Minireview

Roles of co-factors in drug-induced liver injury: drug metabolism and beyond

Ruizhi Gu, Alina Liang, Grace Liao, Isabelle To, Amina Shehu, and Xiaochao Ma

Center for Pharmacogenetics, Department of Pharmaceutical Sciences, School of Pharmacy, University of Pittsburgh, Pittsburgh, Pennsylvania, USA (R.G., A.S., X.M.)

School of Pharmacy, University of Pittsburgh, Pittsburgh, Pennsylvania, USA (A.L., G.L., I.T.)
Running Title: Co-factors in drug-induced liver injury

Corresponding author: Dr. Xiaochao Ma, Department of Pharmaceutical Sciences, School of Pharmacy, University of Pittsburgh; 309 Salk Pavilion, 335 Sutherland Drive, Pittsburgh, PA 15261. Phone: 412-648-9448. Email: mxiaocha@pitt.edu

Number of text pages: 54
Number of figures: 2
Number of tables: 0
Number of references: 193
Number of words in Abstract: 113
Number of words in Introduction: 435
Number of words in Discussion: N/A
Abbreviation list

ADS, antioxidant defense system; APAP, acetaminophen; BSEP, bile salt export pump; BSO, buthionine sulfoximine; CYP, Cytochrome P450; DILI, drug-induced liver injury; EGFR, epidermal growth factor receptor; GSH, glutathione; GST, glutathione S-transferase; HLA, human leukocyte antigens; IL, interleukin; INH, isoniazid; LPS, lipopolysaccharide; MDSC, myeloid-derived suppressor cell; MRP, multidrug resistance-associated protein; NAFLD, nonalcoholic fatty liver disease; NLRP3, NLR family pyrin domain containing 3; Nrf2, nuclear erythroid factor type 2; PXR, pregnane X receptor; RIF, rifampicin; ROS, reactive oxygen species; TB, tuberculosis; TNF-α, tumor necrosis factor-α; TVX, trovafloxacin; UGT, UDP-glucuronosyltransferase; VEGFR, vascular endothelial growth factor receptor
Abstract

Drug-induced liver injury (DILI) remains one of the major concerns for healthcare providers and patients. Unfortunately, it is difficult to predict and prevent DILI in the clinic because detailed mechanisms of DILI are largely unknown. Many risk factors have been identified for both “intrinsic” and “idiosyncratic” DILI, suggesting that co-factors are an important aspect in understanding DILI. This review outlines the co-factors that potentiate DILI and categorizes them into two types: (1) the specific co-factors that target metabolic enzymes, transporters, antioxidation defense, immune response and liver regeneration; and (2) the general co-factors that include inflammation, age, gender, comorbidity, gut microbiota and lifestyle. The underlying mechanisms by which co-factors potentiate DILI are also discussed.

Significance statement

This review summarizes the risk factors for DILI, which can be used to predict and prevent DILI in the clinic. This work also highlights the gaps in the DILI field and provides future perspectives on the roles of co-factors in DILI.
1. Introduction

Drug-induced liver injury (DILI) is an important health problem in the clinic. The incidence of DILI is approximately 14 to 19 cases per 100,000 population, and this number is underestimated because of the limited reporting system (Sgro et al., 2002; Björnsson et al., 2013; Ahmad and Odin, 2017). Most patients who suffer from DILI recover after discontinuation of the drug. However, some patients may develop severe DILI, leading to hospitalization, liver transplantation, and even death (Fontana et al., 2014). In addition, DILI is one of the most common causes of drug withdrawal from the market (Björnsson, 2015; Hoofnagle and Björnsson, 2019). Between 1976 and 2005, 28 drugs had been withdrawn from the market in the USA, among which 6 were removed because of hepatotoxicity (Kaitin, 2005; Babai et al., 2021).

