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Bile acids in drug induced liver injury: key players and surrogate markers

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Abstract

Bile acid research has gained great momentum since the role of bile acids as key signaling molecules in the enterohepatic circulation was discovered. Their physiological function in regulating their own homeostasis, as well as energy and lipid metabolism make them interesting targets for the pharmaceutical industry in the context of diseases such as bile acid induced diarrhea, bile acid induced cholestasis or nonalcoholic steatohepatitis. Changes in bile acid homeostasis are also linked to various types of drug-induced liver injury (DILI). However, the key question whether bile acids are surrogate markers for monitoring DILI or key pathogenic players in the onset and progression of DILI is under intense investigation. The purpose of this review is to summarize the different facets of bile acids in the context of normal physiology, hereditary defects of bile acid transport and DILI.

Introduction

The liver is highly exposed to drugs and xenobiotics, maintains the metabolic homeostasis of the body, and is responsible for bile formation and biliary secretion Thus, it is not surprising that the liver is a major target organ of adverse drug reactions. Drug-induced liver injury can be classified as drug induced cholestasis, drug induced hepatitis and mixed type liver injury [1] and has been the most frequent single cause of safety-related drug marketing withdrawals for the past 50 years [2, 3]. DILI can resemble almost any form of acute or chronic liver disease (*e.g.* bland cholestasis, acute cholestatic hepatitis, acute hepatic

necrosis, acute viral hepatitis-like syndrome, immunoallergic hepatitis, cholestatic hepatitis due to medications, autoimmune hepatitis-like injury, sinusoidal obstruction syndrome, microvesicular steatosis with lactic acidosis and hepatic failure, fatty liver disease, chronic hepatitis, vanishing bile duct syndrome), thus necessitating standardized definitions and diagnostic criteria for DILI [4].

Bile acid biosynthesis and conjugation

Bile acids (BAs) are synthesized in the liver from cholesterol [5]. The classical (neutral) pathway is initiated by CYP7A1 catalyzed 7α-hydroxylation, which represents the rate-limiting step in BA biosynthesis, leading to the formation of the two primary BAs cholic acid (CA) and chenodeoxycholic acid (CDCA). CA biosynthesis involves an additional hydroxylation at steroid ring position 12, catalyzed by CYP8B1, which controls the ratio of CA to CDCA formation. The side-chain is finally oxidized and cleaved by CYP27A1 [6]. This classical pathway produces at least 75 % of total BAs [7]. An alternate (acidic) pathway exists that is initiated by CYP27A1 and CYP7B1, leading to the formation of CDCA only [8, 9] [Figure 1]. In rodents, CDCA can be hydroxylated to α-muricholic acid (αMCA) and further epimerized to βMCA, giving rise to a pool of more polar BAs as compared to human [10]. Within the hepatocyte (and to a lesser extent in cholangiocytes), BAs are subjected to conjugation with glycine or taurine, which is catalyzed by the enzymes bile acid:CoA synthase (BACS) and bile acid:amino acid transferase (BAT) [11]. Dogs are predominant taurine-conjugators, rodents can form taurine and glycine conjugates, and non-human primates and humans show a preference for glycine conjugation. It has been shown that human BAT catalyzes BA conjugation with both glycine and taurine, and the extent to which these two conjugates are formed is determined by substrate availability [12]. This may also apply to other species with mixed taurine and glycine conjugation of BAs [13]. Other conjugation reactions such as sulfation and glucuronidation may occur, but are considered less relevant for the elimination of BAs under normal physiological conditions [14-20].

