Mechanisms of Hepatic Cholestatic Drug Injury

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Abstract

Drug-induced cholestasis represents a form of drug-induced liver disease that can lead to severe impairment of liver function. Numerous drugs have been shown to cause cholestasis and consequently bile duct toxicity. However, there is still lack of therapeutic tools that can prevent progression to advanced stages of liver injury. This review focuses on the various pathological mechanisms by which drugs express their hepatotoxic effects, as well as consequences of increased bile acid and toxin accumulation in the hepatocytes.

Introduction

The liver is the central organ responsible for the selective uptake, metabolism and excretion of endogenous and exogenous compounds, including drugs.1,2 The anatomical location and the activity of uptake transporters that facilitate accumulation of drugs in hepatocytes make this organ susceptible to drug-induced liver injury (DILI).3 DILI is the leading cause of liver transplantation in Western countries due to its role in causing acute fulminant liver failure. It also represents the primary reason for the failure of pharmaceutical agents during drug development.2,3 However, it has been reported that only 10% of jaundiced patients (who already have symptoms of liver disease) with underlying hepatocellular injury will develop acute liver failure.4,5 This indicates that only a small fraction of the overall group experienced the above-mentioned failure.

Nevertheless, DILI represents a serious clinical problem due to its lack of standardized diagnostic criteria, delay in diagnosis, unpredictable nature, and potentially fatal course.6–8 The most severe clinical patterns of DILI are cholestatic and mixed cholestatic and hepatocellular liver injury.2 Regarding clinical patterns, an R value, which is defined as the ratio of serum alanine aminotransferase/upper limit of normal to serum alkaline phosphatase/upper limit of normal can be helpful in separating DILI into the following three patterns of liver injury: cholestatic injury, with \( R < 2 \); mixed injury, with \( 2 < R < 5 \); and, hepatocellular injury, with \( R \geq 5 \).6 Among these three patterns, cholestatic drug injury takes place in 20–40% of cases.5 Additionally, patients with cholestatic injury generally have better outcomes than those experiencing a hepatocellular injury.

Cholestasis can occur as a consequence of impaired formation of bile, or in the case of physical obstruction to the bile flow after it has been secreted from hepatocytes. Drug-induced cholestasis can be caused by inhibition of bile acid (BA) transport (direct or indirect, which includes regulation of transporter localization or expression), hypersensitivity reaction, and direct injury to bile ducts, as well as mitochondrial damage.1 The aim of this review was to evaluate and compare the major pathophysiological mechanisms involved in cholestatic liver injury induced by various drugs.

Epidemiology

The incidence of DILI in general populations was estimated to be between 1/100,000 and 20/100,000 in developed countries,10,11 and was estimated to be responsible for around 10% of acute hepatitis cases.12 Data from France suggested that the annual incidence of DILI was roughly 13.9/100,000. In the USA, idiosyncratic DILI was found to account for approximately 11% of all cases of acute liver failure, and the prevalence of drug-induced cholestasis was reported to be 20% in an elderly population.13,14 Anti-infective drugs, nonsteroidal anti-inflammatory drugs, and herbal and dietary supplements are common causes of DILI in the developed countries of North America and Europe.15 American data showed that herbal and dietary supplements accounted for more than 20% of the causes of DILI.16 Specifically, amoxicillin/clavulanate remains the predominant cause of cholestatic DILI.17 Furthermore, the same drug can cause different types of liver damage.18 Impudent alcohol consumption may increase the risk of DILI.6

Formation of BAs and their toxic potential

Primary BAs, cholic acid (CA) and chenodeoxycholic acid are synthesized from cholesterol in the liver.19 These BAs can be subjected to enzyme-catalyzed conjugation to form taurine-conjugated, glycine-conjugated (GCDCA), glycinecholylcholic acid (GCDCA)
and taurochenodeoxycholic acid. These BAs can also be actively transported out of the liver and into the bile. Bile is a toxic substance, but the toxic effect of bile on hepatocytes and bile duct epithelial cells can be minimized physiologically through various mechanisms, such as high apical membrane cholesterol and sphingomyelin content, bile hydration and alkalization, micellar binding of BAs, mucin formation (in larger ducts and gallbladder), and particularly bile flow, which limits the interaction time of bile with cell membrane domains.

