Köck et al. 2014)
Imipramine	Antidepressant	Moderate to severe	Alterations of hepatobiliary transporters: • MDR3 inhibition (K_i: N.D.) **Other:** • Hypersensitivity • Possible formation of toxic intermediate	(Morrow et al. 1989; He et al. 2015)
Indomethacin	NSAID	Severe to fatal	Alterations of hepatobiliary transporters: • BSEP inhibition (K_i: > 45.1 µM) • MRP3 inhibition (K_i: < 47.7 µM)	(Morgan et al. 2010; Pedersen et al. 2013; Köck et al. 2014)

			• MRP4 inhibition (K_i: < 63.7 µM) **Other:** • Possible formation of toxic intermediate	
Irbesartan	Antihypertensive	Mild	Alterations of hepatobiliary transporters: • BSEP inhibition (K_i: 6.31 µM) • MRP3 inhibition (K_i: 18.4 µM) • MRP4 inhibition (K_i: 79.6 µM) **Other:** • Hypersensitivity	(Morgan et al. 2013)
Isaxonine phosphate	Neurotrophic	Fatal	Unknown	(Fabre et al. 1982)
Isoniazid	Antitubercular	Moderate to fatal	Alterations of hepatobiliary transporters: • Repression of Bsep transcription • Repression of Ntcp transcription **Other:** • Formation of toxic intermediates	(Lauterburg et al. 1985; Guo et al. 2015)
Itraconazole	Antifungal	Moderate to fatal	**Alterations of hepatobiliary transporters:**	(Morgan et al. 2010; Yoshikado et al. 2011; Morgan et al. 2013;

			• BSEP inhibition (K_i: 15.5 µM) • MDR3 inhibition (K_i: N.D.) • Repression of BSEP transcription	Pedersen et al. 2013; He et al. 2015; Mahdi et al. 2016)
Ketoconazole	Antifungal	Mild to fatal	Inhibition of bile acid detoxification: • Inhibition of CYP3A4 expression **Alterations of hepatobiliary transporters:** • BSEP inhibition (K_i: 2.75 µM) • MRP4 inhibition (K_i: 66.0 µM) • MDR3 inhibition (K_i: N.D.) Disturbance of nuclear hormone receptors: • Inhibition of PXR activity	(Lim et al. 2009; Morgan et al. 2010; Dawson et al. 2012; Morgan et al. 2013; Pedersen et al. 2013; He et al. 2015; Mahdi et al. 2016)
Lopinavir	Antiretroviral	Mild to moderate	**Alterations of hepatobiliary transporters:** • BSEP inhibition (K_i: < 45.1 µM) Disturbance of nuclear hormone receptors: • Reduces FXR activity	(Morgan et al. 2010; Pedersen et al. 2013; Garzel et al. 2014)

Lumiracoxib	NSAID	Severe to fatal	Unknown	(Fok et al. 2013)
Medroxyprogesterone acetate	Contraceptive	Mild	**Alterations of hepatobiliary transporters:** • MRP4 inhibition (K_i: 21.6 µM)	(Morgan et al. 2013)
Metformin	Antidiabetic	Moderate to severe	**Alterations of hepatobiliary transporters:** • BSEP inhibition (K_i: > 45.0 µM) Morphological disturbances: • Constriction of bile canaliculi	(Pedersen et al. 2013; Burbank et al. 2016)
Nefazodone	Antidepressant	Fatal	**Alterations of hepatobiliary transporters:** • BSEP inhibition (K_i: 3.98 µM) • MRP4 inhibition (K_i: 68.0 µM) Morphological disturbances: • Constriction of bile canaliculi	(Kostrubsky et al. 2006; Morgan et al. 2010; Dawson et al. 2012; Morgan et al. 2013; Pedersen et al. 2013; Burbank et al. 2016)
Nevirapine	Antibiotic	Moderate to fatal	**Disturbance of bile acid synthesis:** • Increase in BA synthesis	(Terelius et al. 2016)

Nifedipine	Antianginal, antihypertensive	Moderate	Alterations of hepatobiliary transporters: • BSEP inhibition (K_i: 29.1 µM) • MRP3 inhibition (K_i: > 47.7 µM) • MRP4 inhibition (K_i: 15.9 µM) **Other:** • Formation of toxic intermediate	(Dawson et al. 2012; Morgan et al. 2013; Köck et al. 2014)
Nimesulide	NSAID	Fatal	Alterations of hepatobiliary transporters: • Bsep inhibition • Mrp2 inhibition **Other:** • Formation of toxic intermediate	(Zhou et al. 2017)
Nitrofurantoin	Antibiotic	Mild to fatal	**Alterations of hepatobiliary transporters:** • BSEP inhibition (K_i: > 45.1 µM) • MRP4 inhibition (K_i: < 63.7 µM)	(Pedersen et al. 2013; Köck et al. 2014)
Nortriptyline	Antidepressant	Mild	Alterations of hepatobiliary transporters: • MRP3 inhibition (K_i: < 47.7 µM)	(Köck et al. 2014)

