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A Change in Bile Flow: Looking Beyond Transporter Inhibition in the Development of Drug-induced Cholestasis

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Abstract

Background: Drug-induced Liver Injury (DILI) has received increasing attention over the past decades, as it represents the leading cause of drug failure and attrition. One of the most prevalent and severe forms of DILI involves the toxic accumulation of bile acids in the liver, known as Drug-induced Cholestasis (DIC). Traditionally, DIC is studied by exploring the inhibition of hepatic transporters such as Bile Salt Export Pump (BSEP) and multidrug resistance-associated proteins, predominantly through vesicular transport assays. Although this approach has identified numerous drugs that alter bile flow, many DIC drugs do not demonstrate prototypical transporter inhibition, but rather are associated with alternative mechanisms.

Methods: We undertook a focused literature search on DIC and biliary transporters and analyzed peer-reviewed publications over the past two decades or so.

Results: We have summarized the current perception regarding DIC, biliary transporters, and transcriptional regulation of bile acid homeostasis. A growing body of literature aimed to identify alternative mechanisms in the development of DIC has been evaluated. This review also highlights current *in vitro* approaches used for prediction of DIC.

Conclusion: Efforts have continued to focus on BSEP, as it is the primary route for hepatic biliary clearance. In addition to inhibition, drug-induced BSEP repression or the combination of these two has emerged as important alternative mechanisms leading to DIC. Furthermore, there

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has been an evolution in the approaches to studying DIC including 3D cell cultures and computational modeling.

Keywords

Bile salt export pump (BSEP); Drug-induced liver injury (DILI); Drug-induced cholestasis (DIC); inhibition; repression; farnesoid X receptor

1. INTRODUCTION

1.1. Drug Induced Liver Injury

As the major detoxification organ in the body, the liver is challenged by both endogenous and exogenous chemicals daily [1]. Therefore, it is not surprising that Drug-Induced Liver Injury (DILI) has become a significant concern for pharmaceutical industries, regulatory agencies, and the medical community as a whole. Currently, DILI represents the primary cause of drug attrition due to safety, both during development and post-marketing. Historically, there have been over 1000 drugs linked to DILI [2], including antibiotics, central nervous system agents, antineoplastics, immunomodulatory agents and herbal medicines [2–5]. Studies regarding DILI have become heightened topics over the past two decades, ranging from diagnosis and biomarker identification to mechanistic causality and preclinical prediction [6–10]. Notably, despite advances made in DILI research, there are newly approved drugs whose DILI liability was not predicted during drug development and clinical trials that led to liver injury during post-marketing.

DILI is a multifaceted disease, which can manifest similarly to that of acute and chronic liver diseases, complicating its diagnosis and treatment. When DILI is dose-dependent and predictable, it is referred to as intrinsic DILI. The less common but more concerning form of DILI is unpredictable and idiosyncratic, which turns to be the focus of most DILI researches. Annually, more than half of the cases of acute liver failure reported are associated with intrinsic DILI; however, because this is typically caused by exceeding the recommended drug dosages, lowering the dose or complete cessation alleviates symptoms. On the other hand, idiosyncratic DILI may take longer to present clinically, and manifests in a variety of ways, ranging from no symptom (with transient enzyme elevation) to complete liver failure [11]. Idiosyncratic DILI is sometimes referred to as a disease of exclusion, as it is only diagnosed once other forms of liver disease have been ruled out. This is a long, inefficient, and costly process, which has prompted increasing research efforts into the development of quicker and more accurate diagnostic tools. Treatment of idiosyncratic DILI can also be challenging without a clear understanding of the mechanism(s) by which a drug-induced injury occurs. Without dose-dependent predictability, removing the drug completely is typically the safest approach; however, there may be lingering effects of the drug if not caught soon enough, therefore early diagnosis is pivotal.

Identifying DILI in a timely manner is important for drugs already on the market, making their way into the hands of patients; nevertheless, avoiding future hepatotoxic drugs in drug discovery and development is more desirable. The FDA released guidance in 2009 to assist pharmaceutical companies in evaluating the potential of pre-marketed drugs to develop

severe hepatotoxicity [12]. The guidance provides recommendations on how to identify the potential for liver injury in mild serum enzyme elevations, which could result in severe DILI when introduced to the general population. Nevertheless, it is not always feasible to capture the potential of severe DILI in the later stages of development (clinical trials), therefore the burden shifts to developing better predictive means preclinically.