Unfortunately, the detailed mechanisms of DILI are unclear for most drugs, which make it challenging for the prevention of DILI in the clinic. Clinical and preclinical studies have identified many co-factors of DILI, which are patient-specific attributes that increase the patient’s susceptibility to DILI and/or contribute to the deterioration of DILI. Co-factors can affect both intrinsic and idiosyncratic types of DILI. The intrinsic DILI is dose-dependent and predictable (Kullak-Ublick et al., 2017; Hoofnagle and Björnsson, 2019). In addition, intrinsic DILI usually has a short latency period, and is reproducible in animal models (Sandhu and Navarro, 2020). The most well-known example of intrinsic DILI is caused by acetaminophen (APAP), which accounts for >50% cases of acute liver failure in the USA (Reuben et al., 2016). Even though APAP-induced liver injury belongs to the intrinsic type of DILI which is dose-dependent and predictable, there are also co-factors that make patients sensitive to APAP.
hepatotoxicity such as diabetes and alcohol consumption (Zimmerman and Maddrey, 1995; Aubert et al., 2012). Co-factors have also been identified for the idiosyncratic type of DILI, which is dose-independent and unpredictable (Hoofnagle and Björnsson, 2019; Sandhu and Navarro, 2020). For example, isoniazid (INH)-induced idiosyncratic DILI can be potentiated by rifampicin (RIF) (Steele et al., 1991; Chowdhury et al., 2006; Li et al., 2013). In addition, deficiency of N-acetyltransferase 2 also increases the risk of INH hepatotoxicity (Huang et al., 2002; Nicoletti et al., 2021). These data suggest that co-factors play an important role in the development of DILI.

In the current review, we discuss the co-factors that potentiate DILI, which include specific co-factors that target drug metabolizing enzymes, transporters, endobiotic metabolism, antioxidation defense, immune response, and tissue repair; and general co-factors such as inflammation, age, gender, comorbidity, gut microbiota, nutrition, and lifestyle. We also discuss the underlying mechanisms of co-factors that modulate DILI.

2. Co-factors that alter the exposure of drug and its metabolites to the liver and potentiate DILI

2.1. Variations of Phase I drug metabolizing enzymes and DILI

Most drugs are metabolized in the liver through two phases of metabolic process. In Phase I metabolism, parent drugs go through various reactions including oxidation, reduction, and hydrolysis. Oxidation is the most common reaction in Phase I metabolism, which is catalyzed by enzymes including cytochrome P450 (CYP), flavin-containing monooxygenase, and monoamine
oxidase. Among these Phase I enzymes, CYPs are the most common family and have been extensively studied for their roles in DILI.

Induction of CYP enzymes can increase the risk of DILI if the CYP-mediated metabolites are toxic. A series of clinical trials revealed hepatotoxicity in subjects pretreated with RIF followed by ritonavir-containing antiretroviral drugs (Nijland et al., 2008; Haas et al., 2009; Schmitt et al., 2009). RIF is a human specific activator of pregnane X receptor (PXR) that upregulates CYP3A4 (Bertilsson et al., 1998; Lehmann et al., 1998; Goodwin et al., 1999). Using the PXR-humanized mouse model, it was found that RIF increased the CYP3A4-dependent activation pathways of ritonavir to generate toxic metabolites, leading to oxidative stress and hepatocellular injury (Shehu et al., 2019). Cobicistat is an analogue of ritonavir that is also used for antiretroviral therapy. It is generally believed that the safety profile of cobicistat is better than that of ritonavir (Xu et al., 2010; Renjifo et al., 2015). However, activation of the two xenobiotic receptors that induce CYP3A4, PXR and constitutive androstane receptor, potentiates cobicistat hepatotoxicity through the CYP3A4-dependent pathways, suggesting that CYP3A4 inducers are risk factors for cobicistat hepatotoxicity (Shehu et al., 2021). Indeed, CYP inducers as co-factors that potentiate DILI have already been determined for many other drugs, such as valproic acid and APAP (Gopaul et al., 2003; Cheng et al., 2009a).

Other than CYP induction, CYP inhibition can also potentiate DILI if the parent drug is hepatotoxic. Many clinically used drugs as well as chemicals from environment and herbal supplements inhibit CYPs, which decrease the metabolism of the victim drug and increase its
exposure to the liver and potentially lead to hepatotoxicity (Gorski et al., 2004; Lynch and Price, 2007; Causevic-Ramosevac and Semiz, 2013; Wanwimolruk and Prachayasittikul, 2014). For example, atorvastatin hepatotoxicity is boosted by grapefruit juice because it inhibits the CYP3A4-mediated detoxification of atorvastatin (Lilja et al., 1999; Causevic-Ramosevac and Semiz, 2013). Additionally, a dramatic decrease of CYP activity was observed in mice under liver injury condition (Bao et al., 2020; Bao et al., 2021), suggesting that dose adjustment is needed when other drugs are applied, especially for a parent drug that is hepatotoxic.