Disruption of normal hepatobiliary transport may expose cells to the toxic effects of bile, resulting in hepatocellular and bile duct injury. GCDCA and its bile salt glycochenodeoxycholate are major constituents of bile during cholestasis. Several mechanisms have been proposed by which GCDCA executes its toxic effect, including DNA fragmentation, cellular shrinkage and blebbing of the membrane, chromatin margination and condensation, and apoptosis. Additionally, studies have shown that cellular apoptosis is associated with mitochondrial instability and mitochondrial membrane permeability transition pore formation.

Considering all of these facts about BAs, their formation, bile flow, and toxic effect, it is clear that any disturbance in normal bile flow can have significant consequences on the liver. In the following paragraphs various factors involved in bile transport and flow will be discussed as potential targets of drug hepatotoxic effect.

Hepatic canalicular efflux transport proteins

Transport of biliary constituents across the canalicular membrane represents the rate limiting step in bile formation and excretion. This process is regulated by ATP-dependent canalicular transporters, especially the bile salt export pump (known as BSEP and ATP-binding cassette (ABCB11)). BSEP represents a hepatic transport protein that is a member of the ABC gene superfamily, and represents the major determinant of bile salt-dependent canalicular bile secretion. Various drugs involved in cholestatic or mixed cholestatic and hepatocellular liver injury inhibit BSEP-mediated biliary excretion of BAs, consequently leading to an increase in hepatic exposure to cytotoxic effects of BAs in susceptible individuals. As presented in Table 1. Some of these drugs are bosentan, cyclosporine, rifampin, troglitazone, sulindac, erthyromycin, and glibenclamide. Additionally, some drugs, such as taxol and pravastatin, are transported by BSEP, and therefore may be involved in drug-induced cholestasis.

Most of the BSEP inhibitors directly cis-inhibit BSEP. In contrast, estradiol 17β-glucuronide and progesterone metabolites represent indirect trans-inhibitors of BSEP. Prior to trans-inhibition, these drugs are secreted into the bile by another ATP-dependent transporter, the multidrug resistance-associated protein (MRP)-2, and then exert their inhibitory effect from the luminal side of the canalicular membrane. Although BSEP inhibition increases risk for development of liver injury, it is considered that inhibition alone is not enough, and in many cases, the pathological mechanism is more complicated. Cyclosporine can induce cholestatic liver injury through various mechanisms, such as competitive inhibition of ATP transporters, intrahepatic vesicle transport inhibition, targeting of ATP dependent transporters to the apical (canalicular) membrane, and impairment of bile excretion by increasing membrane fluidity without affecting the expression of canalicular transporters.

Other ATP-dependent transporters are MDR1 (ABCB1) and MDR3 (ABCB4), MRP2 (ABCC2), breast cancer resistance protein (ABCG2), cholesterol flippase (ABCG5), and ABCG8. MDR3 represents an ATP-dependent phospholipid flipase that is able to translocate phosphatidylcholine from the inner to the outer leaflet of the canalicular membrane. Phosphatidylcholine is solubilized by canalicular bile salts to form mixed micelles, consequently protecting the biliary epithelium from exposure to the toxic and detergent effects of BAs which could result in cholangiopathy. Various studies confirmed itraconazole as an MDR3 inhibitor which reduces MDR3-mediated efflux of phosphatidylcholine. However, MDR3-mediated BA transport was not affected, suggesting that although inhibition of MDR3-mediated biliary phospholipid excretion may represent a risk factor for drug-induced cholestasis, BA secretion is not altered. In vitro studies showed that drugs, such as verapamil and cyclosporine, are transported by MDR3, potentially leading to competitive inhibition of phospholipid flipase activity and cholestatic injury. Additionally, various genetic variations in BSEP and MDR3 could predispose individuals to drug-induced cholestasis.