1.2. Drug-induced Cholestasis

While DILI can mimic any form of liver diseases, the histopathological manifestations often define the type of DILI experienced. There are five main classes of DILI, as reported by the drug-induced liver injury network, including necrosis, cholestasis, steatosis, vascular injury patterns, and cytoplasmic alterations [13]. However, when considered individually, cholestatic liver injury accounts for most cases of DILI reported [14]. Cholestatic liver injury (which includes mixed hepatocellular/cholestatic type) is the most severe form, with increasing occurrence. Clinically cholestasis is characterized by an elevation in alkaline phosphatase and bilirubin levels in the blood, compared to hepatocellular injury, which typically experiences elevations in alanine aminotransferase levels [15].

A hallmark of Drug-induced Cholestasis (DIC) is the disruption of bile flow, whether metabolically or mechanically by drug exposure that results in the toxic accumulation of bile acids in the liver. While essential to the body, bile acids are amphipathic molecules, which can become toxic if they persist in tissues with high concentration for prolonged periods. Bile acids are derived from the catabolism of cholesterol in the liver [16], and there is a spectrum of bile acids in both human and animals, ranging from primary bile acids formed *de novo*, to their metabolites, the secondary and tertiary bile acids [17]. The two primary bile acids, Cholic Acid (CA) and Chenodeoxycholic Acid (CDCA), are formed solely in the liver via the classical or alternative pathways of cholesterol catabolism [18, 19]. The Cholesterol-7 α -hydroxylase (CYP7A1) is the rate-limiting enzyme responsible for cholesterol breakdown and represents the classical pathway of bile acid formation, alternatively, the mitochondrial sterol 27 hydroxylase (CYP27A1) makes up the rest [19]. Conjugation of CA and CDCA in the liver with glycine or taurine forms Glyco- or tauro-cholic Acid (GCA or TCA) and Glyco- or tauro- chenodeoxycholic Acid (GCDCA or TCDCA) and aids in their excretion from the liver, through the bile canaliculi, to the gallbladder [20, 21].

1.3. Bile Acid Homeostasis

The intracellular bile acid homeostasis is tightly controlled by the balanced biosynthesis and transport. Upon ingestion of food, bile acids are released from the gallbladder into the small intestine, where they can be deconjugated and dehydroxylated by the bacterial flora, creating the secondary bile acids deoxycholic acid (DCA; formed from CA) and lithocholic acid (LCA; formed from CDCA) [19, 22, 23]. Finally, the transformation of LCA in the liver or intestine results in the formation of the tertiary bile acids sulfolithocholic acid and ursodeoxycholic acid [23].

Although bile acids are best known for their role in absorbing vitamins and fats from the intestine, they also play important roles in the regulation of glucose and lipid metabolism,

excretion of endogenous metabolites and xenobiotics, and activation of signaling cascades by binding to various receptors [24, 25]. Many of these functions are physiologically interrelated. For example, activation of the nuclear receptor Farnesoid-x-receptor (FXR) by CA or CDCA leads to the regulation of glucose and lipid metabolism, liver regeneration, and hepatocarcinogenesis [26–31].

The most abundant bile acids in humans are CA, CDCA, and DCA, with the other species contributing minimally to the bile acid pool [32]. Bile acid levels in the body are maintained via the mechanisms of *de novo* synthesis, as discussed above, and enterohepatic circulation. The majority of the bile acid pool (over 90%), which is ~2–3 g in an adult [33, 34], is recycled from the intestines in a process known as enterohepatic circulation, limiting the amount of bile acids synthesized *de novo* each day to 200–600 mg [34]. This recycling of bile acids is vital to ensuring bile acid homeostasis, which is necessary for its variety of endogenous functions.

Enterohepatic circulation maintains the flow of bile acids from the liver where they are synthesized, to the gallbladder for storage, and eventually, the small intestines where they aid in the absorption of dietary fats and vitamins [35, 36]. Once biliary micelles contain these lipids, they are shuttled back to the liver where they can deposit their cargos and start the process again [36]. As the bile acids individually and in the form of micelles can inflict toxic effects, this flow is essential not only to the absorption function but also to the protection of biliary tissue from toxic accumulation of bile acids.