Furthermore, genetic polymorphisms of CYPs can alter CYP function and contribute to DILI. In ticlopidine-induced liver injury, *IH and *IJ haplotypes of CYP2B6 increase the production of toxic metabolites of ticlopidine and boost the risk of ticlopidine hepatotoxicity (Ariyoshi et al., 2010). In another case, a loss-of-function mutation of CYP2B6*6 was found in patients with efavirenz-induced liver injury, which decreased CYP2B6-dependent metabolism of efavirenz and resulted in efavirenz accumulation in the liver (Yimer et al., 2012). Because CYPs are important in drug metabolism and have high individual variations, genetic polymorphisms of CYPs as well as their induction and/or inhibition should be considered before prescribing patients a potential hepatotoxic drug that is metabolized by CYPs.

2.2. Variations of Phase II drug metabolizing enzymes and DILI

Phase II drug metabolizing enzymes include UDP-glucuronosyltransferase (UGT), sulfotransferase, glutathione S-transferase (GST), etc. These enzymes catalyze the conjugation of substrates to hydrophilic groups like UDP glucuronic acid, sulfate, and glutathione (GSH).
Substrates for Phase II enzymes are usually generated from Phase I metabolism or sometimes parent drugs. The products of Phase II metabolism are generally less active and more readily eliminated.

For most drugs, Phase II metabolism is a detoxification process and inhibition of Phase II metabolism leads to adverse outcomes. Pexidartinib is a tyrosine kinase inhibitor approved for treating adults with symptomatic tenosynovial giant cell tumor that is not responsive to surgical treatment (Tap et al., 2019). However, pexidartinib has a boxed warning of hepatotoxicity. Pexidartinib is metabolized by CYP3A4 and UGT1A4 to oxidative and glucuronide metabolites accordingly for elimination. It has been found that probenecid, a nonspecific UGT inhibitor, slows down pexidartinib elimination, leading to its accumulation and liver injury (Lamb, 2019). In addition, phenobarbital and phenytoin potentiate APAP hepatotoxicity partially due to their inhibition on APAP glucuronidation, one of the major detoxification and elimination pathways of APAP (Kostrubsky et al., 2005).

Phase II metabolism may also mediate drug bioactivation and potentiate DILI (Hinson et al., 1995). For example, diclofenac is a widely prescribed analgesic associated with idiosyncratic liver injury. The exact mechanism of diclofenac-induced liver injury is not well understood, but the metabolism of diclofenac by UGT2B7 to form diclofenac acyl glucuronide might contribute to such toxicity (Laine et al., 2009). Indeed, *UGT2B7*2 polymorphic variant is associated with diclofenac hepatotoxicity (Daly et al., 2007). *UGT2B7*2 has an increased glucuronidation
activity which leads to a surge in the formation of diclofenac acyl glucuronide and liver injury (Duguay et al., 2004; Daly et al., 2007).

2.3. Variations of drug transporters and DILI

Hepatic transporters including the solute carrier family and the ATP-binding cassette family are critical for distribution and elimination of drugs and endobiotics like bile acids and bilirubin. Influx transporters, such as organic anion transporting polypeptide 1B1/1B3 and Na\(^+\)-taurocholate co-transporting polypeptide, are members of solute carrier family. Efflux transporters are mainly from the ATP-binding cassette family including multidrug resistance-associated proteins (MRP), bile salt export pump (BSEP), breast cancer resistance protein (BCRP), and P-glycoprotein (Roth and Lee, 2017). Similar to drug metabolizing enzymes, the functions of transporters can be altered by inducers, inhibitors, and genetic polymorphisms.

