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DOI: <https://doi.org/10.1016/B978-0-12-387817-5.00007-8>

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ZORA URL: <https://doi.org/10.5167/uzh-93489>

Book Section

Accepted Version

Originally published at:

Stieger, Bruno; Kullak-Ublick, Gerd A (2013). Role of membrane transport in hepatotoxicity and pathogenesis of drug-induced cholestasis. In: Kaplowitz, N; DeLeve, L D. Drug-Induced Liver Disease. unknown: Elsevier, 123-133.

DOI: <https://doi.org/10.1016/B978-0-12-387817-5.00007-8>

Role of Membrane Transport in Hepatotoxicity and Pathogenesis of Drug-induced Cholestasis

Bruno Stieger and Gerd A. Kullak-Ublick

Department of Clinical Pharmacology and Toxicology, University Hospital, 8091 Zurich, Switzerland

Key words:

organic anion transporting polypeptide, bile salt export pump, acquired cholestasis, transporter inhibition

Abstract

Drug induced liver injury is an important clinical entity. It can be grouped into cholestatic liver injury, hepatocellular liver injury and mixed liver injury. Cholestatic liver injury is characterized by a reduction in bile flow and retention within hepatocytes of cholephilic compounds such as e.g. bile salts that cause hepatotoxicity.

Bile salts are largely taken up in a sodium-dependent manner and to a minor part in a sodium-independent manner (into) by hepatocytes. The former process is mediated by the sodium-(dependent) taurocholate cotransporting polypeptide (NTCP) and the latter by the organic anion transporting polypeptides (OATPs). OATPs have a broad substrate specificity and mediate uptake of a many drugs into hepatocytes. Bile salts are exported from hepatocytes into the biliary tree by the bile salt export pump (BSEP). Inhibition of BSEP by drugs or drug metabolites leads to drug-induced cholestasis. BSEP can be inhibited directly from within the hepatocyte, or indirectly by a mechanism that first requires canalicular secretion of the perpetrator. In cholestatic liver disease, expression of hepatocellular uptake transporters is generally reduced, while the expression of the canalicular export systems tends to be preserved. This adaptation is controlled by nuclear receptors and transcription factors. Genetic variants of BSEP may be a risk factor for drug-induced cholestasis. So far, studies have associated the c.1331T variant of the *ABCB11* gene coding for a V444A substitution in BSEP as a potential risk factor. Additional risk factors may be genetic variants of uptake transporters or other export transporters for drugs or their metabolites.

Introduction

The liver is a key organ in protecting the body from potentially harmful xenobiotics and is central in the metabolism and elimination of xenobiotics. As such, it is constantly challenged by toxic substances and metabolites and it is not surprising that the liver is a major target for adverse drug reactions, which lead to drug induced liver injury (DILI). DILI is a very important clinical entity. The incidence of serious adverse drug reactions in hospitalized patients may be up to 6 % and may contribute up to 0.3 % of all deaths of inpatients (1). Adverse drug reactions account for over 50 % of cases presenting with acute liver failure (2). A recent US study highlighted that in severe cases, transplant-free survival is poor (27 %) and that the overall survival with transplanted patients included is 66 % (3). DILI is categorized into drug-induced cholestasis, drug induced hepatitis or mixed liver injury (4). In medical inpatients, cholestatic and mixed liver injury may account for up to 30 % of cases with DILI

(5). DILI is also a major issue in drug development, where it often leads to attrition of drugs during development or later to removal of drugs from the market (6,7).

Bile formation

Bile formation is a key function of the liver. Disturbances in bile formation lead to a reduction of bile flow, a pathophysiologic process which is called cholestasis. Bile salts are synthesized from cholesterol in a complex series of highly orchestrated biosynthetic steps involving among others peroxisomes in hepatocytes (8). In hepatocytes, new synthesized bile salts are mixed with bile salts entering from the portal blood and secreted via the canalicular membrane into the canaliculi, which are the functional starting point of the biliary tree. The biliary tree ultimately drains into the duodenum. In the small intestine, bile salts are essential for lipid digestion and the absorption of fat and fat soluble vitamins (9,10). Bile salts are absorbed to more than 90 % along the small intestine and transported back by the portal blood to the liver (11), where they are taken up into hepatocytes and again secreted into the canaliculi. This roundtrip of bile salts between the intestine and the liver is termed enterohepatic circulation.

