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Mechanisms and *in vitro* models of drug-induced cholestasis

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Abstract

Cholestasis underlies one of the major manifestations of drug-induced liver injury. Drug-induced cholestatic liver toxicity is a complex process, as it can be triggered by a variety of factors that induce 2 types of biological responses, namely a deteriorative response, caused by bile acid accumulation, and an adaptive response, aimed at removing the accumulated bile acids. Several key events in both types of responses have been characterized in the past few years. In parallel, many efforts have focused on the development and further optimization of experimental cell culture models to predict the occurrence of drug-induced cholestatic liver toxicity *in vivo*. In this paper, a state-of-the-art overview of mechanisms and *in vitro* models of drug-induced cholestatic liver injury is provided.

Key words: Drug-induced cholestasis, liver, mechanisms, *in vitro* models.

Abbreviations: ANIT, α-naphthylisothiocyanate; AOP, adverse outcome pathway; ATF, activating transcription factor; BSEP, bile salt export pump; CIx, cholestatic index; CYP, cytochrome P450; DICI, drug-induced cholestasis index; DILI, drug-induced liver injury; DNA, deoxyribonucleic acid; ECM, extracellular matrix; Egr1, early growth response factor-1; ER, endoplasmic reticulum; FXR, farnesoid X receptor; iPSC; induced pluripotent stem cells; IRE1α, inositol-requiring protein 1α; Keap1, Kelch-like ECH-associated protein 1; LC3, microtubule-associated protein 1 light chain 3; MDR, multidrug resistance protein; MLKL, mixed lineage kinase domain-like; MRP, multidrug resistance-associated protein; NLRP3, nucleotide-binding and oligomerization leucine-rich repeat protein 3; Nrf2, nuclear related factor 2; OATP, organic anion transporting polypeptides; PERK, protein kinase RNA-like endoplasmic reticulum kinase; PCLS, precision-cut liver slice(s); PXR, pregnane X receptor; RIP, receptor interacting protein; ROCK, rho-associated protein kinase; ROS, reactive oxygen species; TLR9, Toll-like receptor 9; UGT, uridine diphosphate glucuronosyltransferase.

1 Introduction

Cholestasis is derived from the Greek words "*chole"* meaning bile and "*stasis"* indicating halting or stopping. Cholestasis denotes any situation of impaired bile flow with concomitant accumulation of noxious concentrations of bile acids in the liver or systemic circulation. A plethora of factors can evoke cholestasis, including gene mutations, metabolic disorders, infections and drugs (European Association for the Study of the Liver 2009; Nguyen et al. 2014; Noor 2015). As such, 2 different types of cholestasis can be distinguished depending on the location of bile flow disturbance, namely intrahepatic and extrahepatic cholestasis. The latter is typically caused by an anatomical obstruction outside the liver, while intrahepatic cholestasis results from a functional defect in bile formation by hepatocytes (Mariotti et al. 2017; Zollner et al. 2006a). Drug-induced cholestasis, either intrahepatic or, less frequently, extrahepatic, can be manifested as an acute or chronic liver disorder. Clinical symptoms, such as jaundice and pruritus, are reversible in acute drug-induced cholestasis and resolve upon drug withdrawal, whereas chronic cholestasis has more persisting symptoms, lasting at least 6 months after the initial cholestatic insult. Other clinical features, depending on the type, include hyperbilirubinemia and increased serum levels of alkaline phosphatase, γ-glutamyl transpeptidase, 5'-nucleotidase, aspartate aminotransferase and alanine aminotransferase (Padda et al. 2011; Vinken et al. 2013). Drug-induced cholestasis constitutes a subtype of drug-induced liver injury (DILI). In fact, cholestatic injury is responsible for as much as 50% of registered DILI cases (Oorts et al. 2015). Cholestatic DILI is elicited by single-prescription medication in approximately 73% of all cases, mainly involving anti-infectious, anti-diabetic, anti-inflammatory and cardiovascular drugs (Bhamidimarri et al. 2013). Current preclinical animal models can only detect about 50% of human DILI, including cholestatic injuries, which is mainly due to interspecies differences in drug metabolism and disposition pathways (Bell et al. 2017; Olson et al. 2000; Ozer et al. 2008). For this reason, along with ethical constraints, there is a tendency to address human-based *in vitro* models to predict DILI. Nevertheless, human hepatic *in vitro* systems available today have their shortcomings and can merely detect as much as 60% of all human clinical DILI cases. The latter can be explained, at least in part, by gaps in the mechanistic understanding of DILI, in particular the cholestatic type (Bale et al. 2014; Laverty et al. 2010). In this

respect, in-depth insight into the mechanisms of DILI may facilitate identification of more suitable biomarkers for prediction of hepatotoxicity, which in turn will assist in increasing the predictive power of human-based hepatic *in vitro* models. In this paper, an assembly of mechanistic scenarios of drug-induced cholestasis is described and a critical overview of presently available *in vitro* models of this type of DILI is provided.

2 Mechanisms of drug-induced cholestasis

Drug-induced cholestasis can be initiated by 3 types of triggering factors, namely (*i*) transporter changes, such as functional inhibition, reduced expression and/or aberrant subcellular localization, (*ii*) hepatocellular changes, including compromised cytoskeletal architecture, disruption of tight junctions and decreased membrane fluidity, and (*iii*) altered bile canalicular dynamics, either dilatation or constriction (Figure 1) (Burbank et al. 2016). These stimuli can induce bile accumulation, which subsequently activates 2 cellular responses, namely a deteriorative response and an adaptive response. Both coinciding responses are characterized by a number of key events (Figure 2). The deteriorative response is typified by the occurrence of mitochondrial impairment (Begriche et al. 2011; Yu et al. 2014), different cell death modes (Afonso et al. 2016; Gao et al. 2014), endoplasmic reticulum (ER) stress with unfolded protein responses (Burban et al. 2018), oxidative stress (Copple et al. 2010; Perez et al. 2009; Tiao et al. 2009) and inflammation (Cai et al. 2017; Gong et al. 2016; Woolbright et al. 2012; 2017). The adaptive response strives to counteract bile acid accumulation *via* activation of a number of nuclear receptors (Halilbasic et al. 2013).