Inhibition of efflux transporters is a common risk factor for DILI. Emodin, an active anthraquinone in herbal medicines, is hepatotoxic and its toxicity can be exacerbated by probenecid, which inhibits the MRP2-mediated excretion of emodin from hepatocytes (Wu et al., 2018). Troglitazone, an antidiabetic and anti-inflammatory drug, was withdrawn from the market because of hepatotoxicity (Funk et al., 2001; Jackson et al., 2018). Troglitazone causes cholestatic liver injury because (1) this drug inhibits BSEP, the major efflux transporter of bile acids (Funk et al., 2001) and (2) troglitazone suppresses the function of farnesoid X receptor, a transcription factor that upregulates BSEP expression (Jackson et al., 2018).
Loss-of-function mutations of efflux transporters are also risk factors for DILI. MRP2 is associated with diclofenac-induced liver injury (Daly et al., 2007). By comparing the genotypes of the patients who encountered diclofenac-induced liver injury to those without DILI, the C-24T variant of MRP2 was identified, which reduces MRP2 expression, leading to the accumulation of diclofenac and its metabolites in hepatocytes (Haenisch et al., 2007). MRP2 is also responsible for bilirubin efflux and genetic deficiency of MRP2 causes Dubin-Johnson syndrome, characterized by hyperbilirubinemia (Keppler, 2014). In addition, the 1331T>C mutation of BSEP increases the susceptibility to contraceptive-induced cholestasis because estrogen and progesterone in contraceptive pill inhibit BSEP-mediated bile acid excretion and the loss-of-function mutation of BSEP exacerbates bile acid accumulation (Meier et al., 2008).

3. Co-factors that interact with the molecular targets of drug and its metabolites in the liver and potentiate DILI

Drugs are designed to specifically target molecules of interest to generate therapeutic effects. However, drugs and/or their metabolites may interact with other molecules leading to off-target effects and even toxicity (Rudmann, 2012). Compared to the therapeutic targets of drugs, which are known for most drugs, the toxic targets of drugs and their metabolites are understudied and largely unknown. For example, the molecular targets in hepatocytes are not clear for most hepatotoxic drugs after they enter hepatocytes (Figure 1).

The applications of omics facilitate the screening of off-targets of drugs (Wang et al., 2017; Paananen and Fortino, 2020). RIF and INH are the first line drugs for the treatment of
tuberculosis (TB) by inhibiting TB RNA polymerase and cell wall synthesis, respectively (Schulz and Zillig, 1981; Quemard et al., 1991). However, co-treatment with RIF and INH causes liver damage (Steele et al., 1991; Chowdhury et al., 2006; Li et al., 2013). Recent metabolomic studies revealed that co-treatment with RIF and INH causes the accumulation of protoporphyrin IX, a hepatotoxin, by inducing delta-aminolevulinate synthase 1 and suppressing ferrochelatase in the liver (Li et al., 2013; Sachar et al., 2016), suggesting that the heme biosynthesis pathway is the off-target of RIF and INH in hepatocytes. INH also interacts with CD38 in Kupffer cells and hepatic stellate cells, leading to the formation of INH-NAD and AcINH-NAD adducts, which potentially contribute to liver injury because of the critical roles of NAD$^+$ in cellular functions (Zhu et al., 2020; Amjad et al., 2021).

Identification of drug off-targets in the liver can be used to prevent DILI by avoiding the co-factors that deliver the second hit on the pathways that are related to the off-targets of the victim drug (Figure 1). Reduced GSH plays an important role in antioxidant defense as well as in regulation of cellular metabolic functions including gene expression, signal transduction, cell proliferation and apoptosis (Aquilano et al., 2014). GSH is the primary target of N-acetyl-p-benzoquinone imine, the reactive metabolite of APAP in the liver, and overdose of APAP leads to GSH depletion and cellular injury (Siegers, 1978; Chiba and Pang, 1995; Li et al., 2011). Therefore, any co-factors that cause GSH deficiency will increase the risk of APAP toxicity, such as buthionine sulfoximine (BSO), a potent inhibitor of GSH synthesis (Tamai et al., 2017). On the other hand, treatment with N-acetylcysteine, a precursor of GSH, rescues APAP hepatotoxicity (Ruffmann and Wendel, 1991). Unfortunately, the off-targets of most DILI drugs in the liver remain unknown (Wang et al., 2017; Paananen and Fortino, 2020). Therefore, there is
an urgent need to explore this field in order to develop mechanism-based approaches to predict and prevent DILI.

4. Co-factors that disrupt cell defense, adaptation, and tissue repair in the liver and potentiate DILI

4.1. Antioxidant defense and DILI

The antioxidant defense system (ADS) combats oxidative stress by reacting with reactive oxygen species (ROS) and prevents ROS-mediated cellular damage (Jaeschke et al., 2012; Ray et al., 2012). ADS consists of peptides and enzymes. GSH, a small peptide, reacts directly with ROS and protects cells against toxicity from excessive amounts of electrophiles, hydroxyl radicals, and superoxide (Birben et al., 2012; Jaeschke et al., 2012; Ray et al., 2012). ADS enzymes include superoxide dismutase and catalase (Birben et al., 2012). In addition, GSTs, a family of Phase II enzymes, also contribute to ADS by catalyzing the conjugation of GSH to a wide variety of electrophilic compounds and protecting cellular macromolecules (Jakoby, 1985; Townsend and Tew, 2003).