Transport of bile acids

After conjugation, BAs are excreted out of the hepatocyte across the canalicular membrane into the bile canaliculi, a process driven mainly by the bile salt export pump BSEP. This transport is ATP-dependent to facilitate BA excretion against a huge concentration gradient [21]. The relative rank order of intrinsic clearance for human BSEP is taurochenodeoxycholic acid (TCDCA) > taurocholic acid (TCA) > tauroursodeoxycholic acid (TUDCA) > glycocholic acid (GCA) [22-24]. In addition, multidrug resistance-associated protein 2 (MRP2) mediates the transport of divalent BA (*e.g.* sulfated and glucuronidated BA conjugated with taurine or glycine), conjugated bilirubin and glutathione-conjugates into bile [25]. P-glycoprotein (MDR1) appears to be capable to transport BAs across liver canalicular membranes to a certain degree [26], as shown in mice. Alternatively, basolateral export of BAs from hepatocytes and cholangiocytes back into the systemic circulation is mediated by multidrug resistance proteins MRP3/MRP4 and organic solute transporters OST-α/OST-β [21, 25, 27-29]. Cholangiocytes, similar to gallbladder epithelial cells, proximal renal convoluted tubule cells and ileal enterocytes, express the sodium-dependent BA transporter ASBT at their apical membrane domain, thereby allowing for cholehepatic shunting of conjugated BAs back to hepatocytes [30].

Cholangiocytes

The amphipathic nature of bile salts drives the solubilisation of phosphatidylcholine and cholesterol in bile canaliculi by forming mixed micelles, thus protecting cholangiocytes from the detergent properties of the bile salts. During its passage through the biliary tree, the primary hepatic bile is further modified by organic anion and electrolyte transport proteins expressed at the surface of biliary epithelial cells [22]. Cholangiocytes secrete significant amounts of bicarbonate, which raises the local pH at the apical membrane. Beuers *et al.* proposed a concept termed the “biliary bicarbonate umbrella” [31] and hypothesized that biliary bicarbonate secretion near the apical surface of hepatocytes and cholangiocytes maintains an alkaline milieu that keeps the predominantly glycine-conjugated BAs in man in their salt form and therefore in a more polar state. Polarity prevents molecules from passive diffusion through

biological membranes, which could therefore protect the integrity of cholangiocytes and periportal hepatocytes from the toxicity of highly concentrated bile salts. Dysregulation or functional impairment of this biliary bicarbonate umbrella may consequently increase the vulnerability of biliary epithelial cells towards biliary bile salts, and therefore contribute to various forms of chronic cholangiopathies/cholestatic liver diseases [31]. Supporting evidence comes from the anion exchanger AE2, which is expressed on the apical membrane of cholangiocytes and secrets bicarbonate into the biliary lumen in exchange for chloride. AE2 deficient knockout mice develop features resembling primary biliary cirrhosis [32]. Human genetic variants in this gene have been shown to influence susceptibility and severity of primary biliary cirrhosis [33]. In vitro experiments with human cholangiocytes in the presence of millimolar concentrations of CDCA and its glycine- and taurine conjugates confirmed the pH- and AE2-dependent uptake and cytotoxicity of BAs. In addition, these experiments suggested a potential protective role of the intact biliary glycocalyx against pH-dependent bile salt toxicity [34, 35].

Enterohepatic cycling of bile acids

In humans, dog and mice, bile is stored in the gallbladder (which is absent in rats) before it is released upon the action of the hormone cholecystokinin (CCK) into the small intestine to facilitate lipid digestion. BA can be deconjugated and further modified (e.g. dehydroxylation) by the intestinal microflora, giving rise to secondary, more hydrophobic BAs. Primary CA derived BAs (CA, GCA, TCA) are converted to the secondary BA deoxycholic acid (DCA), whereas primary CDCA derived BAs (CDCA, TCDCA, glycochenodeoxycholic acid (GCDCA)) form the secondary BA lithocholic acid (LCA). In rats, the primary BA βMCA is transformed to the secondary BA ωMCA by various anaerobic bacteria in a cooperative way [36]. BAs in the intestine are reabsorbed either by passive diffusion (*e.g*. DCA) or by active sodium-dependent transport *via* the ASBT transporter into enterocytes [37]. BAs escaping intestinal absorption are excreted *via* feces accounting for about 10 to 20 % loss per day [7], fecal BAs being mainly secondary BAs. From enterocytes, BAs enter the portal circulation via the OST-α/OST-β transporter heterodimer and are taken up into the liver across the hepatocyte sinusoidal membrane by the sodium-

(Na^+)-taurocholate cotransporting polypeptide (NTCP) or by organic anion transporting polypeptides (OATPs). The reabsorbed BAs mix with *de novo* synthesized BA inside hepatocytes and can be re-conjugated before entering the next enterohepatic cycle [38].