2.1 Triggering factors

2.1.1 Transporter changes

Parent drugs and their metabolites can induce disruption of transport systems in hepatocytes, which results in many cases to drug-induced cholestasis (Pauli-Magnus et al. 2006). Hepatocyte transporters

are responsible for trafficking both bile acids and drugs (Ramboer et al. 2013; Yang et al. 2013). Transporters located at the basolateral membrane play a crucial role in the uptake of chemical substances from the sinusoidal blood (Pauli-Magnus et al. 2006). Canalicular transporters, on the other hand, are in charge of clearance and secretion of drugs, bile acids and other bile constituents across the canalicular membrane of hepatocytes into bile (Morgan et al. 2010; Pauli-Magnus et al. 2006). Any drug-related effect on these transporter systems can therefore lead to accumulation of potentially harmful bile acids or increase of hepatic uptake of xenobiotics, which in turn may evoke liver cell damage (Pauli-Magnus et al. 2006). In this regard, the bile salt export pump (BSEP) and multidrug resistance protein 3 (MDR3) are critical canalicular transporters responsible for bile acid secretion and for maintaining low intracellular levels of noxious bile acids (de Lima Toccafondo Vieira et al. 2014). They are members of the superfamily of the adenosine triphosphate-binding cassette transporters, which also harbors multidrug resistance-associated protein 2 (MRP2). Inhibition of BSEP can result from direct *cis*-inhibition or indirect *trans*-inhibition. The majority of drugs inhibit BSEP *via cis*-inhibition in a competitive manner (Pauli-Magnus et al. 2006). Although BSEP is often considered as the most common transporter associated with drug-induced cholestasis, its inhibition does not necessarily lead to cholestasis and, *vice versa*, cholestasis is not always linked with BSEP modulation. Other transporters, such as MDR3, can be involved in cholestasis as well (de Lima Toccafondo Vieira et al. 2014; Dixon et al. 2000; Kotsampasakou et al. 2017; Morgan et al. 2010; Pauli-Magnus et al. 2006). MDR3 functions as an adenosine triphosphate-dependent phospholipid flippase. This implies that MDR3 facilitates the translocation of phosphatidylcholine from the inner to the outer leaflet of the canalicular membrane (de Vree et al. 1998). Phospholipids are required in the formation of mixed micelles of bile acids and cholesterol to protect apical membranes of cholangiocytes and hepatocytes against the toxic detergent properties of bile acids (Wagner et al. 2009). Another relevant transporter is the anion export pump MRP2, which causes jaundice after inhibition by drugs, such as the antibiotic fusidate (Zollner et al. 2008). Besides canalicular transporters, basolateral transporters also are involved in cholestasis. These basolateral transporters include both efflux transporters, such as MRP3 (Padda et al. 2011; Pauli-Magnus et al. 2006) and MRP4 (Köck et al. 2014; Kotsampasakou et al. 2017) and uptake transporters, like sodium taurocholate cotransporter (NTCP) (Erlinger 2015; Greupink et al. 2012; Padda et al. 2011;

Pauli-Magnus et al. 2006), organic anion transporting polypeptides 1B1, 1B3 and 2B1 (OATP1B1, 1B3 and 2B1) (Padda et al. 2011; Pauli-Magnus et al. 2006). P-glycoprotein, also known as MDR1, is not responsible for transporting bile acids, however, can play an essential role in drug-induced cholestasis. This can be explained by the large number of substrates and inhibitors that can cause drug-drug interactions, which interferes with normal functioning of the hepatocytes (Kotsampasakou et al. 2017; Pauli-Magnus et al. 2006). In addition to inhibition of transporter functionality, genetic mutations as well as downregulation and upregulation of transcription of transporter genes can predispose to cholestasis. As such, mutations and polymorphisms in the *BSEP* gene and diminished transcription of the *MRP2* gene have been described in cholestasis (Lang et al. 2007; Trauner et al. 1997). Furthermore, several drugs that inhibit BSEP activity simultaneously reduce its expression (Garzel et al. 2014). In addition, drugs can influence subcellular localization of transporters. This has been observed for estradiol 17β-glucuronide, an endogenous estrogen metabolite that induces MRP2 and BSEP internalization from the canalicular membrane area. These changes lead to a decrease in bile flow and bile acid secretion in a dose-dependent manner (Mottino et al. 2002; Zollner et al. 2008).

2.1.2 Hepatocellular changes

Drastic alterations in the cytoskeletal architecture of hepatocytes frequently appears as an initiating event of drug-induced cholestasis (Song et al. 1998). The cytoskeleton consists of 3 major components, namely microtubules, microfilaments and intermediate filaments (Fickert et al. 2002). Microtubules assist in the insertion of proteins in polarized membrane domains (Yang et al. 2013). Microfilaments form a supporting network around the bile canaliculus and regulate tight junction permeability (Fickert et al. 2002). Intermediate filaments represent the largest fraction of the cytoskeletal family. They provide structural support and protect against mechanical and non-mechanical stress (Song et al. 1996; Strnad et al. 2008). Drugs can interfere with hepatocyte polarity because of their effects on microtubules and cytokeratin intermediate filaments. This impedes appropriate localization of tethering proteins, which is associated with modified vesicular trafficking of transporters to the canalicular pole of hepatocytes (Fickert et al. 2002; Song et al. 1996, 1998). Rifampicin and estradiol 17-β-glucuronide, known

cholestasis-inducing drugs, affect hepatocyte polarity and additionally disrupt tight junctions (Chen et al. 2009; Mottino et al. 2007). Hepatocyte tight junctions are intercellular barriers between the canalicular and sinusoidal space, shaped by multiple proteins, directly or indirectly anchored to the cytoskeleton. The integrity of hepatocyte tight junctions is of utmost importance for holding back diffusion of bile components from the canalicular spaces to the sinusoidal space (Chen et al. 2009). Deterioration of tight junctions can become apparent when the tight junction structural proteins zonula occludens and occludin are fragmented. This leads to a disturbed bile-to-plasma osmotic gradient and to malfunctioning of the apical-basolateral diffusion barrier. The latter ensues impairment of bile flow, including regurgitation of bile acids into blood and increased paracellular leakage (Mottino et al. 2007; Zollner et al. 2008). The increased permeability of the tight junctions as cause of cholestasis is, however, only convincingly demonstrated for α-naphthylisothiocyanate (ANIT) (Krell et al. 1981) and not for ethinylestradiol (Jaeschke et al. 1983). In fact, changes in the permeability of the paracellular pathway during long-term estrogen treatment are independent of reduction in bile flow (Jaeschke et al. 1987). Thus, only substantial changes of tight junction permeability will have a relevant effect on bile flow. Another hepatocellular effect driven by cholestatic drugs is altered biliary canalicular membrane lipid content, which affects membrane fluidity, thereby disturbing biliary lipid secretion. Membrane fluidity refers to the motional freedom of membrane components, in particular membrane lipids. Cholesterol is a major determinant of membrane fluidity and serves to rigidify membranes. In addition to cholesterol, a high sphingomyelin content plays a role in decreasing membrane fluidity. Increased quantities of free cholesterol and cholesterol esters in plasma membranes are observed in cyclosporin A-induced and estrogen-induced cholestasis (Hyogo et al. 1999; Smith et al. 1987; Yasumiba et al. 2001).