Uptake of bile salts from sinusoidal blood into hepatocytes is predominantly sodium-dependent and to a minor part sodium-independent. The sodium-dependent uptake of bile salts is mediated by the sodium taurocholate cotransporting polypeptide (NTCP, *SLC10A1*), which is expressed in a polar manner at the basolateral plasma membrane of hepatocytes (11,12). NTCP has a preference for conjugated bile salts over unconjugated bile acids. The sodium-independent portion of the bile salt uptake is mediated by organic anion transporting polypeptides (OATPs). OATP1B1 (*SLCO1B1*), OATP1B3 (*SLCO1B3*) and OATP2B1 (*SLCO2B1*) are expressed at the basolateral plasma membrane of hepatocytes, although OATP2B1 appears not to be a bile salt transporter (13-15). OATPs have a preference for unconjugated bile acids over conjugated bile salts (16), which was recently confirmed in genetically modified mice (17,18). Importantly, OATPs mediate uptake of the metabolic endproduct bilirubin (19-21) and of numerous xenobiotics including many widely prescribed drugs (22,23). In contrast, the role of NTCP in the hepatocellular uptake of drugs seems to be minor (12).

After their uptake into hepatocytes, bile salts reach the canalicular plasma membrane by a mechanism that has not yet been characterized in detail, but probably bound to cytoplasmic

proteins (12). The free intracellular bile salt concentration in hepatocytes is probably less than 1 μ M in rats (24) and may be comparably low in human hepatocytes (25). Export into the canaliculus is mediated by the bile salt export pump BSEP against a large concentration gradient. BSEP (*ABCB11*) is a member of the ATP-binding cassette (ABC) transporter super family (12,26,27). The canalicular secretion of bile salts represents the rate limiting step of bile salt transport across hepatocytes (13,28). Due to its strategic position in hepatic bile salt secretion, the proper functioning and regulation of this transporter is essential for hepatocyte viability since bile salts have detergent properties and easily become cytotoxic (29).

In addition to bile salts, bile is rich in phospholipids and organic anions such as bilirubin conjugates. Phospholipids are the main component of biliary lipids and need multidrug resistance protein 3 (*ABCB4*) for entering the canalicular bile (30). This ABC transporter translocates the main biliary lipid phosphatidylcholine from the inner to the outer leaflet of the canalicular membrane. Bile salts, by acting as detergents, release phosphatidylcholine from the outer canalicular leaflet into the canaliculus, where the two constituents form mixed micelles (31). These mixed micelles act as acceptors for poorly water soluble substances, a prototypic example being cholesterol. Cholesterol release from the canalicular membrane is in part mediated by the heterodimeric ABC transporter *ABCG5/ABCG8* (*ABCG5/ABCG8*) (32). In addition to acting as carriers for poorly water soluble substances, mixed micelles are needed to lower the toxic action of biliary bile salts against cholangiocytes to prevent bile salt induced injury of the bile ducts (33,34). Biliary organic anions other than bile salts are mainly conjugates of bilirubin and glutathione. Often, drug metabolites excreted into bile are also organic anions. These compounds are transported by the two ABC transporters multidrug resistance-associated protein 2 (MRP2, *ABCC2*) and *ABCG2* (*ABCG2*).

In summary, bile formation is one of the key functions of the liver and requires an intricate interplay of transport systems expressed at the basolateral and canalicular membrane of hepatocytes. The ABC transporters expressed in the canalicular membrane are able to work against steep concentration gradients and thereby keep the concentration of their respective substrates low within hepatocytes. An interference with these transporters, either by inherited or acquired disturbance of their function can potentially lead to an accumulation of toxic substances in hepatocytes and in turn to liver disease.