The nuclear receptor FXR has been identified as the master regulator for bile homeostasis and enterohepatic circulation [37, 38]. Bile acids represent the predominant class of endogenous FXR ligands with high affinity. When intrahepatic levels of bile acids are elevated, activated FXR induces expression of the Small Heterodimer Partner (SHP), a potent transcriptional repressor, which in turn downregulates the transcription of *CYP7A1* and *CYP8B1* genes [39–41]. Meanwhile, the bile acid-bound FXR also forms a heterodimer with the Retinoic X Receptor (RXR) [42, 43] and directly binds to the promoter of Bile Salt Export Pump (BSEP) to stimulate gene transcription and efflux of bile [44, 45]. Additionally, activation of FXR can induce the expression of the ileal bile acid binding protein, which facilitates the transport of bile acids through enterocytes to the portal blood system [46]. Interestingly, regional activation of FXR in the intestine also increases the level of Fibroblast Growth Factor 19 (FGF19) in the circulation, which facilitates the recruitment of the SHP complex to CYP7A1 promoter and suppresses bile acid synthesis [42, 47].

2. BILIARY TRANSPORT

In order to maintain bile acid homeostasis and facilitate their flow through respective tissues, several biliary transporters are expressed in related cellular compartments. Generally, bile acids are taken up into the liver by the sodium-taurocholate cotransporter polypeptide (NTCP; SLC10A1) and to a lesser extent by the organic anion transporter proteins (OATPs; SLCO 1B1, 1B3 and 2B1) located on the basolateral (sinusoidal) membrane of hepatocytes [48, 49] (Fig. (1)). Serving as supportive biliary uptake transporters, OATPs typically transport unconjugated bile acids and other molecules with molecular weights 350

Daltons, including drugs bound to albumin [50]. On the other side, the primary route of canaliculi elimination of bile acids from the liver is through BSEP, which is expressed on the apical (canalicular) membrane of hepatocytes. Another transporter found at the apical membrane is the multi-drug resistance-associated protein 2 (MRP2). In addition to transporting sulfated bile acids, MRP2 is important in excreting bilirubin and glutathione into the bile canaliculi, both essential components of bile [51, 52]. Similarly, multi-drug resistance protein 3 (MDR3) and the p-type ATPase ATP8B1 flippases contribute to bile flow by ensuring phospholipids present in the bile canaliculi [53–55]. MRP3 and MRP4, located on the basolateral membrane, partially contribute to the excretion of bile acids back to the blood-stream [56–58]. Organic cations are needed for bile formation and are transported by the Multi-drug Resistance Protein 1 (MDR1) [59]. Mixed micelles are formed from the bile constituents once they are transported to the bile canaliculi and eventually facilitate lipid absorption from intestines.

From the liver, bile travels through the bile ducts to the gallbladder for storage. The gallbladder releases bile salts into the small intestine upon consumption of a meal to initiate absorption of dietary lipids [60]. The major transporters in the small intestines and colon responsible for biliary transport are the apical sodium-dependent bile-salt transporter (ASBT; SLC10A2) and organic solute transporter α and β heterodimer (OST α/β) [61–63]. Once recycled through the small intestine, bile acids travel back to the liver through the portal vein. The liver plays an essential role in bile acid homeostasis, as enterohepatic circulation primarily begins and ends in the liver. As mentioned above, there are various hepatic and intestinal transporters (Fig. (1) which coordinately balance the flow of bile acids and all other constituents that make up the bile [59]. Among others, two hepatic transporter proteins, NTCP and BSEP, located on the basolateral and canalicular membrane of hepatocytes, respectively, represent the most predominant force controlling the trafficking of bile acids in and out of the liver. Disruption of the hepatic bile acid flow through these transporters is a key characteristic of DIC.

2.1. NTCP and Bile Acid Homeostasis

The sodium-dependent taurocholate cotransporter polypeptide, NTCP, was first identified in 1978 and is the first member of its family of transporters [64]. As the name suggests, NTCP requires the selective transport of sodium ions in order to transport bile acids [65]. Specifically, two sodium ions must be present for biliary transportation, and transport occurs in the same direction. While NTCP is selective for sodium-dependent transport of taurocholate, it can co-transport a range of substrates, including hormones and drugs. The affinity of NTCP for drugs allows competitive inhibition of bile acid transport, in addition to potential allosteric inhibition away from the active site [65, 66]. NTCP is responsible for uptaking approximately 80% of conjugated bile acids, therefore disruption of NTCP activity can have significant effects on bile acid homeostasis [66].