2.1.3 Bile canalicular changes

The frequency of myosin light chain 2 phosphorylation and dephosphorylation concomitant with spontaneous rhythmic contractions of bile canaliculi are essential for bile flow. Myosin light chain 2 phosphorylation and dephosphorylation are controlled by regulators of the rho-associated protein kinase (ROCK)/myosin light chain kinase/myosin pathway (Sharanek et al. 2016). Cholestatic drugs can

interfere with bile canaliculi dynamics at a very early stage, even before transporter inhibition, leading to alterations in overall contractile movement and disturbed bile removal. These alterations rely on constriction or dilatation of bile canaliculi (Burbank et al. 2016). Cyclosporin A and chlorpromazine initiate constriction due to early ROCK activity and myosin light chain 2 phosphorylation. These effects are considered irreversible, since the constriction introduces a terminal step of cell death. Flucloxacillin, on the other hand, induces dilatation of bile canaliculi, which merely triggers reversible cell damage and does not impede cell survival (Burban et al. 2017; Burbank et al. 2016; Sharanek et al. 2016). Flucloxacillin activates protein kinase C/p38, resulting in the phosphorylation of heat shock protein 27. This essential protein elicits phosphatidylinositol-4,5-biphosphate 3-kinase activation and protein kinase B phosphorylation, concomitant with ROCK inhibition and hence dilatation of bile canaliculi (Burban et al. 2017). Other chemicals, such as bosentan and ANIT, induce bile canalicular dilatation by hindering myosin light chain kinase and by reducing myosin light chain 2 phosphorylation (Sharanek et al. 2016).

2.2 Deteriorative response

2.2.1 Mitochondrial impairment

Mitochondrial defects underlie several liver diseases (Arduini et al. 2011; Hassanein 2004; Wei et al. 2008). In chronic drug-induced cholestasis, mitochondrial dysfunction occurs as a consequence of the loss of membrane potential, decrease in respiratory chain activity, fatty acid oxidation and ketone body formation (Arduini et al. 2011; Palmeira et al. 2004; Spivey et al. 1993). Specifically, accumulation of bile salt glycochenodeoxycholate was found to induce rapid fragmentation of mitochondria, leading to an increased cell death rate (Yu et al. 2014). Glycochenodeoxycholate binds to the outer membrane of mitochondria and evokes detachment of the inner membrane. Subsequently, mitochondrial membrane permeability transition is induced, being a decisive event in the induction of cell death. Mitochondrial membrane permeability transition involves an unspecific increase in inner mitochondrial membrane permeability, inducing structural changes, such as swelling of mitochondria, followed by rupture of the mitochondrial outer membrane. These events activate the release of cytochrome c and other cytotoxic

mitochondrial proteins that cause cell death (Begriche et al. 2011; Schulz et al. 2013). Next to affecting mitochondrial membrane permeability, bile acids can play a role in mitochondrial fission, thereby contributing to the production of reactive oxygen species (ROS), hepatocellular injury and fibrosis (Yu et al. 2014). The overall severity of these bile acid-induced mitochondrial effects depends on species, dose and time. Sequential mitochondrial destruction suggests that rat hepatocytes exposed for short periods of time or to low concentrations of hydrophobic bile acids undergo apoptosis driven by limited mitochondrial impairment. By contrast, mitochondria exposed to high concentrations of bile acids for extended periods of time, particularly in addition to elevated calcium concentrations, are more prone to necrosis due to irreversible mitochondrial destruction (Schulz et al. 2013). It is important to keep in mind that these hydrophobic bile acids are more relevant to the human pathophysiology rather than the rodent pathophysiology of cholestasis (Woolbright et al. 2015). However, in most of these studies regarding the mechanistic basis of cholestasis, mainly high concentrated unconjugated or glycine-conjugated hydrophobic bile acids are being used on rodent hepatocytes, while rodents are never exposed to these bile acids at such high concentrations *in vivo*. The most abundant rodent bile acids are taurocholic acid, β-muricholic acid and tauro-β-muricholic acid, which are not cytotoxic in rodent hepatocytes (Zhang et al. 2012).

2.2.2 Endoplasmic reticulum stress

Various stress-sensing and signaling functions are fulfilled by the ER, in particular those related to folding and posttranslational modifications of proteins destined for the secretory pathway. In addition, the ER maintains an efficient oxidized and calcium-rich folding environment for cells. During pathophysiological conditions, such as hypoxia, ER homeostasis is compromised and protein folding processes are hampered, resulting in an imbalanced protein folding/load capacity ratio, a condition called ER stress (Bhat et al. 2017). The accumulation of misfolded proteins is recognized by the glucose-regulated protein 78, which elicits a stress reaction known as the unfolded protein response. This response is characterized by the activation of 3 signaling pathways mediated by inositol-requiring protein 1α (IRE1α), protein kinase RNA-like ER kinase (PERK) and activating transcription factor

(ATF) 6α. The IRE1α signaling pathway starts by autophosphorylation of IRE1α. This activates RNase activity that splices mRNA of x-box binding protein 1 into an active transcription factor. Likewise, IRE1α activation leads to induction of the stress kinase c-Jun *N*-terminal kinase. The second pathway mediated by PERK attenuates global mRNA translation, but increases the translation of specific mRNAs, including transcription factor ATF4 and CCAAT-enhancer-binding protein homologous protein. The third pathway involves ATF6α, which moves to the Golgi compartment, where it is cleaved to yield an active soluble transcription factor. Overall, these unfolded protein response pathways promote protein folding, transport and degradation of misfolded proteins, and concurrently attenuate the load of new proteins entering the ER (Malhi et al. 2011). The primary function of the unfolded protein response is to re-establish ER homeostasis and to promote cell survival. However, a switch occurs from a prosurvival mode towards a prodeath response in case of excessive ER stress *via* activation of the intrinsic apoptosis pathway or through autophagy (Bhat et al. 2017). Cholestatic chemicals, such as cyclosporin A and ANIT, elevate the expression of ER stress genes (Sharanek et al. 2014; Szalowska et al. 2013; Tamaki et al. 2008; Yao et al. 2016). The critical role of ER stress in the initiation and progression of drug-induced cholestasis has been shown for several drugs, including cloxacillin, nafcillin, fluoroquinolones, levofloxacin, erythromycin and penicillinase-resistant antibiotics (Burban et al. 2018). Nonetheless, other data suggest that ER stress suppresses the primary bile acid synthetic pathway and promotes the removal of excess bile acids from the liver by means of activation of hepatic transporters. This points to a rescue mechanism instead of a deteriorative role for ER stress (Henkel et al. 2017).