Clinical Features of Drug Induced Cholestasis

Diagnosis of DILI and DIC depends on a detailed clinical assessment of the patient and individual drugs, which can lead to a specific clinical phenotype (35-37). Diagnosis of DILI includes chronological criteria, exclusion of other causes and positive clinical criteria (1,35-40). A classification system such as e.g. established by the Council for International Organization of Medical Sciences is certainly helpful for identifying patients with drug-induced cholestasis among the many other forms of DILI (4). Bland cholestasis is associated with elevated alkaline phosphatase and elevated bile salts in plasma and usually rapidly reverts upon discontinuation of the culprit drug. Mild forms of DILI may be asymptomatic and vanish as a consequence of adaptation (41).

Hepatocellular Drug Uptake and Intracellular Drug Concentrations

NTCP is a secondary active transporter and can mediate uptake of its substrates against a concentration gradient, leading to a potentially higher intracellular concentration than in the sinusoidal blood plasma. The driving force of OATPs is so far not fully understood and remains controversial (15,36). OATPs are most likely bidirectional transporters that exchange anions against each other. Evidence that intracellular anions are effluxed upon OATP-mediated substrate uptake has so far been presented for glutathione (42), glutathione-conjugates (43) and bicarbonate (44,45). In addition, transport activity of almost all OATPs tested so far is activated by a low extracellular pH (45). As there is an in to out gradient of glutathione over the hepatocellular plasma membrane, uptake in exchange for glutathione could potentially work against a concentration gradient leading to an intracellular accumulation of OATP substrates, e.g. drugs. For example, in rats glibenclamide was observed to be fifty times higher in the liver compared to serum indicating a concentrative uptake mechanism (46). As coadministration of the OATP-inhibitor rifampicin with glibenclamide leads to an increase of glibenclamide exposure in humans (47), glibenclamide is a potential substrate for OATPs. Higher hepatocellular concentration of glibenclamide compared to plasma is in line with the observation that indocyanine green, which is transported into hepatocytes by OATP1B3 and also by NTCP (48) shows ten-fold higher concentrations in hepatocytes than in the plasma of rats (49). Furthermore, the transport activity of OATPs may be modulated by endo- and xenobiotics, such as prostaglandins (50), estrone-3-sulfate (51), estradiol-17 β -glucuronide (52), clotrimazole (53) or herbal extracts (54,55). This modulation can, at least in part, be due to different substrate binding sites of different OATPs (54). All these different transport mechanisms may individually play a role in OATP-mediated drug uptake into hepatocytes and preclude a prediction of the intracellular

drug concentration from serum values. It is however clear that DILI injury including drug-induced cholestasis correlates with the dose of the drug, which in turn impacts intracellular drug concentrations (56).

In addition to uptake, metabolism and efflux of drugs from hepatocytes are also important steps in overall drug disposition that affect intracellular drug concentrations. In the canalicular membrane, multidrug resistance protein 1 (MDR1, *ABCB1*) is a major contributor to hepatocellular export of drugs into the canaliculus. The substrate specificity of MDR1 is very broad and includes numerous drugs (57,58). The substrates of MDR1 are electrically neutral or positively charged organic molecules and include anticancer drugs, immunosuppressants, antifungals, CNS drugs, antihistamines, antihypertensives, protease inhibitors and xenobiotics. Since MDR1 is expressed at many organ boundaries, it is a key determinant in drug and xenobiotic disposition and in the toxicity of such compounds (59). MRP2 exports drugs, xenobiotics and numerous phase II metabolites (58,60,61) and ABCG2 transports a long list of drugs and also toxins (62-64). In hepatocytes, drug and drug metabolite excretion also occurs back into the portal plasma. This step is mediated by MRP3 and MRP4, which mediate export of drugs including some antimetabolites and drug metabolites across the basolateral plasma membrane of hepatocytes (65).

In summary, the hepatocyte is equipped with an intricate network of uptake and efflux transporters for drugs and their metabolites. Drug metabolism both biotransforms and generates substrates of these transporters. Consequently, transport and metabolism are linked in a complex network via substrate levels and processes that regulate gene expression (66).