Cholestasis can be defined as bile acid accumulation in the liver or blood, indicating a disruption to bile flow. When referring to the hepatic accumulation, it is expected that efflux transporters are the major players; however, accumulation of bile acids in the blood could indicate interference in hepatic uptake of bile acids. Bile acids are primarily taken up into

the liver by NTCP on the basolateral membrane, therefore inhibition of this transporter has been studied as a potential mechanism of DIC [67]. Although there may not be a direct correlation between NTCP inhibition and DILI potential [68], as seen with efflux biliary transporters, there may be more than one mechanism contributing to the development of cholestasis, and the impact of NTCP inhibition should not be ignored.

2.2. BSEP and DIC

The longtime pursuit of identifying a selective canalicular bile salt transport has led to the cloning of the full-length of bile salt export pump (BSEP, ABCB11) in rats in 1998 [69]. It turns out to be a “sister of P-glycoprotein” and shares 88% sequence homology with MDR1 when the sequence was characterized. As a member of the ATP-binding Cassette (ABC) superfamily of the transporter, BSEP requires ATP as an energy source for active biliary excretion of bile acids against a steep concentration gradient. Similar to other ABC family members, the BSEP protein contains twelve transmembrane domains, which form two motifs referred to as the Walker A and B motifs [69, 70]. Transport of bile acids occurs through the tunnel-like structure created from the two six-transmembrane domain motifs. Substrates preferred by BSEP include the conjugated monovalent bile acids with high specificity, and a handful of drugs such as pravastatin and vinblastine, which could also function as competitive inhibitors for BSEP though generally with low affinity in comparison to the endogenous bile acids [71]. As the rate-limiting mechanism for the biliary excretion of the major bile acid species from hepatocytes, BSEP represents a major target for numerous cholestatic drugs.

2.2.1. Inhibition of BSEP Activity—Direct inhibition of the efflux activity of BSEP by drugs and their metabolites is generally regarded as one of the molecular mechanisms for the development of DIC. Many drugs such as bosentan, erythromycin, troglitazone, cyclosporine A, and nefazodone, demonstrating clinical cholestatic features, are experimentally associated with inhibition of BSEP activity [67, 72, 73]. Using membrane vesicles harvested from BSEP-transfected insect cells, Morgan *et al* assessed 200 pharmaceutical compounds including marketed and withdrawn drugs for their capacity of BSEP inhibition [74]. In this study, a strong correlation between the potency for BSEP inhibition and human liver injury was observed and nearly all drugs with an IC₅₀ value of 25 µM are associated with liver liabilities in humans reported elsewhere. Building upon these observed correlations between BSEP inhibition and DILI, Ogimura *et al.* further investigated whether drug-induced BSEP dysfunction indeed results in hepatotoxicity by using sandwich-cultured hepatocytes treated with test drugs in the presence or absence of bile acids [75]. Interestingly, many of the test compounds that are known BSEP inhibitors such as ritonavir, cyclosporine A, simvastatin, and troglitazone were not cytotoxic at the tested concentrations when bile acids were absent. These BSEP inhibitors, however, demonstrated significant cytotoxicity in hepatocytes in the presence of bile acids, suggesting drug-induced BSEP dysfunction could lead to cholestatic liver injury.

2.2.2. Repression of BSEP Expression—As the primary path of biliary excretion from hepatocytes, expression of BSEP is tightly controlled at both transcriptional and post-translational levels. At approximately the same time that BSEP was cloned, a number of

single nucleotide mutations of this gene were linked with the incidence of Progressive Familial Intrahepatic Cholestasis 2 (PFIC2). PFIC2 is characterized by early-onset cholestasis soon after birth with high serum bile acid concentration and low biliary bile salts [76]. Further analysis revealed that these mutations identified in PFIC2 patients are often associated with the unexpected introduction of stop codons that result in early termination of protein synthesis (*e.g.* R575X, R1057X) or nucleotide insertion/deletion, which lead to a frameshift in BSEP translation (*e.g.* c.2783_2787dup5) [77]. Notably, while severe impairment of BSEP expression/function could lead to PFIC2, this type of genetic variants are relatively rare; most BSEP Single Nucleotide Polymorphisms (SNPs) only moderately influence the expression/activity of BSEP and carriers of these SNPs are generally normal without cholestatic drug challenge. Accumulating evidence, however, indicates that these alleles could be additional risk factors that predispose carriers to DIC [78, 79]. At the transcriptional level, nuclear receptors such as FXR, Nuclear-factor-erythroid-2-like 2 (NRF2), Peroxisome Proliferator-activated Receptor- α (PPAR α), Pregnane X Receptor (PXR), NAD-dependent deacetylase Sirtuin-1 (SIRT1), and Liver Receptor Homolog 1 (LRH1) have previously been reported to regulate the inductive expression of BSEP in the liver, with FXR being the most well-characterized [44, 80–84]. Activation of FXR by endogenous bile acids creates a feed-forward mechanism of BSEP regulation, as excess levels of bile acids induce BSEP expression, allowing it to facilitate hepatic bile acid homeostasis [70]. Interestingly, although bile acids such as CDCA, DCA, CA, and their conjugates are known agonists of FXR, with CDCA being the most potent one, the secondary bile acid, LCA functions as an antagonist of FXR [85]. In human primary hepatocytes and HepG2 cells, exposure to LCA effectively repressed CDCA- and GW4064 (a synthetic FXR agonist)-induced FXR activation and BSEP expression. This finding may provide mechanistic insights for the long-standing question of LCA-induced cholestasis in which down-regulation of BSEP leads to the toxic effects of LCA [85].