			• MRP4 inhibition (K_i: > 63.7 µM) **Other:** • Formation of toxic intermediate	
Omeprazole	Anti-secretory	Severe	Unknown	(Sánchez et al. 2007)
Perhexiline	Prophylactic antianginal	Severe to fatal	**Morphological disturbances:** • Constriction of bile canaliculi	(Burbank et al. 2016)
Phenylbutazone	NSAID	Severe to fatal	Unknown	(Benjamin et al. 1981)
Pioglitazone	Antidiabetic	Moderate to severe	**Alterations of hepatobiliary transporters:** • BSEP inhibition (K_i: 0.28 µM) • MRP4 inhibition (K_i: 47.4 µM)	(Morgan et al. 2010; Dawson et al. 2012; Morgan et al. 2013; Pedersen et al. 2013; Köck et al. 2014)
Pirprofen	NSAID	Fatal	Unknown	(Bentata et al. 1986)
Pyrazinamide	Antitubercular	Fatal	Disturbance of nuclear hormone receptors: • FXR inhibition **Other:** • Formation of toxic intermediate	(Guo et al. 2016)

Quinine	Antimalarial	Moderate to severe	Alterations of hepatobiliary transporters: • MRP4 inhibition (K_i: > 63.7 µM) **Other:** • Hypersensitivity	(Farver et al. 1999; Köck et al. 2014)
Repaglinide	Antidiabetic	Mild	Alterations of hepatobiliary transporters: • BSEP inhibition (K_i: 19.0 µM) • MRP2 inhibition (K_i: 39.1 µM) • MRP3 inhibition (K_i: 14.6 µM) • MRP4 inhibition (K_i: 51.6 µM) **Other:** • Formation of toxic intermediate	(Morgan et al. 2013; Pedersen et al. 2013)
Rifampicin	Antibiotic	Mild to fatal	Alterations of hepatobiliary transporters: • BSEP inhibition (K_i: 11.9 µM) • MRP2 inhibition (K_i: 28.7 µM) • MRP3 inhibition (K_i: 60.1 µM) • MRP4 inhibition (K_i: 40.1 µM) • Repression of BSEP transcription	(Stieger et al. 2000; Byrne et al. 2002; Morgan et al. 2010; Dawson et al. 2012; Morgan et al. 2013; Garzel et al. 2014; Köck et al. 2014)

			Morphological disturbances: • Altered integrity of tight junctions **Other:** • Formation of toxic intermediate	
Ritonavir	Antiretroviral	Mild to severe	**Alterations of hepatobiliary transporters:** • BSEP inhibition (K_i: 1.55 µM) • MRP3 inhibition (K_i: 9.67 µM) • MRP4 inhibition (K_i: 32.5 µM) • MDR3 inhibition (K_i: N.D.) • Repression of BSEP transcription	(Morgan et al. 2010; Morgan et al. 2013; Pedersen et al. 2013; Köck et al. 2014; He et al. 2015)
Rosiglitazone	Antidiabetic	Moderate to severe	**Alterations of hepatobiliary transporters:** • BSEP inhibition (K_i: 6.06 µM) • MRP4 inhibition (K_i: 20.1 µM)	(Morgan et al. 2010; Dawson et al. 2012; Morgan et al. 2013; Pedersen et al. 2013; Köck et al. 2014)
Saquinavir	Antiretroviral	Mild	**Alterations of hepatobiliary transporters:** • BSEP inhibition (K_i: 4.22 µM)	(Morgan et al. 2010; Morgan et al. 2013; Pedersen et al. 2013; Garzel et al. 2014; He et al. 2015)

			• MRP3 inhibition (K_i: 36.6 µM) • MRP4 inhibition (K_i: 56.5 µM) • MDR3 inhibition (K_i: N.D.) • Repression of BSEP transcription **Other:** • Formation of toxic intermediate	
Simvastatin	Antilipemic	Moderate to severe	**Alterations of hepatobiliary transporters:** • BSEP inhibition (K_i: < 45.1 µM) • MRP3 inhibition (K_i: < 47.7 µM) • MRP4 inhibition (K_i: < 63.7 µM) • Repression of BSEP transcription	(Morgan et al. 2010; Pedersen et al. 2013; Garzel et al. 2014; Köck et al. 2014)
Sulfamethoxazole-trimethoprim	Antibiotic	Mild to fatal	**Other:** • Hypersensitivity	(Cario et al. 1996)
Sulindac	NSAID	Severe	Alterations of hepatobiliary transporters: • BSEP inhibition (K_i: 214 µM) • MRP3 inhibition (K_i: < 47.7 µM)	(Rabinovitz et al. 1992; Bolder et al. 1999; Dawson et al. 2012; Pedersen et al. 2013; Köck et al. 2014)