MRP2 plays an important role in detoxification and chemoprotection by transporting into bile a wide range of compounds, especially conjugates of lipophilic substances with glutathione (GSH), glucuronate, and sulfate (phase II products of biotransformation). Additionally, MRP2 is able to cotransport uncharged compounds with GSH, consequently modulating the pharmacokinetics of numerous drugs. MRP2 regulates bile salt-independent bile flow by excretion of glutathione. Accordingly, Fauassier et al. showed that bosentan (competitive antagonist of endothelin-1) stimulates MRP2-dependent bilirubin secretion and bile salt-independent bile flow, whereas phospholipid and cholesterol excretion were markedly inhibited and uncoupled from bile salt excretion. Alpha-naphthylisothiocyanate forms a labile GSH adduct in hepatocytes, which is afterwards transported into alkaline bile and finally dissociates, with a potential to induce cholestatic bile duct injury. It has been shown that rats with a mutation in MRP2 are unable to pump the adduct into bile and, therefore, are protected from alpha-naphthylisothiocyanate-induced cholestatic injury.

Drug metabolites, such as troglitazone sulfate and troglitazone glucuronide are both eliminated by MRP2 into bile, suggesting that canalicular elimination by MRP2 may represent an important factor in the pathogenesis of troglitazone-induced cholestatic injury. Additionally, direct competition of the above-mentioned troglitazone metabolites with conjugated bilirubin at the level of MRP2 could result in conjugated hyperbilirubinemia. These metabolites are also capable of cis-inhibiting BSEP and, therefore, inducing the cholestasis and hepatotoxicity. A previously mentioned drug, cyclosporine, is proposed to reduce expression of GSH synthetizing enzymes, and the MRP2 system, consequently leading to decreased bile salt-independent bile flow and hepatotoxicity. Co-administration with rapamycin can exacerbate the cholestatic effect of cyclosporine.

The effects of various drugs on the function of hepatic efflux transport proteins are very diverse. Some affect efflux of bile to the canalculus, while others involve protection of biliary epithelium. Inhibition of any of these functions predisposes liver tissue to toxic effects of bile. However, there are many compensatory mechanisms which can reverse the
Compensatory basolateral efflux prevents hepatic toxicity of drugs. One of the major mechanisms involves canalicular efflux transporters.

**Hepatic basolateral efflux transport proteins**

Inhibition of each one of the above-mentioned hepatic canalicular efflux transporters represents a risk factor for drug-induced cholestasis. Nevertheless, as explained before, not all drugs that inhibit these transporters cause cholestasis because there are several compensatory mechanisms of bile transport present in the hepatocytes. In contrast to BSEP, MRP2, breast cancer resistance protein, and MDR1, MRP3 and MRP4 transporters are expressed on the basolateral (vascular) side of the polarized cells, which means that in the liver they efflux their substrates into the blood rather than into the bile. Under the normal conditions, these transporters play a minor role in BA efflux, whereas under cholestatic conditions their expression is induced in order to compensate for impaired biliary excretion. Compensatory basolateral efflux prevents hepatic BA accumulation, leading to increased renal elimination of BAs. Therefore, impaired function of MRP3 and MRP4 by various drugs may result in accumulation of toxic BAs in hepatocytes. Substrates of MRP3 transporters include endogenous compounds (estradiol-17β-glucuronide, leukotriene C4, and monovalent bile salts such as cholate and glycocholate), chemotherapeutic agents, acetaminophen-glucuronide, morphine-3-glucuronide, and fexofenadine.

Several drugs are inhibitors of MRP3, including tenofovir, indomethacin, furosemide, probenecid, non-nucleoside reverse transcriptase inhibitors (delavirdine, efavirenz, and nevirapine), and nucleoside reverse transcriptase inhibitors (emtricitabine and lamivudine).