Regulation of BSEP through FXR, the primary bile acid sensor, has been mechanistically linked to the activation of SIRT1, a deacetylase that mediates hepatic energy homeostasis and bile acid metabolism [86, 87]. Under metabolic disease conditions and DIC, acetylation of lysine 217 of FXR was often abnormally elevated, which inhibits its DNA binding capacity and transactivation activity [86]. Activation of SIRT1 by small molecules has been shown to efficiently deacetylate FXR and reverse certain metabolic disorders as well as DIC [87–89]. Hepatocyte specific deficiency of SIRT1, in contrast, was reported to repress the expression of FXR by limiting Hepatic Nuclear Factor1 (HNF1)-mediated transcription [90]. In α -naphthyl isothiocyanate- and thioacetamide-induced cholestatic models, celastrol, a herbal activator of SIRT1, efficiently alleviated the liver damage, while co-administration of SIRT1 inhibitors diminished such protective effects [91]. Although most studies thus far support a beneficial role of SIRT1 activators in DIC management, the SIRT1-FXR signaling is a tightly-controlled and finely-tuned process in the liver. Overexpression of SIRT1 and sustained SIRT1 activation, on the other hand, may lead to ubiquitination/proteasome degradation of FXR, and cholestatic liver injury thereafter [92].

Previously, we analyzed the mRNA expression of 30 drugs with a BSEP IC₅₀ 25 μ M, selected from Morgan's report [74], in sandwich-cultured human primary hepatocytes [93]. Our results indicate that BSEP inhibitors that also reduce the expression of BSEP are often

associated with severe manifestations of DILI, leading to black box warnings and drug withdrawal from the market. This data suggest that similar to protein inhibition, repression of *BSEP* gene expression can lead to diminished overall biliary efflux of bile acids. In another study assessing protein expression in liver biopsies from 23 patients with DILI, Zollner *et al.* observed that 8 out of 12 patients with DIC exhibit marked reduction of BSEP expression [94]. It is worth mentioning that although direct inhibition of BSEP function represents a hallmark mechanism of DIC, inhibition alone cannot fully explain BSEP-associated cholestatic DILI. Indeed, both cholestatic BSEP non-inhibitors such as chlorpropamide and tolbutamide as well as non-cholestatic BSEP inhibitors such as finasteride and oxybutynin were reported previously, suggesting additional mechanisms including transcriptional repression of BSEP may contribute significantly to DIC [74, 95, 96].

2.2.3. Internalization of BSEP—Like all other membrane proteins, post-translational modification is required for the proper localization and function of BSEP in the canalicular domain of hepatocytes. To date, many signaling molecules including Cyclic Adenosine Monophosphate (cAMP), Phosphoinositide-3-kinase (PI3K), Protein Kinase C (PKC), and Mitogen-activated Protein Kinases (MAPKs) have been implicated in the regulation of BSEP protein cellular localization [97–99]. Internalization of hepatic BSEP is another mechanism associated with DIC. For instance, in addition to inhibiting BSEP activity through direct competition, cyclosporine A triggers internalization of BSEP from the canalicular membrane into the cell [100]. In another study, treatment of rats with the cholestatic estradiol-17beta-D-glucuronide (E₂17G) resulted in endocytic internalization of BSEP accompanied by an impairment of bile salt excretion [101]. Further analysis revealed that activation of both PKC/p38 and PI3K/ERK1/2 signaling pathways by E₂17G is involved in the cholestatic effects of this estradiol metabolite [102, 103]. Additionally, a number of protein partners such as HS1-associated protein X-1 (HAX-1) and α - μ 2-adaptin can directly bind to BSEP and influence its cellular localization. Disruption of their association with BSEP by dominant negative expression or RNA interference inhibits BSEP internalization [104, 105]. Together these findings provide compelling evidence that drugs can induce cholestatic injuries through internalization of canalicular BSEP by affecting intracellular signaling cascades.