Mechanisms of BSEP Inhibition

Inhibition of BSEP leads to a reduction of biliary bile salt secretion and bile flow as well as to accumulation of bile salts within hepatocytes. If the inhibition persists, BSEP inhibition leads to acquired clinical cholestasis, often of the bland type. Drug examples originally shown to inhibit human and rat BSEP/Bsep are cyclosporine, rifampicin, rifamycin, glibenclamide or bosentan (67-71). All these drugs have been shown to lead to acquired cholestasis in susceptible patients. The cholestatic mechanism of the dual endothelin receptor antagonist bosentan has been worked out in considerable detail. Bosentan and its main metabolite enter hepatocytes via uptake through OATP1B1 and OATP1B3 and bosentan is eliminated by biliary excretion (72). During clinical trials, bosentan was found to cause reversible,

asymptomatic transaminase elevations in some patients (73). In the same patients, plasma bile salt levels increased in proportion to the dose of bosentan. In rats, bosentan treatment also leads to an elevation of plasma bile salt levels, which is more pronounced after coadministration of glibenclamide (73). Both, rat and human Bsep/BSEP are competitively inhibited by bosentan, which strongly suggests that in vivo - in patients with elevated bile salts - bosentan has the same principle of action. In these patients, no elevation of bilirubin was observed (73), which supports the concept of a specific bosentan - BSEP interaction. Further investigation of bosentan-induced cholestasis in rats revealed a more complex mechanism of bosentan-induced cholestasis. In rats, administration of bosentan leads to a stimulation of bile flow (74) This is not in line with the basic concept of cholestasis, which is defined by a reduction of bile flow. In this animal study, no impact of bosentan on bile salt output was observed, but a reduction in biliary phospholipid secretion (74). Increased canalicular bile flow with constant bile salt output leads to a lower intracanalicular bile salt concentration. As biliary phospholipid secretion is critically dependent on canalicular bile salt concentrations (31), a reduction in biliary lipid secretion might adversely alter the lipid asymmetry of the canalicular membrane, which in turn could lead to an intracellular accumulation of bile salts (75). This choleric effect of bosentan in rats is dependent on the presence of functional Mrp2 in the canalicular membrane (74). Indeed, while the transport activity of rat and human BSEP is inhibited in Sf9 cell vesicles by bosentan, this drug stimulates the transport activity of rat and human MRP2 (76). The list of drugs that have until now been demonstrated to inhibit BSEP is long (77,78). Morgan and coworkers demonstrated that drugs with IC₅₀ values in the low μM range towards BSEP are known to have clinical liabilities (77). Dawson and coworkers found no clear distinction between unbound plasma C_{max} values, IC₅₀ values and DILI in man (78), supporting the concept that other factors such as for example drug uptake into hepatocytes contribute to the development of acquired cholestasis.

Troglitazone is a drug, which was withdrawn from the market as a consequence of severe hepatotoxicity. The detailed mechanism of troglitazone toxicity remains somewhat elusive, but involvement of mitochondrial toxicity has emerged as an agreed concept (79,80). In rats, the main metabolite of troglitazone is troglitazone sulphate, which is eliminated into bile (81). Administration of troglitazone to rats leads to a reduction of bile flow, i.e. cholestasis (82). Troglitazone and its sulphated metabolite are potent inhibitors of rat Bsep (83). Consequently, troglitazone, which is also an inhibitor of dog and human Bsep/BSEP (84), can lead to an

accumulation of bile salts in hepatocytes, which in turn are toxic to mitochondria (29). This process aggravates the negative impact of troglitazone to mitochondria and leads to mixed (hepatocellular and cholestatic) liver injury. The thiazolidinediones, rosiglitazone and ciglitazone, inhibit bile salt transport into rat canalicular plasma membrane vesicles, suggesting a class effect on Bsep inhibition (85). Indeed, rosiglitazone is also associated with liver injury (86).

Oral contraceptives may lead to acquired cholestasis (87,88) and steroid metabolites are associated with the onset of intrahepatic cholestasis of pregnancy (87,89-92). Application of estradiol-17 β -glucuronide and progesterone sulfate leads to acute cholestasis in rats (93,94). Both steroid metabolites do not inhibit Bsep expressed in Sf9 cells, but require the coexpression of Mrp2 (67,94,95), indicating *trans*inhibition of Bsep. An alternative explanation for this finding is an interaction of Mrp2 with Bsep in the canalicular membrane that is fostered by estradiol-17 β -glucuronide and leads to Bsep inhibition (96). The epidermal growth-factor receptor (HER1/HER2) inhibitor PKI166 has been reported to exhibit a similar pattern of inhibition (97).