Regulation of bile acid homeostasis

The enterohepatic FXR-FGF15/FGF19-FGFR4/βKlotho pathway assigned a new endocrine role to BAs [39]. Within enterocytes BAs bind to the farnesoid X nuclear receptor FXR and activate the transcription of fibroblast growth factor FGF15/19. FGF19 in humans, and FGF15 in rodents, is released into the portal blood from where it reaches the liver and binds to and activates the membrane-bound FGFR4/βKlotho receptor complex [40]. Activation of this receptor tyrosine kinase triggers a complex intracellular signaling cascade that finally results in the suppression of CYP7A1 - the key regulatory enzyme of *de novo* BA synthesis in hepatocytes. By this enterohepatic feedback circuit BAs regulate their own biosynthesis in an endocrine manner [41]. Additional feedback-mechanisms exist in the liver for controlling BA homeostasis, both at the transcriptional and posttranscriptional levels. FXR is also expressed in hepatocytes and regulates hepatic BA biosynthesis and transport proteins that are involved in hepatocellular uptake (NTCP, OATP) and excretion (BSEP, MRP2, OST-α/OST-β, MDR1). Activation of FXR by BAs leads to suppression of both CYP7A1, CYP8B1 and the hepatic influx transporters NTCP and OATP (human only), and to an upregulation of the hepatic efflux transporters BSEP, MRP2, OST-α/OST-β, thus preventing accumulation of cytotoxic BAs within the hepatocytes under normal physiologic conditions [38, 42-45]. The relative potency of individual BAs to activate FXR was determined as CDCA>DCA>LCA. The corresponding glycine- and taurine conjugates were also shown to activate FXR when BA transporters were co-expressed in cellular *in vitro* test systems. Free and conjugated CA, ursodeoxycholic acid (UDCA) and α/βMCA had only minimal or no effect on FXR [46, 47].

BA in health and disease

The concept that BAs could be key players in DILI is derived from the fact that BAs are amphiphilic molecules with strong emulsifying detergent properties that may impair cell membrane integrity and thus exert cytotoxic effects. The liver is exposed to extremely high concentrations of BAs (canaliculus and biliary ductules: 20 to 50 mM, gallbladder: ≤ 300 mM), which makes this organ particularly vulnerable to BA-induced toxicity [48].

The lipophilicity of individual BAs is believed to be a determinant of cytotoxicity, since it is the main driver for passive diffusion of BAs through biological membranes. In addition, the pH of the surrounding environment together with the pKs of the individual BA determines whether the molecule exists in its uncharged, protonated or salt form, which makes a significant difference for its polarity and capability to penetrate membranes [49, 50]. Unconjugated DCA, LCA, CDCA, but also conjugated GCDCA and TCDCA were reported to impair the functionality of mitochondria isolated from rat liver, which may in part mediate cytotoxicity [51]. Structure-activity analysis of BAs showed that the cytotoxicity of naturally occurring BAs can be ranked according to DCA≥LCA≥CDCA>>CA based on assessment in an oesophageal (HET-1A) and hepatic carcinoma (Huh7) cell line, and overall this rank order correlates with lipophilicity. However, the respective taurine and glycine conjugates had no cytotoxic effect on HET-1A cells, which lack BA transporters [52]. Conjugated BAs are fully ionized at physiological pH and are therefore considered membrane impermeable without active transport. Thus, intracellular toxicity mediated by conjugated BAs only occurs when transporters facilitate their uptake into the cell [48] or if their efflux is inhibited leading to intracellular accumulation. It consequently appears appropriate to consider transporter-mediated flux of conjugated BAs into different compartments along with potential interconversion reactions that may affect their cytotoxicity profile when discussing the role of BAs in DILI. Figure 2A illustrates the differences in the BA profiles in plasma, bile, feces and urine of dogs as determined by LC-MS/MS in our laboratory.