In contrast to the previously mentioned MRP1 and MRP2 transporters, MRP3 expresses a higher affinity for glucuronide conjugates compared to GSH conjugates, and unlike MRP1 and MRP2 MRP3 does not require GSH to transport substrates. Inhibition of each one of the above-mentioned hepatic canalicular efflux transporters represents a risk factor for drug-induced cholestasis. Nevertheless, as explained before, not all drugs that inhibit these transporters cause cholestasis because there are several compensatory mechanisms of bile transport present in the hepatocytes. In contrast to BSEP, MRP2, breast cancer resistance protein, and MDR1, MRP3 and MRP4 transporters are expressed on the basolateral (vascular) side of the polarized cells, which means that in the liver they efflux their substrates into the blood rather than into the bile. Under the normal conditions, these transporters play a minor role in BA efflux, whereas under cholestatic conditions their expression is induced in order to compensate for impaired biliary excretion. Compensatory basolateral efflux prevents hepatic BA accumulation, leading to increased renal elimination of BAs. Therefore, impaired function of MRP3 and MRP4 by various drugs may result in accumulation of toxic BAs in hepatocytes. Substrates of MRP3 transporters include endogenous compounds (estradiol-17β-glucuronide, leukotriene C4, and monovalent bile salts such as cholate and glycocholate), chemotherapeutic agents, acetaminophen-glucuronide, morphine-3-glucuronide, and fexofenadine.

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Table 1. List of the most important hepatic transporters, their locations, functions and drugs that act as their inhibitors

| Hepatic transporter | Location in hepatocytes | Function | Inhibitors |
|---------------------|-------------------------|----------|------------|
| BSEP                | Canalicular membrane    | ATP-dependent transport of bile salts, taxol and pravastatin | Bosentan, cyclosporine, rifampin, troglitazone, sulindac, erythromycin, glibenclamide, progesterone metabolites, estradiol 17β-glucuronide |
| MDR3                | Canalicular membrane    | ATP-dependent excretion of phosphatidylcholine | Itraconazole, chlorpromazine, imipramine, haloperidol, ketoconazole, clotrimazole, troglitazone |
| MRP2                | Canalicular membrane    | ATP-dependent efflux of numerous drugs, organic anions and bile acids, bile salt-independent bile flow by GSH transport | Cyclosporine, efavirenz, benz bromarone, probenecid |
| MDR1                | Canalicular membrane    | ATP-dependent efflux of numerous drugs, organic cations and bile acids | Cyclosporine, carvedilol, clarithromycin, amiodarone, itraconazole, lapatinib, verapamil |
| BCRP                | Canalicular membrane    | ATP-dependent efflux of anticancer drugs | Gefitinib, mesylate, 17β-estradiol, ritonavir, omeprazole, cyclosporine |
| MRP3                | Basolateral membrane    | ATP-dependent efflux of drug glucuronide conjugates | Tenofovir, indomethacin, furosemide, probenecid, non-nucleoside reverse transcriptase inhibitors (delavirdine, efavirenz, and nevirapine), and nucleoside reverse transcriptase inhibitors (emtricitabine and lamivudine) |
| MRP4                | Basolateral membrane    | ATP-dependent efflux of sulfated drugs and bile acids | Troglitazone sulfate, NSAIDs, phosphodiesterase inhibitors, verapamil, losartan, silymarin, probenecid |
| NTCP                | Basolateral membrane    | Uptake of bile-salts from portal blood, transport of rosuvastatin | Cyclosporine, bosentan, troglitazone, propranolol, furosemide, ketoconazole, renin inhibitors and a somatostatin analogue, rifamycin |
| OATPs               | Basolateral membrane    | Sodium-independent uptake of bile salts, organic anions, and numerous drugs | Itraconazole, nefazodone, nifedipine, reserpine, diazepam, sulfasalazine |

Data from Yang et al.,1 solvobiotech.com,44 Kim et al.,48 and Kalgren et al.51

Abbreviations: BCRP, Breast cancer resistance protein; BSEP, bile salt export pump; MDR, multidrug resistance; MRP, multidrug resistance-associated protein; NSAID, nonsteroidal anti-inflammatory drugs; NTCP, sodium-taurocholate cotransporting polypeptide; OATPs, organic anion transporting polypeptides.
Hepatic uptake transport proteins

The uptake transport proteins sodium-taurocholate cotransporting polypeptide (NTCP) and organic anion transporting polypeptides (OATPs) are involved in taking up BAs from the portal vein into hepatocytes\(^1,51\) (Fig. 1). Basolateral uptake transporters play major roles in controlling the response to hepatic and systemic exposure to drugs and toxins. The hepatic accumulation of BAs is regulated by both uptake and efflux (basolateral and canalicular) processes. Thus, inhibition of hepatic BA uptake could diminish intracellular bile salt concentrations in hepatocytes and consequently lower the cytotoxic action of bile salts.\(^1,51\)