3. *IN VITRO* STRATEGIES TO ASSESS DIC

Comparing to progress made in our understanding of the molecular mechanisms and pathogenesis of DIC, effective prediction of this disorder is significantly lagging behind. Challenges to this dilemma include but not limited to: 1) Many DICs exhibit idiosyncratic features with the lack of dose-dependence and often affect only susceptible individuals. 2) Species-specific composition of bile acid pools and distinct enzyme/protein features between humans and animals limit the value of experimental animal models in DIC assessment. And 3) most routinely used approaches do not fully reflect human physiological and pathophysiological conditions. Although quantitative and accurate prediction of DIC continues to be challenging, accumulating evidence supports that novel mechanism-based human *in vitro* models are promising tools for improved assessment of DIC.

3.1. Inhibition of Efflux Transporter in Membrane Fraction

The most common approach to study the DIC potential of drugs remains the examination of a direct interaction of the drug with biliary transporters. There are a few *in vitro* model systems for investigating transporter inhibition by using cell membrane vesicles obtained from insect oocytes and immortalized cell lines overexpressing selected transporters or from cultured primary hepatocytes.

Insect membrane vesicles provide a simplified system to study drug transport; transporters of interest can be over-expressed and their function be monitored specifically, without other interfering factors [96]. This approach is typically the first effort to identify transporter inhibitors and can subsequently be validated with more complicated systems. Although overexpression in insect membrane vesicles is selective for a transporter of interest, the physiologically relevant interactions of the transporter with its natural environment cannot be investigated. Comparatively, membrane vesicles isolated from human cell lines and liver tissue retain a physiologically appropriate microenvironment for transporter functions allowing more quantifiable measurement of interactions to be obtained. Draw-backs, however, to this system include difficulty in obtaining and procurement of human liver resections, impediment in purifying hepatic plasma membrane with proper orientation, and the inherited variability in the expression and function of transporters from one individual to another [106]. Currently, membrane vesicle systems obtained from both insect and mammalian cells are widely used at the early stage of assessing DIC potentials.

Given the importance of BSEP in bile salt export, inhibition of BSEP function is the key mechanism investigated in the study of DIC. Disruption of bile flow from the liver can result in the accumulation of bile acids, which left untreated, can result in toxicity, including cell death and liver failure. Inhibition of BSEP protein is a straightforward approach often used in drug development and liver toxicity evaluation. Numerous studies have utilized insect membrane vesicles with BSEP over-expression as a foundational technique in identifying potentially cholestatic drugs. The findings from these studies have served as the building block for a host of subsequent investigations. Additionally, although inhibition of MRP2 alone has been linked to drug-induced hyperbilirubinemia and has implications for drug-drug interactions, when combined with BSEP inhibition, it contributes to DIC potentials [107, 108]. The same phenomenon applies to the basolateral efflux transporters, MRP3 and MRP4. Served as compensatory routes of bile acid elimination from the liver, MRP3 and MRP4 are often expressed at low levels, as their direction of efflux is contradictory to typical bile flow. As a result, the consequence of MRP3 and MRP4 inhibition is less severe under normal conditions. When bile acids begin to accumulate in the liver as a result of BSEP inhibition, the compensatory routes turn essential to alleviate bile acid-associated hepatotoxicity. Co-inhibition of these transporters with BSEP often leads to severe DIC [95, 109]. Studies have shown that there are many BSEP inhibitors, which also inhibit MRP3 and/or MRP4 [110].

3.2. Inhibition of Efflux Transporter in Cultured Cells

A well-accepted model for investigating the cholestatic potential of drugs, in a physiologically relevant environment, has been the use of primary hepatocytes in a

sandwich-cultured format. As previously mentioned, properly cultured hepatocytes retain the structure and expression profile of major drug-metabolizing enzymes, transporters, and essential transcription factors in the liver, while affording the benefit of an *in vitro* model. Sandwich-cultured hepatocytes form a configuration that maintains bile canalicular networks and apical localization of efflux transporters for days [111, 112]. Further study revealed that tight junctions, the membrane joints forming the canalicular channel between hepatocytes, could be disrupted in a calcium-dependent manner [113, 114]. Subsequently, this model has been extensively used for *in vitro* investigation of intrinsic biliary clearance and transporter-based drug-drug interactions [115–117].