2.2.3 Oxidative stress

Oxidative stress is a critical determinant in the pathogenesis of cholestasis, characterized by increased ROS formation and impaired antioxidant systems. ROS are naturally generated from molecular oxygen by every living cell with a normal cellular metabolism (Birben et al. 2012). Hepatocytes are prone to develop oxidative stress, as they contain hundreds of mitochondria, which are a major source of ROS in the liver, in which electron transport can be easily disturbed (Copple et al. 2010; Perez et al. 2009; Tiao

et al. 2009). Hydrophobic bile acids fulfill a critical function in oxidative stress in humans by generating ROS, while elevating cytosolic free calcium levels. The latter facilitates opening of the mitochondrial membrane permeability pore. The subsequent electron leakage stimulates additional ROS formation (Perez et al. 2009; Tiao et al. 2009). The resulting oxidative stress will activate several antioxidants, such as glutathione, ubiquinone-9 and ubiquinone-10. Deprivation of the antioxidant reservoirs renders the cells more vulnerable to additional oxidative stress and thus to cell death (Copple et al. 2010; Perez et al. 2009). Limited ROS formation may cause a preconditioning effect to oxidative stress rather than cell death. The preconditioning effect induced by a subtoxic stress stimulus increases resistance to more severe inflammatory oxidative stress. By doing so, limited levels of oxidative stress activate the Kelch-like ECH-associated protein 1 (Keap1)-nuclear related factor 2 (Nrf2)-antioxidant response element signaling pathway. This pathway responds to oxidative stress by regulating a large number of defense genes that protect against acute and chronic injury. This consists of the stress sensor Keap1, which is responsible for keeping Nrf2 in the cytosol for proteolysis. When Keap1 undergoes structural modifications due to oxidation/conjugation of its cysteine residues, Nrf2 dissociates from Keap1. Consequently, Nrf2 translocates to the nucleus and binds to antioxidant response elements. This activates the gene transcription of a number of genes that encode detoxifying or antioxidative proteins, such as heme oxygenase-1, superoxide dismutase-1 and glutathione peroxidase (Jaeschke 2011). Cyclosporin A was found to upregulate the expression of Nrf2, heme oxygenase-1 and superoxide dismutase-2 in hepatocytes, presumably as an oxidative stress-mediated response during acute cholestasis (Sharanek et al. 2014).

2.2.4 Inflammation

ER stress, mitochondrial injury, changes in mitochondrial membrane potential and release of cytochrome c may be the result of bile acid accumulation. Mitochondrial damage that coincides with the release of mitochondrial deoxyribonucleic acid (DNA) is identified as damage-associated molecular patterns, capable of triggering Toll-like receptor 9 (TLR9). TLR9 is an intracellular DNA receptor involved in the innate immune response that, upon activation, stimulates production of inflammatory

cytokines and chemokines (Woolbright et al. 2017). The transcription factor early growth response factor-1 (Egr1) is known to be an additional important determinant in inflammation that is induced by bile acids (Allen et al. 2010; Kim et al. 2006). Egr1 is a master regulator of proinflammatory molecules in hepatocytes. Increased concentrations of bile acids activate mitogen-activated protein kinase signaling, which upregulates Egr1, in turn affecting the expression of certain chemokines. These chemokines attract and activate neutrophils (Allen et al. 2011; Kim et al. 2006), causing neutrophil-dependent liver injury (Gujral et al 2003). Furthermore, excessive amounts of bile acids, such as chenodeoxycholic acid, are believed to trigger inflammasome activation through promotion of ROS production and potassium efflux in Kupffer cells and liver macrophages. Active inflammasomes initiate inflammatory responses through activation, maturation and secretion of proinflammatory cytokines, including interleukin-1β (Gong et al. 2016). The nucleotide-binding and oligomerization leucine-rich repeat protein 3 (NLRP3) inflammasome can be activated by a broad range of stimuli, among which bile acid accumulation. In response to these stimuli, the NLRP3 complex assembles the adaptor molecule apoptosis-associated speck-like protein, which contains a caspase recruitment domain and the effector molecule procaspase-1 (Li et al. 2017; Rathinam et al. 2012; Stehlik et al. 2003). Once cleaved, activated caspase 1 stimulates NLRP3 and thereafter cleaves pro-interleukin-1β and pro-interluekin-18 yielding their active forms (Li et al. 2017).

2.2.5 Cell death

Bile acid accumulation burgeons into cell death. However, there is still quite some discussion regarding the nature of cell death in cholestasis (Woolbright et al. 2012). Serum biomarkers and histopathological features in cholestatic mice indicate early cholestatic liver injury through inflammatory necrosis rather than apoptosis (Gujral et al. 2004; Mitchell et al. 2011; Woolbright et al. 2013). Necrotic cell death in rodents can be induced by neutrophil-derived oxidative stress involving hypochlorous acid (Gujral et al. 2003). The resulting cholestatic liver injury is a pure inflammatory liver insult. Dominant rodent bile acids, such as taurocholic acid and tauro-muricholic acid, are not cytotoxic, but act as inflammagens in this model (Allen et al. 2011; Zhang et al 2012). Interestingly, if the bile acid composition is modified