It is well known that different species show different BA pool compositions (Figure 2B), which adds additional complexity to the interpretation of preclinical data in the context of human safety assessment during drug development. Rodents, for example, have a higher portion of polar BAs due to the synthesis of a higher proportion of hydroxylated MCAs, which makes their BA pool generally less cytotoxic. Interestingly, genetically modified mouse models susceptible towards developing progressive familial intrahepatic cholestasis (PFIC) display only a mild phenotype compared to human patients. However, when the BA pool of these PFIC susceptible mice was "humanized" by liver-specific disruption of the cytochrome P450 reductase gene resulting in reduced CYP450 activity and cholic acid supplementation, a more hydrophobic BA pool was established and mice rapidly developed pronounced cholestasis with histologic liver injury [53]. These data might therefore indicate that BA pool composition and changes thereof could be a critical factor for the susceptibility to liver injury.

The mutual effects of BAs on the intestinal microflora and *vice versa* have repeatedly been reported [54]. The composition of the gut microbiota and their metabolic capacity strongly affects the composition of the BA pool, notably its hydrophobicity, cytotoxicity and size [55]. Treatment of mice with probiotics resulted in enhanced deconjugation and fecal excretion of BAs, affecting ileal BA absorption and subsequent stimulation of hepatic *de novo* BA synthesis via repression of the enterohepatic FXR-FGF15/FGF19-FGFR4 pathway [56]. Hara and coworkers reported that obesity induced changes of the gut microbiota, leading to increased levels of the secondary BA DCA in the enterohepatic circulation. DCA, being a relatively cytotoxic BA, provoked DNA damage and cellular senescence in hepatic stellate cells, which consequently released inflammatory and tumor-promoting factors in the liver, facilitating hepatocellular carcinoma development in mice previously exposed to a chemical carcinogen [57]. Drugs that impair the intestinal microflora can therefore indirectly affect the liver by altering the BA profile and BA induced cellular effects [58, 59].

Various liver diseases are associated with impaired BA homeostasis. Genetic variations in the genes for FXR, BSEP and MDR3 confer susceptibility to intrahepatic cholestasis of pregnancy (ICP) - a transient

cholestasis in pregnant women – in association with elevated circulating levels of estrogens and progesterone [22]. Estrogen and progesterone metabolites, *e.g.* estradiol-17beta-glucuronide, were shown to trans-inhibit BSEP after its secretion into bile canaliculi by MRP2 [60, 61]. Increased serum levels of sulfated progesterone metabolites were reported to affect FXR functionality in addition to inhibiting the hepatocellular BA uptake and efflux transporters NTCP and BSEP [62-64], thus mechanistically linking the cholestatic syndrome in susceptible women to the hormonal changes arising during pregnancy. Consistently, drug therapy with oral contraceptives and anabolic steroids such as 17α-alkylated androgens were also associated with cholestasis, depending on the individual susceptibility of the recipient [65].

Progressive familial intrahepatic cholestasis (PFIC) is an inherited early-onset cholestatic syndrome with progression to end-stage liver disease caused by mutations in canalicular transporter genes. Less severe forms of cholestasis such as benign recurrent intrahepatic cholestasis (BRIC) are linked to mutations in BSEP or MDR3, likely impairing expression or function of the respective transport protein [22]. Treatment of ICP patients with the secondary BA UDCA was reported to reduce plasma concentrations of sulfated steroid metabolites [66] and exert other beneficial effects [67]. These and various other examples illustrate the importance of intact BA homeostasis for the integrity of the liver.

Bile acids as key players in DILI:

Consistent with the role of BA transporter impairment in various liver diseases, inhibition of BSEP by drugs or their metabolites is considered an important mechanism of drug induced cholestasis and has been reported for cyclosporine A, rifampicin, bosentan, troglitazone and various other compounds [68].