Impact of Elevated Intracellular Bile Salt Levels on Transporter Expression

Inhibition of BSEP by drugs in susceptible patients may lead to bland cholestasis or may aggravate cholestasis with subsequent mixed liver injury. The mechanism of BSEP inhibition can either be competitive - i.e. direct - or may require the action of an additional transporter, such as Mrp2 and may consequently be indirect. Any impairment of BSEP will lead to an intracellular accumulation of bile salts. Within hepatocytes, bile salts activate a bile salt sensor, the nuclear transcription factor farnesoid-X-receptor (FXR, *NRIH4*) (98,99). FXR acts as a key regulator of metabolic processes (100). Generally, activation of FXR by increased bile salt concentrations in hepatocytes down-regulates the uptake systems for bile salts and maintains or upregulates bile salt efflux systems. (101). FXR upregulates the expression of small heterodimer partner (SHP), which in turn directly represses the expression of rat Ntcp (102) and down-regulates the expression of human NCTP indirectly via the glucocorticoid receptor (103). The impact of FXR on the expression of *SLCO1B1* has been reported with conflicting results. While an earlier publication reported HNF1-mediated down-regulation of OATP1B1 expression after activation of FXR (104,105), thereby explaining the decrease in OATP1B1 mRNA expression that was found in liver tissue from patients with cholestatic liver disease (106), another group reported a direct activation of the expression of OATP1B1

after pharmacologic activation of FXR (107). To our knowledge, FXR dependent regulation of the expression of OATP2B1 has not yet been reported and unpublished data from our group showed a complex response element within the 5' flanking region of the human *SLCO2B1* gene that appear to be repressed by monomeric FXR (Eloranta and Kullak-Ublick, unpublished) (108). The promoter of *SLCO1B3* coding for the third hepatocellular OATP is directly transactivated by FXR (109). The *ABCB11* gene is also subject to direct transactivation by FXR (110) and *ABCC2* transcription also appears to be responsive to FXR activation (111). The ligand binding to FXR is rather specific and to date, only a limited number of synthetic agonists exist (112,113). Bile salts are derived from cholesterol, which in turn may also be metabolically converted to oxysterols. (114). Oxysterols activate the liver X receptors (LXR,) LXR α (NR1H3) and LXR β (NR1H2) (112). The promoter of *SLCO1B1* has been shown to contain a LXR α response element (107). Expression of BSEP is also upregulated by oxysterols, but this regulation involves FXR rather than LXR (115). Drugs and xenobiotics are ligands of the pregnane X receptor (PXR, *NR1I3*) and/or of the constitutive androstane receptor (CAR, *NR1I2*) (112,113,116). Next to activation of phase I and phase II detoxification processes, these nuclear receptors also regulate transporter expression. So far, direct activation by PXR could only be shown for the rat *Slco1a4* gene (117). However, OATPs mediate uptake of a considerable number of PXR or CAR ligands and as such are indirectly involved in the regulation of drug and xenobiotic metabolism in hepatocytes (118). At the transcriptional level, the expression of MRP2 is positively regulated by both PXR and CAR (111,119). This may help to protect hepatocytes from potentially toxic metabolites of drugs and xenobiotics.

Data on alterations of transporter expression levels in human liver tissue from patients with drug-induced cholestasis have not been published. However, information on transporter expression in other forms of liver disease, including cholestatic liver disease has been investigated. Patients suffering from progressive familial intrahepatic cholestasis, a severe cholestatic liver disease, show unchanged NTCP expression at the mRNA level but reduced protein levels (120). In patients with biliary atresia, a form of obstructive cholestasis, NTCP mRNA is decreased, but increases after restoration of bile flow (106,121,122). In primary biliary cirrhosis (PBC), NTCP expression is not affected in early stages, but is decreased in late stages (106,123,124). Whereas patients with chronic hepatitis C do not display altered NTCP expression, cholestatic hepatitis leads to reduced NTCP expression (106). After liver transplantation, both the expression of NTCP mRNA as well as hepatic bile salt output are