by lithocholic acid feeding, the now dominant hydrophobic bile acids can cause hepatocellular death directly and independent of inflammatory cells (Woolbright et al. 2014). In humans, hydrophobic glycine-conjugated bile acids are also observed to cause directly necrotic cell death during cholestasis (Woolbright et al. 2015). In addition, necroptosis can be involved in cholestasis. Necroptosis is a well-regulated type of necrosis that depends on receptor interacting protein (RIP) 3 kinase activity (Afonso et al. 2016). Necroptosis is initiated by the binding of specific ligands to their cell death receptors, including tumor necrosis factor α. Subsequently, RIP1 forms a complex with RIP3. This intracellular complex assembles the necrosome, a filamentous amyloid protein complex that acts as a transducer of the necroptotic signal. Downstream of RIP3 locates a mixed lineage kinase domain-like (MLKL) that can be phosphorylated by RIP3 (Afonso et al. 2016; Linkermann et al. 2014). Increased levels of RIP1/3 and MLKL, indicative of necroptosis, are observed in cholestasis patients (Afonso et al. 2016). Autophagy is a regulatory process that degrades and recycles intracellular proteins and damaged organelles to maintain energy homeostasis and to promote cell survival following stress (Sasaki et al. 2015). This regulatory process is characterized by the formation of double-membraned autophagosomes. Closure of the autophagosomal membranes requires microtubule-associated protein 1 light chain 3 (LC3). These autophagosomes fuse with lysosomes and break down the autophagosome-enclosed content (Manley et al. 2014). Autophagy is manifested in cholestatic mice, where this process serves a protective role against hepatic injury induced by ROS. Moreover, compromised autophagy is believed to contribute to the pathogenesis of cholestasis. Indeed, bile acids increase hepatic levels of LC3-II and p62, which can be associated with an impaired autophagic process. In particular, bile acids are thought to interfere with the fusion of autophagosomes and lysosomes (Gao et al. 2014). It should be noted that ER stress is also associated with autophagy (Manley et al. 2014).

2.3 Adaptive response

Cholestatic liver damage is accompanied by remarkable hepato-protective mechanisms aimed at counteracting the disturbed bile acid homeostasis. These defense mechanisms are part of the adaptive response. Besides their toxic properties, bile acids also have a protective function as signaling molecules

during the adaptive response. A number of transcriptionally coordinated mechanisms can be activated by bile acids, leading to a decrease in their cellular uptake, while increasing their cellular efflux. Enhanced hydroxylation and conjugation of bile acids by means of phase 1 and phase 2 biotransformation reactions promote detoxification of bile acids and creates an alternative way of elimination *via* urine (Wagner et al. 2009; Zollner et al. 2006a). These transcriptionally regulated mechanisms rely on the actions of specific nuclear receptors (Halilbasic et al. 2013; Zollner et al. 2006a; 2006b). Nuclear receptors are activated through ligand binding and act as transcription factors. As part of their activation, nuclear receptors undergo conformational changes. These changes facilitate coactivator recruitment and dissociation of corepressors, and thus stimulate gene transcription (Cuperus et al. 2014). The nuclear receptors farnesoid X receptor (FXR), pregnane X receptor (PXR) and vitamin D receptor are most frequently associated with hepatobiliary homeostasis and bile secretion. Other nuclear receptors, such as the peroxisome proliferator-activated receptors, glucocorticoid receptor and constitutive androstane receptor, behave as regulators of inflammation, fibrosis and energy homeostasis. They can also alter bile acid homeostasis and therefore underlie cholestatic injuries (Cuperus et al. 2014; Halilbasic et al. 2013). Activated FXR can exert its transcriptional activity by forming a heterodimer with the retinoid X receptor (RXR). The resulting FXR-RXR heterodimer directly binds to an inverted repeat 1 sequence of the gene promotor and initiates transcription (Halilbasic et al. 2013). FXR promotes bile acid excretion through induction of the expression of BSEP, MDR3, MRP2 and organic solute transporter α/β (Cuperus et al. 2014; Halilbasic et al. 2013; Li et al. 2015). Moreover, FXR protects hepatocytes against bile acid-induced injury through detoxification of bile acids by inducing the expression of cytochrome P450 (CYP) 3A4, sulfotransferase 2A1 and uridine diphosphate glucuronosyltransferase (UGT) 2B4. These enzymes mediate hydroxylation and conjugation of bile acids, resulting in more hydrophilic and less toxic compounds (Cuperus et al. 2014; Halilbasic et al. 2013). PXR induces the production of sulfotransferase 2A1, UGT isozymes, MRP2 and organic anion transporting polypeptide 2 upon cholestatic stress. Furthermore, FXR enhances the expression of the atypical nuclear receptor short heterodimer partner. In contrast to FXR-RXR heterodimers, the short heterodimer partner does not bind to DNA, but interferes with other nuclear receptors as part of transcriptional repression (Cuperus et al. 2014; Halilbasic et al. 2013; Li et al. 2015). The short

heterodimer partner represses transcription of the genes encoding the bile salt uptake transporter sodium-taurocholate co-transporting polypeptide and enzymes essential in the biosynthetic pathway, such as CYP7A1 and CYP8B1 (Cuperus et al. 2014). Importantly, the adaptive response to cholestatic insults is not restricted to the hepatocytes, but also occurs in the intestine, kidneys and epithelia of the bile duct (Wagner et al. 2009). In this respect, proliferation of cholangiocytes leads to corrugations of the luminal duct surface. Accordingly, the surface area increases by 5-fold, ducts elongate, branches sprout and loops are formed. Alterations in the bile duct morphology strive to maintain the proximal position of the bile duct relative to the portal vein, which is essential for bile acid transport. In addition, this remodeling process enhances resorption of bile acids from the bile duct lumen and transportation to the portal vein (Jansen et al. 2017; Vartak et al. 2016).

3 *In vitro* models of drug-induced cholestasis

In vitro models circumvent a number of constraints encountered with animal testing, in particular regarding the ethical aspects (Russell and Burch 1959). Among many advantages, *in vitro* models provide the possibility for higher-throughput screening of potentially cholestatic drugs (Soldatow et al. 2013) and for gaining mechanistic insight without dealing with the challenge of a complex environment. Absence of this dynamic surrounding can, however, also hamper *in vitro*-to-*in vivo* extrapolation of results (Das 2018). As such, 2 groups of liver-based *in vitro* models are available today, namely liver-derived *in vitro* models and stem cell-derived *in vitro* models. Liver-derived *in vitro* models eligible for testing cholestatic drugs include monolayer cultures of human hepatoma HepaRG cells (Woolbright et al. 2016), sandwich cultures of primary human hepatocytes (Chatterjee et al. 2014; Oorts et al. 2016), spheroid cultures of primary human hepatocytes or HepaRG cells (Bell et al. 2016; Hendriks et al. 2016) and freshly isolated liver slices (Vatakuti et al. 2017). The other group consists of *in vitro* models derived from stem cells, such as human adult stem cells, human embryonic stem cells and human induced pluripotent stem cells (iPSC) (Snykers et al. 2011; Soldatow et al. 2013).