While drug induced cholestasis without hepatitis can be transient and is commonly less severe for the patient, severe irreversible drug-induced liver failure can be fatal or require organ transplantation. Troglitazone (TGZ) is an anti-diabetic drug that was withdrawn from the market due to acute severe idiosyncratic drug-induced liver injury in some patients [69-71]. Clinical observations and *in vivo* rat studies suggested, at least in part, a cholestatic mechanism for TGZ-induced hepatotoxicity, in addition to

a role of a reactive quinone metabolite [72, 73]. Both in human and in rat, TGZ is efficiently metabolized to the major metabolite TGZ-sulfate, which has a high potential to competitively inhibit BSEP and accumulate in the hepatocytes (apparent Ki for rat BSEP inhibition were determined as 1.3 μM for TGZ and 0.23 μM for TGZ-sulfate) [74]. Rat studies with TGZ demonstrated significantly reduced bile flow and a rapid and dose-dependent increase in plasma BA concentrations [74, 75]. A mechanism leading to cytotoxic intracellular bile salt accumulation due to BSEP inhibition was therefore also discussed for humans. Despite the disturbances in BA homeostasis, rat models failed to predict TGZ-induced hepatotoxicity, potentially due to interspecies differences in BA composition and the less cytotoxic BA pool in rodents [76].

Jemnitz *et al*. investigated the effect of cholestatic drugs on TCA transport in human and rat sandwich-cultured hepatocytes and found significant differences in TCA elimination: in human hepatocytes, equal fractions of TCA were eliminated via basolateral (likely by MRP3/4 efflux pumps) and canalicular (mainly BSEP) efflux (34.8% and 34.4 %, respectively) with a similar portion remaining within the cell (30.5%). In contrast, basolateral transport (71.7%) was dominant in rat hepatocytes and intracellular TCA accumulation was low (6.9%). Concordantly, inhibition of BSEP by TGZ treatment resulted in a significantly higher relative increase in intracellular TCA concentrations in human *versus* rat hepatocytes [77]. The higher rate of basolateral BA efflux in the rat in combination with the lower intracellular concentrations and the lower toxicity of the rodent BA pool could have a protective role in the rat and explain species differences in response to cholestatic or hepatotoxic drugs.

Recently, a system pharmacology model was described that could predict species differences in TGZ-induced hepatotoxicity due to altered BA homeostasis [76]. The model simulated a human population, taking into account drug/metabolite disposition, BA physiology/pathophysiology (transporter inhibition), hepatocyte life cycle and liver injury biomarkers and predicted TGZ-induced delayed increases in serum alanine transaminase and bilirubin levels. Such physiologically based pharmacokinetic models could be of great value for assessing adverse outcome effects due to impairment of BA homeostasis. Implementation

of additional compensatory regulatory networks, *e.g.* FXR-mediated transcriptional up- or downregulation of hepatic BA transporters and biosynthesis enzymes, or the endocrine FXR-FGF19/FGFR4 pathway, could add another valuable dimension.

The drug bosentan, an endothelin receptor antagonist approved for pulmonary arterial hypertension, contains a black-box warning for hepatotoxicity. Similarly to TGZ, a mechanism involving inhibition of BSEP with subsequent intracellular accumulation of cytotoxic BAs and induction of liver cell damage was discussed [78]. Despite the inhibitory potential on rat BSEP leading to elevated serum BAs, bosentan did not induce hepatotoxicity in rats. Lesli *et al.* showed that bosentan differentially inhibits both rat and human NTCP in addition to BSEP. The authors concluded that the rat might be less sensitive to hepatotoxicity since bosentan showed stronger inhibitory potential towards rat NTCP compared to human, resulting in less intrahepatocyte accumulation of BAs in rats [79]. By applying a mechanistic model of DILI Woodhead *et al*. simulated whether - in response to bosentan - BA accumulation can reach toxic levels, both in a human and in a rat population, or not. Although the incidence of bosentan toxicity was underestimated, the system predicted a mild hepatocellular ATP decline and serum ALT elevation in human but not in rats, which was accounted for by the less toxic BA pool in rats [80].

A recent case report described two Japanese patients who had initially received bosentan monotherapy and experienced liver toxicity. After dose reduction or discontinuation, liver function was normalized and patients received retreatment with a combination of bosentan and UDCA. Subsequent liver function tests did not show any abnormalities after the combined therapy [81], indicating compensatory effects by the treatment with UDCA.