For drugs that damage hepatocytes via oxidative stress, co-factors that promote the formation of ROS or impair ADS will potentiate DILI. BSO inhibits glutamate cysteine ligase (GCL), a critical enzyme in GSH synthesis, and thus suppresses GSH production. Methimazole is a widely used antithyroid drug, but it can cause hepatotoxicity by unknown mechanisms. Studies found that pretreatment with BSO depletes GSH in the liver and potentiates methimazole hepatotoxicity in mice (Mizutani et al., 1999; Mizutani et al., 2000). BSO also amplifies
APAP-induced liver injury (Tamai et al., 2017). In addition, genetic polymorphisms of ADS encoding genes potentiate DILI. C to T substitution mutation at position 47 of superoxide dismutase and GST-M1 null genotype increase the risk of anti-TB drug-induced liver injury (Huang et al., 2007). The loss-of-function mutations of GST also increase the risk of tacrine and troglitazone-induced liver injury (Simon et al., 2000; Okada et al., 2011).

The nuclear erythroid factor type 2 (Nrf2) regulates ADS (Enomoto et al., 2001; Li and Kong, 2009; Telkoparan-Akillilar et al., 2019). Nrf2 activation induces the expression of ADS enzymes and promotes the detoxification and cytoprotective process. On the other hand, Nrf2 inhibitors undermine ADS and potentiate DILI. In mice co-treated with APAP and brusatol, a Nrf2 inhibitor, more serious liver injury together with a lower GSH level and a higher ROS level were observed when compared to that of APAP alone group (Chan et al., 2001; Li et al., 2019).

4.2. Immune response and DILI

Apart from direct damage, abnormal activation of immune system also potentiate DILI. Small molecular drugs are unlikely to trigger immune response (Mak and Uetrecht, 2017). However, some reactive metabolites of drugs can bind to endogenous proteins and the drug-protein adducts trigger immune response (Mak and Uetrecht, 2017). Immune tolerance counteracts the immune response activated by neoantigen resulting in no apparent or mild liver injury in most patients (Uetrecht, 2007). There are mainly two mechanisms for immune tolerance in the liver: (1) antigen presenting cells in the liver produce various inhibitory cytokines like interleukin (IL) 10 and transforming growth factor beta 1, resulting in poor T cell activation (Erhardt et al., 2007;
Crispe, 2014); and (2) a suppressive network can be generated by myeloid-derived suppressor cells (MDSCs), which activate regulatory T cells and inhibits effector T cells (Chen et al., 2011). However, a small percentage of patients still develop strong immune response and cause severe liver injury, which might be due to additional environmental or genetic factors that modulate immune response.

Factors that impair immune tolerance can exacerbate immune-mediated DILI. An immune tolerance impaired mouse model has been generated by depleting the MDSCs cells (Chakraborty et al., 2015). Treatment with halothane, a drug that induces allergic hepatitis, developed hepatotoxicity in MDSCs depleted mice, but not in wild-type mice (Chakraborty et al., 2015), suggesting the protective role of immune tolerance in DILI. In addition to MDSCs, immune checkpoints CTLA-4 and PD-1 also modulate immune tolerance. Immune checkpoint inhibitors used in cancer therapy can suppress immune tolerance and thus increase the risk of immune-mediated DILI (Sznol et al., 2017). In patients co-treated with nivolumab (anti-PD-1) and ipilimumab (anti-CTLA-4), a high rate of hepatic adverse events occurred (Sznol et al., 2017). Preclinical studies also provided evidence for the role of immune tolerance inhibitors in DILI. In PD-1-null mice co-treated with amodiaquine and anti-CTLA4 antibody, a more severe liver injury was noted when compared to amodiaquine monotherapy group (Metushi et al., 2015).