3.1 Liver-derived *in vitro* models

3.1.1 Monolayer cultures of human hepatoma HepaRG cells

Cell lines are featured by their continuous growth, almost unlimited lifespan, ease of use and stable phenotype, allowing them to be used for long-term testing purposes. Moreover, cell lines can be easily standardized among different laboratories, thereby expediting interlaboratory reproducibility (Bell et al. 2017; Castell et al. 2006). However, several human hepatoma-derived cell lines, in particular HepG2 cells, show limited functional expression of drug metabolizing mediators, including CYP enzymes (Bell et al. 2017; Donato et al. 2013), thus restricting their use for reliable detection of cholestatic effects. Nevertheless, human hepatoma HepaRG cells present many hallmarks of differentiated hepatocytes, including functional expression of phase I and II biotransformation enzymes, drug transporters and nuclear receptors (Castell et al. 2006; Gripon et al. 2002). In addition, HepaRG cells show susceptibility to bile acid toxicity and mimic mechanisms of cell death similar to freshly isolated primary human hepatocytes (Woolbright et al. 2016). These features make the HepaRG cell line popular for use in studying cholestatic effects induced by drugs, such as chlorpromazine (Bachour-El Azzi et al 2014) and cyclosporin A (Sharanek et al. 2014). As such, the involvement of oxidative stress both as a primary causal process and aggravating event in intracellular bile acid accumulation has been demonstrated in conventional monolayer cultures of HepaRG cells treated with chlorpromazine (Anthérieu et al. 2013). Bile acid pool concentrations typically increase up to 50-fold in serum of cholestasis patients (Humbert et al. 2012; Tagliacozzi et al. 2003). Hence, to better resemble cholestatic liver injuries *in vivo*, cultures of HepaRG cells are often simultaneously exposed to cholestatic drugs and a 30-60 times concentrated mixture representative of the most abundant bile acids in human serum (Sharanek et al. 2017). Adding such bile acid mix enables HepaRG cells cultured in a monolayer configuration to distinguish potentially cholestatic drugs from those rarely causing cholestasis in clinic settings by assessing the *in vitro* intracellular accumulation of unconjugated forms of hydrophobic bile acids (Sharanek et al. 2017). Moreover, since HepaRG cells are bipotential, resulting in the formation of 2 separate morphologically distinct cell types, namely cholangiocyte-like cells and hepatocyte-like cells (Parent et al. 2004), they

may be used to investigate bile acid-evoked injury on hepatocytes as well as on cholangiocytes (Woolbright et al. 2016).

3.1.2 Sandwich cultures of primary human hepatocytes

The golden standard in the field of liver-based *in vitro* modeling is primary hepatocytes and their cultures derived from freshly isolated liver tissue, as they provide a good reflection of the hepatic *in vivo* situation (Fraczek et al. 2013; Godoy et al. 2013; Soldatow et al. 2013). Primary human hepatocytes are typically isolated from resected liver tissue by means of the 2-step collagenase perfusion technique (Seglen et al. 1976). Alternatively, cryopreserved primary human hepatocytes can be used in order to overcome the ubiquitous limited availability of fresh human material for toxicity testing purposes (Hengstler et al. 2000). When seeded on a plastic cell culture dish, whether or not coated with a layer of extracellular matrix (ECM), primary human hepatocytes rapidly lose their typical *in vivo* morphological and functional differentiation properties (Soldatow et al. 2013; Zeilinger et al. 2016). This dedifferentiation process can be counteracted by culturing the cells between 2 layers of ECM, yielding a so-called sandwich culture system. In this configuration, the cultured hepatocytes maintain their polarized phenotype with apical and basolateral domains, the latter being in direct contact with an ECM scaffold (Du et al. 2008; Vinken et al. 2006). Collagen type I is the most common ECM compound used to set up sandwich culture systems (Du et al. 2008). Other ECM scaffolds addressed for this purpose include extracts from murine Engelbreth-Holm Sward tumors, such as Geltrex™ and Matrigel™ (Hasirci et al. 2001). Sandwich cultured hepatocytes are metabolically competent, with properly localized drug transporters and a functional bile canalicular network. For this reason, they are often used when assessing hepatobiliary disposition of xenobiotics and drug transporter-based drug-drug interactions (Annaert et al. 2005; De Bruyn et al. 2013; Yang et al. 2016). Renewal of the ECM overlay in a sandwich configuration every 3-4 days was shown to extend cultivation regimes up to 14 days (Parmentier et al. 2013). Hence, they represent suitable *in vitro* tools for long-term hepatotoxicity studies, including drug-induced cholestasis (Bell et al. 2018; Oorts et al. 2016). In order to quantify the cholestatic potential and evaluate the cholestatic risk of compounds tested in sandwich cultures, the

drug-induced cholestasis index (DICI) was introduced. This parameter reflects the functionality of hepatocytes co-exposed to a cholestatic compound and a concentrated bile acid mixture compared to treatment with the cholestatic compound alone. Compounds with a DICI equal to or lower than 0.8 are considered to bear a cholestatic risk (Oorts et al. 2016). A safety margin for drug-induced cholestasis can be further calculated by dividing the lowest incubation concentration yielding a DICI equal to or lower than 0.8 by the reported mean peak therapeutic plasma concentration in humans. The safety margins obtained in the sandwich cultures of primary human hepatocytes seem to correlate well with clinical incidence data on drug-induced cholestasis. As such, drugs that show a safety margin of 30 or lower are considered to pose a significant risk in causing cholestasis (Chatterjee et al. 2014; 2018; Oorts et al. 2016). Besides their usefulness for hazard identification purposes, DICI values can also be used to study donor-dependent differences in the response of primary human hepatocytes to cholestatic toxicity of various compounds (Parmentier et al 2018).

3.1.3 Spheroid cultures of HepaRG cells or primary human hepatocytes

A scaffold-free approach to delay dedifferentiation of primary hepatocytes as well as to enhance differentiation of HepaRG cells relies on promoting hepatocyte reaggregation by cellular self-assembly, generating a 3D configuration in so-called spheroid cultures (Godoy et al. 2013; Kelm et al. 2003, 2004; Takahashi et al. 2015). Spheroid cultures can be obtained by seeding cells on a non-adherent substratum to prevent attachment, which results in spontaneous aggregation of hepatocytes within a few days (Landry et al. 1985). Self-aggregation can also be induced *via* gravity-enforced cellular assembly in the hanging drop technique (Keller et al. 1995; Kelm et al. 2003) or by subjecting a cell suspension to continuous controlled centripetal forces in a rotary culture (Moscona et al. 1961), rocked culture (Brophy et al. 2009), perfused bioreactor (Tostões et al. 2012) or microchip (Fukuda et al. 2006). Spheroid cultures of HepaRG cells or primary human hepatocytes remain functionally stable for at least 3 or 5 weeks, respectively, and maintain endogenous hepatic functions, including drug metabolizing competence (Bell et al. 2016; Gunness et al. 2013; Messner et al. 2018; Vorrink et al. 2017; 2018). Functional formation of bile ducts and proper cellular polarity have also been reported in spheroid