increased (125). In patients with advanced PBC or icteric cholestasis OATP1B1 shows lower expression than in controls (106,124). OATP1B1 and OATP1B3 are also decreased in late stages of PBC (123) and OATP1B1 was found to be reduced in primary sclerosing cholangitis (PSC)(126). In patients suffering from obstructive cholestasis predominantly as a consequence of cancer of the biliary tract or of the pancreas, successful percutaneous drainage leads to a mildlyfuzzy canalicular staining, while poorly drained patients show reduced and fuzzy canalicular BSEP staining and a slight decrease of BSEP mRNA (127). Pediatric patients with biliary atresia have no change of BSEP protein, but BSEP mRNA is decreased to an extent that depends on the stage of the disease (128). BSEP is down-regulated in patients with inflammatory cholestatic liver disease (106), but appears to be preserved in PBC (123,124). In fact, induction of BSEP has even been reported in PBC (129). The organic anion and drug-metabolite efflux pump MRP2 is down-regulated in primary sclerosing cholangitis primary sclerosing cholangitis (126) and is decreased in late stages of PBC (123,130) but was found to be preserved in other studies (124). Icteric cholestasis lowers expression of MRP2, while its mRNA was found to be unchanged (106), a finding that could not be confirmed in a different study (129) and was found to be variable in a third study (131).

Taken together, uptake systems in hepatocytes tend to be down-regulated in human hepatocytes under various forms of liver disease, while canalicular export pumps usually are preserved in such conditions. These findings in humans are mirrored in many animal studies with models for liver disease and have been summarized elsewhere (132).

Susceptibility Factors for Drug Induced Cholestasis

While drug induced cholestasis (DIC) is a relevant clinical entity, the risk for incurring (DIC) is very low (133). However, identification of risk factors that render patients susceptible to DIC bears the potential to further reduce the incidence of DIC, which in turn will improve the outcome and the quality of life of patients and lower the financial burden to the health care systems. Our understanding of the mechanisms of DIC in humans has advanced considerably in recent years. For ethical reasons, any hypothesis developed can only be tested in animals, which may or may not apply to the human situation. This is exemplified by progressive familial intrahepatic cholestasis type 2 (PFIC2). These patients have mutations in the *ABCB11* gene coding for BSEP (134) and develop severe liver disease. In contrast, mice with a disrupted *Abcb11* gene have only a mildly impaired liver function (135).

BSEP is a likely susceptibility factor for DIC, as impaired BSEP function is an underlying molecular mechanism of hepatotoxic adverse drug events. Analysis of the protein expression of several canalicular ABC transporters revealed a considerable interindividual variability of the expression of BSEP in healthy human liver tissue (136). Individuals with lower protein levels of BSEP could be at risk for developing DIC (27). The hypothesis behind this concept is supported by a patient with benign recurrent BSEP deficiency syndrome. This patient is a compound heterozygote for two mutations in the *ABCB11* gene, E297G and R432T (137). In Sf9 cells, both mutations show only residual transport activity. Thus, although functional BSEP capacity may have sufficed to uphold bile salt excretion under normal physiologic situations, the threshold for inhibition of BSEP activity and bile salt export from hepatocytes was lower. One of the cholestatic episodes of this patient was putatively linked to nonsteroidal anti-inflammatory drugs, which are known to be a challenge for hepatocytes. While multiple studies on *ABCB11* gene polymorphisms and variants have been published, the nonsynonymous c.1331T>C and c.2029A>G polymorphisms in exons 13 and 17, respectively are consistently reported with frequencies greater than 0.5 % in independent cohorts (138-142). Genotyping of the individuals contributing to the cohort of liver samples used for expression analysis revealed that individuals carrying at least one c.1331C allele tended to have lower BSEP expression levels (136). As individuals homozygous for the c.1331C allele were all found in the low range of BSEP expression, this allele could potentially be a susceptibility factor for DIC and acquired cholestasis. Kinetic characterization of the two BSEP variants in the Sf9 cell expression system revealed no difference (140). An association of the c.1331C allele with acquired cholestasis was confirmed in patient cohorts with DIC (140) and cholestasis of pregnancy (143,144). Interestingly, the same polymorphism correlates with progression to cirrhosis in hepatitis C genotype 2 and 3 patients (145) and shows an association with a lower sustained viral response for hepatitis C genotype 2 and 3 (146). The underlying mechanism could be a positive correlation between intracellular bile salt levels and hepatitis virus C replication (101). An association of the c.1331C allele with acquired forms of cholestasis has also been found in several case reports (12,70). However, a study on a Swedish cohort of women presenting with cholestasis of pregnancy found no association with common *ABCB11* haplotypes (147). Methodologically, this study analyzed haplotype distribution, while all the previous studies investigated single nucleotide polymorphisms.