Impairment of BA homeostasis by a single insult, *e.g.* inhibition of a single hepatic BA transporter, might be compensated by activation of regulatory feedback pathways involving nuclear receptors (*e.g.* FXR), leading to up- or down-regulation of hepatic BA transport systems. Additionally, alternative BA efflux systems or detoxifying BA conjugation reactions could prevent intracellular accumulation of cytotoxic BAs. However, additional insults by a drug impairing the integrity of multiple compensatory pathways

could exceed the protective capacity of the liver and become critical for liver function. Glucuronidation of BAs, for example, appears to be of little relevance under normal physiologic conditions [82]. However, it was reported that glucuronidation may become more important under severe cholestatic conditions, since it allows the conversion of cytotoxic BAs into non-toxic glucuronides, which also facilitates MRP3-mediated alternative efflux from hepatocytes into the blood for urinary elimination [16, 83-85]. Sulfation of BAs by sulfotransferase (*e.g.* SULT2A1) is another important mechanism to detoxify BAs. Sulfated BAs exhibit decreased toxicity and enhanced fecal and urinary excretion since they cannot be reabsorbed by the intestine, as exemplified by LCA sulfates. Sulfation of BAs is increased during cholestatic disease and might therefore play an important role under pathologic conditions [86].

Although there are various indications that BAs might be key players in the onset of DILI, the initiation and progression of liver injury is usually multifactorial. Aleo *et al*., for example, assessed various compounds with Most-DILI-, Less-DILI- and No-DILI-concern and found that drugs with dual potency as mitochondrial and BSEP inhibitors were highly associated with more severe human DILI and more restrictive product safety labeling related to liver injury [87]. In the case of TGZ, reactive metabolite formation and mitochondrial dysfunction are considered to be critical steps in DILI pathogenesis in addition to inhibition of BSEP [73, 74, 88]. Inflammatory processes significantly contribute to DILI, which may often be triggered by reactive metabolite mediated hapten formation. However, BAs were also shown to induce inflammatory genes in hepatocytes and in mouse liver after bile duct ligation [89], which may promote hepatic inflammation during cholestasis and contribute to DILI. Besides the potential contribution of impaired BA homeostasis, mechanisms such as mitochondrial dysfunction, cytotoxicity, reactive metabolite formation, immune-mediated hypersensitivity reactions, dose and lipophilicity of a drug itself and combinations thereof [90, 91] seem to be important factors in the onset of acute liver failure in combination with individual risk factors.

Bile acids as surrogate markers

Circulating total BAs are well-established clinical markers to detect liver injury and reflect a disturbance of normal hepatic excretory function. Changes in total BAs are usually indicative for hepatocellular and/or hepatobiliary injury. With the availability of new analytical multiplexing methods based on LC-MS/MS and GC/MS, circulating BA profiles are currently being evaluated as biomarkers for hepatotoxicity [92]. In an attempt to identify potential biomarkers for improved DILI detection by untargeted metabolomics, significant elevations of a panel of BAs in plasma and urine of rats treated with different types of hepatotoxins were found [93]. Other groups reported changes in metabolites derived from energy [94, 95], urea and BA metabolism in urine and blood of mice or rats treated with a single dose of acetaminophen (APAP), which strongly correlated with the development of hepatic necrosis [96]. Luo *et al.* evaluated by a targeted LC-MS/MS approach CA, GCA and TCA as potential biomarkers of liver injury in rodent models [97]. The authors reported that animals with histopathological signs of hepatocellular necrosis showed increases in all three BAs in serum, whereas animals with signs of bile duct hyperplasia displayed increases in only the conjugated forms GCA and TCA. They suggested that individual serum BAs might be a potential diagnostic marker to differentiate between different forms of liver injury [97] and characterized individual BA profiles in patients with liver injury and healthy volunteers [98]. Their data showed that total and individual BAs were strongly increased in samples from hepatic injury subjects with the exception of the secondary BA DCA. In addition, conjugated BAs were higher than unconjugated BAs in the patient group. The portion of free and conjugated CA in serum was also higher in subjects with liver injury compared to healthy controls. The changes induced by APAP with respect to individual BAs in urine and blood based on clinical and non-clinical data and their potential use as biomarkers of liver injury were recently reported [99].