cultures (Tostões et al. 2012). Spheroid cultures are used for the detection of long-term cholestatic effects. In fact, long-term repeated dosing drastically enhances the sensitivity of primary human hepatocytes cultured in a spheroid model to hepatotoxins (Bell et al. 2016). Reminiscent of the DICI, the cholestatic index (CIx) was introduced for spheroid cultures to classify the cholestatic risk of test compounds. Unlike DICI, this value reflects the viability rather than the functionality of hepatocytes co-exposed to a cholestatic compound and a concentrated bile acid mixture compared to treatment with the cholestatic compound alone (Hendriks et al. 2016). Besides the identification of cholestatic compounds, spheroid models have also been successfully exploited to recapitulate and identify underlying mechanisms of drug-induced cholestasis (Bell et al. 2018; Hendriks et al. 2016). Incorporating non-parenchymal cells into a primary hepatocyte spheroid culture more closely resembles the hepatic micro-environment *in vivo*, in particular heterotypic cell-cell contacts. Accordingly, non-parenchymal liver cells, such as liver sinusoidal endothelial cells, hepatic stellate cells and Kupffer cells, provide support and improve functionality of primary hepatocytes, while contributing to inflammatory responses in spheroid cultures. Spheroid co-cultures therefore qualify to exploit the role of the immune system in drug-induced cholestasis as well as to study the mechanisms involved in idiosyncratic DILI (Baze et al. 2018; Messner et al. 2013; Sison-Young et al. 2015).

3.1.4 Liver slices

A plethora of techniques has been introduced to obtain liver slices with a uniform thickness, including using razor blades. However, liver slices obtained by following these procedures suffered from multiple shortcomings, among which low reproducibility and high sensitivity to hypoxia, leading to acute necrosis. From 1980 onwards, this technique boomed in popularity due to the implementation of mechanical tissue slicers. This method allows to produce thin and minimally damaged liver slices, which provide adequate nutrient and oxygen exchanges, referred to as precision-cut liver slices (PCLS) (Hengstler et al. 2014; Lerche-Legrand 2000). PCLS represent a solid model characterized by the presence of all liver cell types in their natural habitat as well as sustained intercellular and cell-ECM interactions (Starokozhko et al. 2017a). The presence of all cell types in this model is of great value for

toxicological studies, as Kupffer cells, endothelial cells, hepatic stellate cells and bile duct epithelial cells are involved in the pathogenesis of many adverse outcomes, including inflammation, necrosis, fibrosis and cholestasis (Godoy et al. 2013). PCLS are therefore applicable for multicellular acute toxicity studies (Vatakuti et al. 2016). Incubating PCLS with cholestatic compounds together with bile acids is used as a classification model. Gene expression profiling is hereby applied to distinguish hepatotoxicants that induce cholestasis from those inducing necrosis with high accuracy. Furthermore, transcriptomic analysis of PCLS exposed to cholestatic drugs reveals several aspects of the machinery of events involved in cholestasis, including both the *in vivo*-like deteriorative response, with processes such as oxidative stress, ER stress and the unfolded protein response, and the adaptive response. This renders PCLS a suitable model for identification of cholestatic biomarkers, while exploring the mechanisms associated with cholestatic toxicity (Vatakuti et al. 2016; 2017). As a matter of fact, by using this model, the roles of nuclear receptor FXR in cholesterol metabolism pathways and ER stress in cholestasis have been substantiated (Vatakuti et al. 2016). Long-term exposure or chronic toxicity testing in fresh PCLS were initially considered not feasible because of the occurrence of cell necrosis 48-72 hours after setting up the model as well as the decreasing activity of (xenobiotic) metabolizing enzymes after 6-72 hours (Hengstler et al. 2014; Soldatow et al. 2013). Nonetheless, a recent study showed human PCLS cultured in a specific cell culture medium to exhibit extended viability and improved functionality up to 5 days, allowing the model to be used for prolonged toxicity testing (Starokozhko et al. 2017b). Unfortunately, like freshly isolated primary human hepatocytes, human PCLS are restricted in availability, which limits their use in screening large amounts of drugs (Vatakuti et al. 2017).

3.2 Stem cell-derived *in vitro* models

Stem cell-derived *in vitro* models provide several characteristics that may overcome certain drawbacks of primary human hepatocytes and hepatoma-derived cells (Guguen-Guillouzo et al. 2010a; Ni et al. 2016). Stem cells display a self-renewing capacity *in vitro*, while maintaining the possibility to differentiate in multiple cell types, and are capable of *in vivo* reconstitution of a given tissue (Hannan et

al. 2013; Kia et al. 2013; Lakshmipathy et al. 2005). Based on their origin and differentiation potential, stem cells can be divided into 2 major groups, namely embryonic stem cells and adult stem cells (Lakshmipathy et al. 2005; Zeilinger et al. 2016). In 2006, a state of pluripotency was induced for the very first time in an adult cell, yielding so-called iPSC. Stem cells can be differentiated *in vitro* to hepatocyte-like cells by sequential exposure to growth factors, cytokines and other factors according to liver embryogenesis *in vivo*. (Cai et al. 2012; Guguen-Guillouzo et al. 2010b; Snykers et al. 2009). Stem cell-derived *in vitro* models have shown effective in modeling different types of DILI, including phospholipidosis and steatosis (Natale et al. 2018; Rodrigues et al. 2013; 2015; 2016), as well as in modeling familial hereditary cholestasis (Imagawa et al. 2017). However, pronounced differences are found between the transcriptomic signature of human iPSC and that of primary human hepatocytes cultured in spheroids. In particular, expression of most transcripts encoding enzymes involved in xenobiotic and endogenous metabolism are significantly lower, whereas levels of fetal CYP enzymes and transporters are considerably increased in iPSC. Chlorpromazine-induced and troglitazone-induced cholestatic injury can be detected by iPSC-derived hepatic *in vitro* models, albeit with lower sensitivity compared to spheroid cultures of primary human hepatocytes, and requiring much higher concentrations than clinically relevant (Bell et al. 2017). So-called human-induced hepatocytes are produced by direct reprogramming of human fibroblasts into a hepatic cell lineage, with avoidance of the intermediate pluripotent state. These human-induced hepatocytes are thought to correlate well with primary human hepatocytes in predicting potential drug-induced BSEP inhibition. Of note, these human-induced hepatocytes express significantly lower levels of BSEP compared to primary human hepatocytes. In addition, they also produce less intercellular conjugated bile acids and display diminished susceptibility to conjugated bile acid-induced hepatotoxicity (Ni et al. 2016).