NTCP, an electrogenic transporter, is responsible for sodium-dependent bile salt uptake, and it transports two sodium ions together with one bile salt molecule.\(^52,53\) Because it is an energy-dependent transporter, NTCP is capable of taking up bile salt from the portal blood into hepatocytes against a concentration gradient.\(^51\) It has greater affinity of transporting trihydroxy and conjugated as opposed to dihydroxy and unconjugated BAs. However, NTCP is also able to mediate the transport of drugs and toxins.\(^1,51\) Regulation of NTCP is associated with changes in bile salt load to hepatocytes; the main goals are to prevent entry of cytotoxic bile salts during liver disease and to maintain BA homeostasis inside hepatocytes.\(^51\)

NTCP is a target of inhibition by numerous drugs, such as cyclosporine, bosentan, troglitazone, propranolol, furosemide, ketoconazole, renin inhibitors and a somatostatin analogue, as well as rifamycin.\(^54\) Additionally, differential effects of drugs on individual BAs accumulation should be considered when predicting hepatotoxicity in humans.\(^1\) Although, inhibition of NTCP should lead to an increase of serum bile salt levels, the effects may be inconsistent because the liver also expresses sodium-independent bile salt transporters, such as the OATPs, which can partly compensate for the inhibition of NTCP.\(^51\) Nevertheless, these drugs often have the capability of inhibiting OATPs in addition to NTCP, as demonstrated for rifampicin and rifamycin and for cyclosporine.\(^55,56\)

Many drugs are substrates of the OATPs, such as statins, bosentan, and angiotensin-converting enzyme inhibitors. Drugs that may inhibit OATP transporters are itraconazole, nefazodone, nifedipine, reserpine, diazepam, sulfasalazine, etc.\(^57\) Additionally, there are some individual differences between different drugs in inhibiting a specific OATP transporter, considering that different types of OATP transporter are present (OATP1B1, OATP1B3, and OATP2B1).\(^57\) Hepatic uptake is another mechanism for maintaining BA homeostasis in liver cells. Therefore, any drug that disturbs this function, particularly when liver injury is already present, can result in deterioration of liver function.

Metabolic defects

To date, nine congenital errors of BA synthesis have been reported. Congenital errors in BA metabolism have been identified by analysis of abnormal BA metabolites and mutations or enzyme deficiencies that can be caused by a primary defect in the enzymes involved in the BA biosynthetic pathways. Congenital errors of BA synthesis can produce abnormal BA metabolites, with modified steroid side chains or steroid nucleus structure. Such metabolites can be toxic and capable of causing cholestatic liver disease and progressive neurological disease.\(^58,59\)

BAs are crucial physiological agents, signaling molecules and metabolic regulators that activate nuclear receptors and G protein-coupled receptors, such as farnesoid X, pregnane X, constitutive androstane receptors, and vitamin D receptor signaling to control hepatic lipid, glucose, and energy homeostasis and maintain metabolic homeostasis.\(^60,61\) Disturbances in BA metabolism can cause cholestatic liver diseases, dyslipidemia, fatty liver diseases, cardiovascular diseases, and diabetes. However, acid-activated nuclear and G protein-coupled receptor signaling protects against inflammation in the liver and intestines, and macrophages.\(^59\) AMP-activated protein kinase is a critical player in the pathogenesis of

**Fig. 1. Localization of the most important hepatic transporters in hepatocytes.** Abbreviations: MRP3, multidrug resistance-associated protein 3; MRP4, multidrug resistance-associated protein 4; NTCP, sodium-taurocholate cotransporter; OATPs, organic anion transporting polypeptides; BSEP, bile salt export pump; MRP2, multidrug resistance-associated protein 2; MDR3, multidrug resistance protein-3; BCRP, breast cancer resistance protein; MDR1, multidrug resistance protein 1; OA, organic anion; BA-G, bile acid glucuronide; BA-S, bile acid sulfate; NA, sodium; BA, bile acid; OC, organic cation; PC, phosphatidylcholine.
cholestatic liver injury that induces suppression of the nuclear receptor, disturbs BA homeostasis, and facilitates inflammation at the late stages. Potentially, AMP-activated protein kinase is a therapeutic target for the treatment of cholestatic liver injury.62