Commercially available cell lines derived from hepatoma cells are typically allowed for long period culture with continuous passaging capacity. These models provide a relatively stable platform for experiments, making comparisons between assays more reliable. The HepaRG™ cell line, developed from a human liver tumor, has been terminally differentiated to mimic the physiology of human primary hepatocytes [118, 119]. Studies have shown that these cells have the functional benefit of primary hepatocytes, maintaining similar cytochrome P450 and transporter expression and localization [116], while providing the proliferation feature of an immortalized cell line, making them a promising cellular model for cholestatic studies. In one study, BSEP wild-type and knockout HepaRG™ cell lines were exposed to known cholestatic drugs bosentan and troglitazone. Interestingly, BSEP wild-type cells show only marginal effects, while the knockout cells exhibit a more significant disruption to bile acid homeostasis when exposed to these drugs [120].

In addition to HepaRG™, other functional hepatocyte-like cells have been derived using different approaches. Fa2N-4 cells (Pfizer) and HepatoCells (Corning) were immortalized by transducing the simian virus 40 large T antigen into hepatocytes obtained from a 12-year-old and a 9-year-old female donor, respectively [121, 122]. Further studies revealed that these immortalized cells exhibit a number of hepatocyte-featured drug metabolism and transport functions [123, 124]. Multiple human induced functional hepatocytes have also been generated from fibroblasts by ectopically expressing hepatic fate conversion factors such as HNF1A, HNF4A, HNF6, activating transcription factor 5, prospero homeobox protein 1, and CCAAT/enhancer-binding protein alpha [125–127]. The transcriptional factor-induced hepatocytes express adequate functional drug-metabolizing enzymes and transporters that are comparable to human primary hepatocytes [128]. Recently, Burkard *et al* successfully expanded mature human primary hepatocytes up to 40 doublings by introducing a combination of human papilloma virus genes E6 and E7 and oncostatin M [129]. Under optimized culture conditions, these cells display polarization and the formation of canalicular lumen for bile flow [130]. Overall, while each of these cell models has its own advantages and disadvantages, collectively they offer alternative *in vitro* systems in compensating the rather high-cost human primary hepatocytes.

3.3. Repression of Transporter in Cell Culture

Sandwich-cultured human primary hepatocytes can retain the expression and function of a full spectrum of drug-metabolizing enzymes and transporters for days. Thus, these cells are also frequently used for evaluation of drug-induced expression of metabolism and transport

proteins. While enhanced expression of BSEP does not lead to a severe adverse effect on the liver, reduced expression of this transporter has been linked closely to PFIC and other cholestatic conditions [76, 131, 132]. Previously, we have shown that dual inhibition and repression of BSEP is often associated with severe clinically reported DILI, suggesting that in addition to functional inhibition, repression of BSEP expression may play an important role in DIC [93]. Most recently, we have studied metformin, reportedly a cholestatic BSEP non-inhibitor [95], in sandwich-cultured human primary hepatocytes. We found that metformin concentration-dependently repressed the expression of BSEP at both mRNA and protein levels. Intriguingly, while metformin does not directly inhibit BSEP activity in a prototypical short term B-clearance assay (in agreement with Kock's 2014 observation), 72-hour pretreatment of hepatocytes with metformin prior to monitoring biliary clearance, reduced the clearance of [3H]-TCA to 19.22% compared to the vehicle control value of 81.52% (Unpublished data). This finding suggests that while metformin does not inhibit BSEP through direct substrate competition, longer exposure of metformin reduces the expression of BSEP and affects bile clearance thereafter. Thus, we propose that a modified inhibition experiment (repression assay), which includes a 3-day drug pretreatment as depicted in Fig. (2), can be used as a novel approach for functional validation of pure repressors of BSEP as well as other transporters.