4 Conclusions and perspectives

Drug-induced cholestasis is one of the main causes of DILI (Noor 2015) and is a complex pathological process. Several factors can trigger drug-induced cholestasis, including transporter changes (Pauli-Magnus et al. 2006; Ramboer et al. 2013; Trauner et al. 1997; 2003; Yang et al. 2013), hepatocellular

changes (Fickert et al. 2002; Hyogo et al. 1999; Smith et al. 1987; Song et al. 1996; 1998; Yasumiba et al. 2001) and bile canalicular changes (Burban et al. 2017; Burbank et al. 2016; Sharanek et al. 2016). Collectively, this leads to accumulation of bile acids, which in turn activates a deteriorative response characterized by mitochondrial impairment (Begriche et al. 2011; Yu et al. 2014), ER stress/unfolded protein responses (Burban et al. 2018), oxidative stress (Copple et al. 2010; Perez et al. 2009; Tiao et al. 2009), inflammation (Cai et al. 2017; Gong et al. 2016; Woolbright et al. 2012; 2017) and cell death (Afonso et al. 2016; Gao et al. 2014). At the same time, an adaptive response is triggered, which mainly relies on nuclear receptor activation (Halilbasic et al. 2013). As such, in-depth knowledge of the molecular mechanisms of cholestasis is crucial to ensure a more accurate prediction of this type of DILI. In this regard, current *in vitro* prediction of drug-induced cholestasis typically relies on testing single parameters, such as BSEP inhibition, or using single techniques, like microarrays. This has been shown problematic due to poor predictivity (Aleo et al. 2017; Ali et al. 2017; Köck et al. 2014) or low sensitivity (Kawamoto et al. 2017; Van den Hof et al. 2014). In the last decade, significant efforts have been focused on the development of *in vitro* systems to study drug-induced cholestatic liver injury. A number of state-of-the-art *in vitro* models to monitor DILI are now available and belong to 2 classes, namely liver-derived *in vitro* models and stem cell-derived *in vitro* models. Stem cell-derived *in vitro* models have shown their value for studying different types of liver toxicity, including accumulation of lipids (Natale et al. 2018; Rodrigues et al. 2013; 2015; 2016). However, although some studies demonstrated their promise (Imagawa et al. 2017; Natale et al. 2018; Rodrigues et al. 2013; 2015; 2016), hepatocyte-like cells derived from stem cells have been reported not to be the most appropriate *in vitro* systems for the detection of cholestatic chemicals (Bell et al. 2017, Ni et al. 2016). Liver-derived *in vitro* models, such as HepaRG cells cultured in a classical monolayer configuration, sandwich cultures of primary human hepatocytes, spheroid cultures of primary human hepatocytes or HepaRG cells, and liver slices, are still considered the most appropriate systems to identify cholestatic drugs (Hendriks et al. 2016; Oorts et al. 2016; Qiu et al. 2016). Each of these liver-derived *in vitro* models has its own strengths and flaws (Table 1-2), therefore it is recommended to combine these systems when studying and predicting drug-induced cholestasis. Likewise, optimal combinations of biomarkers should be used instead of focusing on isolated endpoints. In addition, *in vitro* testing should be ideally complemented with computational

modeling of drug-induced cholestasis. *In silico* modeling represents a time-efficient and cost-efficient approach to quickly detect hepatotoxicity based on structural and/or physico-chemical parameters (Przybylak et al. 2012; Zhu et al. 2017). Further exploration of such combined *in vitro* and *in silico* test batteries will undoubtedly pave the way for more accurate prediction of cholestatic effects induced by candidate pharmaceuticals in the early drug development process.

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Conflict of interest statement

The authors have no conflicts of interest to declare.

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Figure and table legends

Figure 1

Triggering factors resulting in drug-induced cholestasis. Multiple triggering factors or molecular initiating events can lead to drug-induced cholestasis. The first group consists of transporter changes (1), namely direct inhibition, internalization and/or reduced expression of bile transporters. In particular, bile salt export pump (BSEP), multidrug resistance protein 3 (MDR3), multidrug resistance-associated protein 2-4 (MRP2-4), sodium taurocholate cotransporter (NTCP), organic anion transporting polypeptides 1B1, 1B3 and 2B1 (OATP1B1/1B3/2B1) and P-glycoprotein (MDR1) represent frequent targets in drug-induced cholestasis (Pauli-Magnus et al. 2006; Lang et al. 2007; Trauner et al. 1997). The second group are hepatocellular changes (2) and consists of drug-induced perturbance of the cytoskeletal architecture *via* disturbing intermediate filaments and microtubules (Song et al. 1996, 1998), disruption of tight junctions (Mottino et al. 2007) and decreased membrane fluidity by increasing levels of free cholesterol and cholesterol esters in the plasma membrane (Hyogo et al. 1999). Finally, drugs can interfere with bile canaliculi dynamics by inducing constriction or dilatation (3), which leads to changes in overall bile contractile movement and bile removal (Burbank et al. 2016).

Figure 2

Mechanistic basis of drug-induced cholestasis. Drug-induced cholestasis can be initiated by 3 types of triggering factors namely, transporter changes, hepatocellular changes and bile canaliculi changes (Figure 1). These stimuli induce bile accumulation, which simultaneously activates 2 cellular responses, namely a deteriorative response and an adaptive response. The deteriorative response is typified by the occurrence of mitochondrial impairment (Begriche et al. 2011; Yu et al. 2014), different cell death modes (Afonso et al. 2016; Gao et al. 2014), endoplasmic reticulum (ER) stress with unfolded protein responses (UPR) (Burban et al. 2018), oxidative stress (Copple et al. 2010; Perez et al. 2009; Tiao et al. 2009) and inflammation (Cai et al. 2017; Gong et al. 2016; Woolbright et al. 2012; 2017). The adaptive cellular response strives to counteract this deteriorative response, and thus bile acid accumulation,

through activation of a number of nuclear receptors (Halilbasic et al. 2013). (ROS, reactive oxygen species).

Table 1

Advantages and disadvantages of liver-based in vitro models for studying and detecting drug-induced cholestasis. (CYP, cytochrome P450; ECM, extracellular matrix; iPSC, induced pluripotent stem cells).

Table 2

Expression of CYP enzymes and bile acid transporters in liver-based in vitro models. (CYP, cytochrome P450; iPSC, induced pluripotent stem cells; ND, not determined).