			• MRP4 inhibition (K_i: < 63.7 µM) • Ntcp inhibition **Other:** • Hypersensitivity	
Tacrolimus	Immunosuppressant	Mild	**Alterations of hepatobiliary transporters:** • BSEP inhibition (K_i: 6.19 µM) • MRP2 inhibition (K_i: 21.8 µM) Morphological disturbances: • Dilatation of bile canaliculi	(Morgan et al. 2013; Burbank et al. 2016)
Tamoxifen	Antiestrogen	Mild to severe	Alterations of hepatobiliary transporters: • BSEP inhibition (K_i: > 45.1 µM) • MRP3 inhibition (K_i: < 47.7 µM) • MRP4 inhibition (K_i: < 63.7 µM) **Other:** • Formation of toxic intermediate	(Pedersen et al. 2013; Köck et al. 2014)
Ticlopidine	Antiplatelet	Moderate to severe	Alterations of hepatobiliary transporters:	(Dawson et al. 2012; Pedersen et al. 2013;

			• BSEP inhibition (K_i: 70.1 µM) • MRP4 inhibition (K_i: > 63.7 µM) **Other:** • Hypersensitivity	Köck et al. 2014)
Tienilic acid	Uricosuric	Severe	Unknown	(Schumacher et al. 1981)
Tolbutamide	Antidiabetic	Severe to fatal	Alterations of hepatobiliary transporters: • BSEP inhibition (K_i: > 45.1 µM) • MRP3 inhibition (K_i: < 47.7 µM) **Other:** • Hypersensitivity	(Köck et al. 2014)
Tolcapone	Antiparkinsonian	Fatal	Alterations of hepatobiliary transporters: • BSEP inhibition (K_i: 31.6 µM) • MRP3 inhibition (K_i: 74.0 µM) • MRP4 inhibition (K_i: 16.0 µM) Morphological disturbances: • Dilatation of bile canaliculi	(Smith et al. 2003; Morgan et al. 2013; Burbank et al. 2016)

			Other: • Formation of a toxic intermediate	
Troglitazone	Antidiabetic	Moderate to severe	Disturbance of bile acid synthesis: • Inhibition of BAAT • Inhibition of BACS • Inhibition of CoA-BA translocation Inhibition of bile acid detoxification: • Inhibition of SULT2A1 **Alterations of hepatobiliary transporters:** • BSEP inhibition (K_i: 2.56 µM) • MRP2 inhibition • MRP3 inhibition (K_i: 27.0 µM) • MRP4 inhibition (K_i: 58.4 µM) • MDR3 inhibition (K_i: N.D.) • NTCP inhibition (K_i: N.D.) Disturbance of nuclear hormone receptors:	(Morgan et al. 2010; Dawson et al. 2012; Morgan et al. 2013; Garzel et al. 2014; Köck et al. 2014; He et al. 2015; Sharanek et al. 2017)

			• Reduces FXR activity Morphological disturbances: • Constriction of bile canaliculi	
Verapamil	Antiarrhythmic	Mild	Alterations of hepatobiliary transporters: • MRP3 inhibition (K_i: < 47.7 µM) • MRP4 inhibition (K_i: > 63.7 µM) • MDR3 inhibition (K_i: N.D.) **Other:** • Hypersensitivity	(Hare et al. 1986; López-Morante et al. 1993)

	Can be used to determine alterations of BA transporters	**Can be used to investigate perturbations of BA homeostasis**	**Can be used to study disturbances of hepatocyte morphology**	**Endogenous BA production?**	**Formation of bile canaliculi?**	**Accuracy to predict DIC**	**References**

Isolated canalicular liver plasma membrane vesicles (cLPM)	Yes	No	No	NA	No	Low	(Horikawa et al. 2003)
Membrane vesicles originating from BSEP-overexpressing cell lines	Yes	No	No	NA	No	Low	(Dawson et al. 2012)
Sandwich-cultured hepatocytes	Yes	Yes	Yes	Yes	Yes	High	(De Bruyn et al. 2013)
HepaRG	Yes,	Yes	Yes	Yes	Yes	Moderate	(Sharanek et

	although certain transporters have low expression levels						al. 2015)
HepG2	Yes, although certain transporters have low expression levels	Yes	Yes	Yes	Yes	Moderate	(Everson et al. 1986; Sormunen et al. 1993)
Transfected cell lines	Yes	No	No	No	No	Low	(Woolbright et al. 2015)

Spheroids	Yes	Yes	Yes	Depending on cells used	Yes	High	(Bell et al. 2016)
Microfluidic systems	Yes	Yes	Yes	Depending on cells used	Depending on cells used	High	(Lin et al. 2016)
Liver slices	Yes	Yes	Yes	Yes	Yes	High	(Yoneyama 2001; Starokozhko et al. 2017)

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