To our knowledge, up to now no studies analyzing specifically the role of MRP2 variants in the susceptibility to DIC have been reported. However, *ABCC2* haplotypes have been associated with liver injury induced by drugs or herbal remedies (148) and with diclofenac induced hepatotoxicity (149). These findings make it likely that an association of MRP2 variants with susceptibility to DIC is possible. MRP2 variants have also been associated with altered drug disposition (150). With respect to *ABCG2*, MRP3 and MRP4, pharmacogenetic studies on the role of *ABCG2*, *ABCC3* and *ABCC4* in drug disposition or efficacy showed conflicting results (150). Hence, a potential role of *ABCG2*, MRP3 and MRP4 variants in DIC remains to be investigated. Its capacity to transport a wide variety of drugs and its impact on the disposition of its substrates has led to many studies that have investigated the pharmacogenetics of MDR1 in relation to drug disposition (150). The results of these studies have been highly inconsistent, in some cases contradictory. A majority of recent meta-analyses found no impact of the synonymous c.3435C>T polymorphism of *ABCB1* on pharmacokinetics and pharmacodynamics as well as susceptibility to diseases (150). Consequently, it seems fair to assume that MDR1 is not an important susceptibility factor for DIC. Additional genetic risk factors for DIC are genes coding for phase I and phase II enzymes of drug metabolism (2,37,151).

Even the strongest known associations between genotype and the phenotype of DILI (e.g. the HLA-B*5701 genotype in flucloxacillin-induced liver injury (152) ultimately account for only a small fraction of the overall risk for the phenotype in question to occur. Additional factors such as drug doses, preexisting liver disease, sex, age and lifestyle constitute additional susceptibility factors for DIC (2,56,153,154). The low overall incidence of this adverse drug reaction makes the assessment of the relative importance of each individual risk factor difficult. This is where systematic pharmacoepidemiological studies will be of value, since they combine an extensive survey of patient risk constellations with the clinical appraisal of individual cases, in tribute to the observation that no two cases of DILI or DIC are ever the same.

Outlook

The impairment of membrane transport is now recognized as an important mechanism in the pathogenesis of DILI, notably drug-induced cholestasis. Several hepatotoxic drugs have been shown to inhibit BSEP, although additional mechanisms such as mitochondrial toxicity and immune-mediated liver damage are usually required for clinically severe liver injury to occur.

Of all the transporters characterized to date, BSEP is the best characterized in connection with a cholestatic pattern of injury. The V444A genetic variant, which is common in the population, appears to confer a risk for drug-induced and other forms of acquired cholestasis, although other BSEP variants have also been identified. The role of the phospholipid translocator MDR3 in drug-induced cholestasis is still unclear. A functional *in vitro* assay for MDR3 transport activity is not available to date, making inhibition studies difficult. It seems likely that bile duct injury inflicted by drugs may in some cases be caused by inhibition of MDR3 mediated phospholipid excretion into the bile ducts, but this area of research remains a challenge for the future. In addition to the expression and genotype of transporters themselves, the intricate network of transcriptional and epigenetic regulatory mechanisms that affect transporter activity are under intense investigation and new mechanisms by which drugs can adversely affect membrane transport are likely to emerge from this line of study. To date, functional testing of BSEP and MRP2 inhibition by drugs is commonly required by the FDA in the context of liver signals during drug development, but interpretation of the results still requires an exact analysis of the individual clinical presentation in connection with the *in vitro* findings.

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