While alterations in BA profiles can be the consequence of hepatotoxicity, functional inhibition of BA transporters *in vivo* may also lead to shifts in BA composition in the circulation without being the consequence of cellular damage of the liver. In an exploratory study with male Sprague-Dawley rats

treated with 20 mg/kg/day cyclosporine A (CsA) by oral gavage for 7 days, we found significant changes in individual plasma BA components. The unconjugated primary BAs CA, CDCA, and α/βMCA, which accounted for approximately 60% of the total BAs in control plasma, were significantly increased with CsA. While DCA plasma levels were not affected by the treatment, the unconjugated secondary BAs ωMCA, γMCA and UDCA tended to be increased in absolute concentrations. LCA, though present only in traces in plasma, was decreased. Both primary and secondary taurine-conjugated BAs were generally less abundant in treated animals when compared to controls. The sum of all individual BAs measured increased approximately 3-fold with CsA, which was mainly driven by the increase of the unconjugated primary BAs CA, CDCA and αMCA (Figure 2C). This complex pattern of changes in circulating BAs observed with CsA in rats is likely the consequence of inhibition of multiple hepatic transporters. CsA has the potential to inhibit both rat influx and efflux transporters in the lower micromolar range as determined in primary cultured rat hepatocytes and membrane vesicles, respectively (NTCP IC_{50} = 8.63 μM, OATPs IC_{50} = 4.55 μM, BSEP IC_{50} = 1.75 μM, MRP2 IC_{50} = 9.2 μM) [100]. Although the individual contribution of these transporters to CsA-induced changes in BA profiles is difficult to assign, it seems that not all BAs were affected in the same way. To further shed light into the complex system of BA homeostasis, it would be advantageous to systematically generate structure-activity relations of BAs for their various BA transporters. Such data could be valuable to better simulate potential BA-mediated adverse effects in humans, taking the different species' BA profiles into account.

The IMI SAFE-T consortium, a public-private partnership between industry, health authorities and academia, is currently evaluating BA profiles as potential markers for drug-induced BA transporter inhibition and liver injury [101]. The complexity of this endeavor lies in the fact that different mechanisms can lead to changes in BA profiles with overlapping effects. Many drugs inhibit more than one BA transporter and/or have effects on BA biosynthesis. In addition, the transition from BAs as early functional markers to assess BA transporter inhibition to surrogate biomarkers for liver injury may be difficult to differentiate based on thresholds. Preclinical studies can be very helpful since they allow an optimal study design with controlled conditions and appropriate sampling. However, the differences between species in

BA composition and physiology need to be considered for proper data interpretation and subsequent translation or extrapolation to humans. Human biosamples with a clearly defined pathology are mandatory for validating circulating individual BAs as biomarkers for monitoring liver integrity.

Summary and conclusion

Accumulation of intrahepatic cytotoxic BAs due to drug-induced disturbances of BA homeostasis appears to be an important contributing factor to the onset of DILI in susceptible individuals in combination with other key adverse events impacting liver physiology, *e.g.* mitochondrial dysfunction, reactive metabolite formation and immune-mediated responses. Circulating individual BAs' concentrations might become promising sensitive and selective early markers to monitor functional impairment of the liver, allowing a prognostic assessment before irreversible damage occurs.

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Figure legends

Fig. 1: Schematic overview of hepatic BA biosynthesis, conjugation, transport and enterohepatic cycling. Regulatory pathways of BA homeostasis, *e.g.* the endocrine FXR-FGF15/19-FGFR4 pathway and intrahepatic regulation of BA transporters by FXR, are shown. The rodent specific MCAs are depicted in italic

Fig. 2: A: BA profiles in dog plasma, bile, feces and urine. B: Differences in BA composition in plasma of mouse, rat, monkey and human. C: Changes in individual plasma BA concentrations in rats treated with 20 mg/kg/day Cyclosporine A for 7 days. Levels of statistical significance *versus* controls were calculated using a student`s *t* test: * $p>0.05$; ** $p>0.01$; *** $p>0.001$. Unconj.1stBA: sum of unconjugated primary BAs. T-1stBA: sum of taurine conjugated primary BAs. T-2ndBA: sum of taurine conjugated secondary BAs. All data were obtained in-house by a multiplexing LC-MS/MS method enabling the analysis of 17 BAs.

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