3.4. 3D Models for DIC Prediction

Although sandwich-cultured human primary hepatocytes demonstrate promising capacity in evaluating hepatic bile acid homeostasis and DIC, the assays often need be completed within one week and condition of hepatocytes starts deteriorating thereafter [72, 75, 133]. To overcome such shortages, a plethora of physiologically more relevant hepatocyte models have been developed in recent years including three-dimensional (3D) oriented and microfluidics-based hepatocyte cultures, as well as liver-on-chip models [134–137]. Among others, 3D hepatic spheroid culture has emerged as a promising new model that could be easily adopted by many laboratories. To date, a variety of hepatic cells, including primary human hepatocytes and HepaRG™ cells, have been cultured in such a 3D conformation that the cells will exhibit enhanced liver phenotype, increased cell-to-cell contact and extracellular matrix formation, and improved metabolic activity and cell polarity [138, 139]. Importantly, hepatic spheroids can be cultured in 96- and 384-well platforms and maintain their hepatic functions over 28 days in culture. Through a comprehensive evaluation of 110 drugs in 3D spheroids and 2D primary hepatocyte cultures, Proctor *et al* showed that 3D spheroids displayed increased sensitivity in toxicant identification, while comparable specificity when compared with 2D primary hepatocytes [140]. More specifically, Studies by Hendricks and colleagues have shown that hepatocytes and HepaRG™ in a spheroidal configuration can replicate the cholestatic condition and exhibit this phenotype when challenged with known cholestatic drugs but not with non-cholestatic compounds [141]. Notably, prolonged exposure from 8 to 14 days further increased the synergistic toxicity of cholestatic compounds with a nontoxic level of bile acids via a mechanism by which both BSEP inhibition and repression were involved. Most recently, the predictive power of 3D hepatic spheroids in detecting hepatotoxic compounds was further evaluated by analyzing the hepatotoxicity of 123 drugs including 70 clinically liver toxic associated compounds and 53 non-hepatotoxic compounds [142]. This study demonstrated that a chemically defined

spheroid culture of primary human hepatocytes was capable of accurately identifying 69% of all known hepatotoxic drugs without false positives, which seems to be superior than all previously published *in vitro* evaluation regarding the sensitivity and specificity though additional confirmation investigations are needed. These results, combined with other DILI studies using spheroids, demonstrate they are capable of detecting alterations in bile acid homeostasis and can accurately portray, at an *in vitro* level, what may be happening *in vivo*.

CONCLUSION

Drug-induced cholestasis has been a long-term concern of the pharmaceutical field, stimulating an abundance of new research in the past two decades. Although drugs appear to be safe during development and clinical trials, upon release into the market, cases of cholestasis are unexpectedly observed. Many factors contribute to the poor prediction of drug-induced liver injury, including low sample sizes during clinical testing, species differences between pre-clinical *in vivo* models and humans, and incomplete understanding of the mechanisms that contribute to toxicity. In this review, we analyze the emergence of alternative mechanistic pathways and experimental approaches that have contributed significantly to our understanding of DIC and future drug development. While cholestatic screenings previously focused solely on the inhibition of efflux transporters such as BSEP, future studies should take into consideration of both activity and expression of BSEP, as well as the coordinated consequences from the fluctuation of multiple transporters simultaneously or consequently. In addition to deliberating new drug targets, the physiological relevance and accessibility of cellular models used for these studies have been greatly improved. Applying multiple approaches and techniques to capture the wide range of mechanisms responsible for DIC provides the greatest potential for avoiding drug toxicity in pharmaceutical development. With improved understanding of the mechanisms underlying DIC and promising development of analytic techniques, only time will tell whether the recent advances made will lead to a safer pharmaceutical future.

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LIST OF ABBREVIATIONS

ASBT	Apical Sodium-dependent Bile-salt Transporter
BSEP	Bile Salt Export Pump
CA	Cholic Acid
CDCA	Chenodeoxycholic Acid
CYP	Cytochrome P450

DCA	Deoxycholic Acid
DILI	Drug Induced Liver Injury
DIC	Drug Induced Cholestasis
FGF19	Fibroblast Growth Factor 19
FXR	Farnesoid-X-activated Receptor
LCA	Lithocholic Acid
HNF	Hepatocyte Nuclear Factor
NRF2	Nuclear Factor (erythroid-derived 2)-like 2
MDR1	Multidrug Resistance Protein 1
MRP	Multidrug Resistance-associated Protein
NTCP	Na ⁺ -taurocholate Cotransporting Polypeptide
OATP	Organic-anion-transporting Polypeptide
SHP	Small Heterodimer Partner
SIRT1	NAD-dependent Deacetylase Sirtuin-1
RXR	Retinoid X Receptor

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Fig. (1).
Schematic illustration of transporters control bile flow in the liver and intestine.

Fig. (2).

Schematic illustration of cell-based inhibition and repression assays. In both assays are performed after four-day culture of primary hepatocytes. Drug exposure is brief (10 minutes) and executed during the assay itself (inhibition) or pre-incubated for 72 h prior to the start of the assay (repression).