Mitochondrial damage

Mitochondrial biogenesis is a complex process, wherein the peroxisome proliferator-activated receptor G coactivator 1a is a key regulator.63,64 Mitochondria serve as the main energy producers in hepatocytes through their role in fatty acid oxidation, pyruvate oxidation, and the oxidative phosphorylation system, as well as the electron transport chain. Because of its importance in energetics, it is not surprising that mitochondria are also at the center of signaling pathways that mediate hepatocyte damage.66,67 Genetic factors, hormones, post-infectious factors and drugs can all result in harmful effects on mitochondrial function.68

DILI affecting the mitochondrial function is common. When mitochondrial injury results in canalicular membrane damage or transporter dysfunction, cholestatic liver injury can result. Hepatotoxic drugs cause mitochondrial dysfunction through different mechanisms, like membrane permeabilization, oxidative phosphorylation system impairment, and mitochondrial DNA depletion; subsequently, some drugs directly inhibit mitochondrial respiration and the mitochondrial β-oxidation of fatty acids. The conversion of a nontoxic drug into a chemically reactive metabolite is the most frequent mechanism of DILI.66,68,69 Some marketed drugs have received Black Box warnings from drug agencies due to mitochondrial dysfunction and related hepatotoxicity; as well, some examples of DILI resulting from mitochondrial dysfunction have prevented the marketing of some compounds, led to the repeal of diethylnicotinoxyhexestrol, nefazodone, troglitazone and troxafloxicin, and led to restricted use of perhexiline, as well as the early therapeutic misadventures with tetracyclines and valproic acid.

BAs are the major organic solutes in bile. Retention of hydrophobic BAs during cholestasis plays an important role in liver injury through apoptosis or necrosis of hepatocytes. Hepatocyte injury caused by the retention of hydrophobic BAs and mitochondrial dysfunction has been associated with liver cholestasis. Toxic bile salt accumulation can lead to chronic injury with mitochondrial damage, increase in reactive oxygen species, and apoptosis which can result in liver dysfunction.70,71 Induction of the mitochondrial permeability transition (MPT) is known to play a key role in cell death, and mitochondrial permeability transition induction is involved in BA cytotoxicity.72,73 Mitochondrial dysfunction can occur in death receptor-mediated apoptosis, and also by DCA-triggered apoptosis in reduced mitochondrial membrane potential and modifications in Bax subcellular distribution.74 Pretreatment with cyclosporin A, a specific and potent inhibitor of the mitochondrial permeability transition, protects cells from cell death, prevents mitochondrial swelling, and diminishes the efflux of cytochrome c induced by DCA, a hydrophobic BA.74,75 High concentrations of BAs cause disruption of plasma membrane integrity. In chronic cholestasis, mitochondrial calcium homeostasis can be severely damaged. In cholestatic liver diseases, ursodeoxycholic acid is used as a therapeutic agent because of its ability to modulate hydrophobic BA-induced damage in hepatocytes.73,76 However, this does not address the underlying mechanism of cholestatic disease. Cholestasis and its related complications still remain therapeutic challenges, and new effective agents are much needed. At present, aside from liver transplantation, there is no method to reverse injury due to hepatic mitochondria or to replace them. Recent studies, however, have suggested that targeted mitochondrial transplantation in hepatocytes is possible.77

Conclusions

Drugs represent an important cause of severe liver injury and acute fulminant liver failure. Despite the progress that has been made in our understanding of the mechanisms involved in drug-induced cholestatic liver injury, adequate therapeutic measures are lacking. The fact that drugs can act through many different mechanisms to cause cholestasis makes prompt diagnosis and effective therapy a challenge.

Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Conceived the article outline (GYW), critical revision, funding, administration, and technical support (GYW, MS), writing of the manuscript (TOK, VN), literature searches and text update (RS).

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