Polymorphisms of human leukocyte antigens (HLA) have been identified as risk factors for immune-mediated DILI (Castiella et al., 2014). *HLA-B*5701 was found to be significantly
associated with flucloxacillin-induced liver injury (Daly et al., 2009). A later study confirmed the role of \textit{HLA-B*5701} and suggested that \textit{HLA-B*5703} is also associated with flucloxacillin hepatotoxicity (Nicoletti et al., 2019). More associations between \textit{HLA} polymorphisms and DILI have been revealed such as \textit{HLA-DRB1*0701} for lapatinib hepatotoxicity and \textit{HLA-DRB1*1501} for lumiracoxib hepatotoxicity (Singer et al., 2010; Spraggs et al., 2011). These specific associations may result from certain \textit{HLA} haplotypes having a high genetic predisposition to both the adaptive immune response and presentation of hapten-protein adducts required in DILI (Castiella et al., 2014).

\subsection*{4.3. Tissue repair and DILI}

The liver has a remarkable regenerating capacity. After partial hepatectomy in mice, typically removal of two-thirds of the liver, hepatic mass is back to the original size in ~ 7 days (Michalopoulos, 2010). The APAP overdose mouse model that causes hepatocellular necrosis is frequently used to study liver regeneration in DILI, in which compensatory hepatocellular regeneration begins in the necrotic zones and newly proliferated cells replace the dead cells (Jaeschke et al., 2012; Bhushan et al., 2014). It remains unclear how injured hepatocytes communicate with their neighborhood healthy cells, but the involvement of extracellular vesicles has been proposed (Cho et al., 2018; Umbaugh and Jaeschke, 2021). The regeneration is critical for the final outcome and if regeneration fails, liver failure occurs (Mehendale, 2005). However, there are factors that can inhibit liver regeneration and thus potentiate DILI.
Growth factors play a critical role in liver regeneration. Epidermal growth factor receptor (EGFR) is involved in liver regeneration and its ligands act as mitogens that trigger hepatocyte proliferation (Michalopoulos and Khan, 2005; Michalopoulos, 2007). Inhibition of EGFR by canertinib at 12 hours post APAP treatment impaired liver regeneration which led to progression of injury and increased fatality in mice (Bhushan et al., 2017). In addition to EGFR, inhibition of vascular endothelial growth factor receptor (VEGFR) also results in impairment of liver regeneration (Donahower et al., 2006a). The expression of VEGF-A protein and its receptor was elevated after the initiation of APAP-induced liver injury in mice, suggesting the VEGFR signaling pathway contributes to liver regeneration (Donahower et al., 2006b; Papastefanou et al., 2007; Kato et al., 2011). A follow-up study revealed that VEGFR inhibitor SU5416 diminished hepatocyte regeneration in APAP-induced liver injury mouse model, which provided further evidence for the role of VEGFR in liver regeneration (Donahower et al., 2006a). These data suggest that growth factor inhibitors should be cautiously used when combined with hepatotoxic drugs.

In addition to growth factors, the regeneration process in the liver is also modulated by cytokines and chemokines, such as IL-6 and macrophage inflammatory protein-2 (Hogaboam et al., 1999; James et al., 2003). Knockout of IL-6 or macrophage inflammatory protein-2 receptor CXCR2 abrogates the protective effects against liver damage and increases the mortality in APAP overdose mouse model (Hogaboam et al., 1999; James et al., 2003), indicating that co-factors that decrease IL-6 level or act as CXCR2 antagonists may exacerbate DILI. Furthermore, the sympathetic nervous system also regulates liver regeneration (Oben and Diehl, 2004). By analyzing the clinical cases of APAP-induced liver injury in the FDA database, it was found that
co-administration of APAP with sympathetic stimulants was associated with the increased likelihood of fatality (Suzuki et al., 2009), suggesting that sympathetic stimulants should be considered as co-factors for DILI.

5. General co-factors that potentiate DILI

5.1. Inflammation and DILI

Co-existing inflammation and the onset of an inflammatory episode increase DILI susceptibility (Shaw et al., 2010). Inflammation is usually triggered by bacteria or virus infection, as well as pathophysiological changes including necrotic cell death. The most common inflammatory agent is lipopolysaccharide (LPS), the endotoxin from gram-negative bacteria (Hamesch et al., 2015). Trovafloxacin (TVX), an antibacterial quinolone agent, causes hepatotoxicity by unknown reasons. Interestingly, an inflammatory episode increases the risk of TVX-induced liver injury (Shaw et al., 2010). In mice and rats pre-treated with a nontoxic dose of TVX followed by a non-toxic dose of LPS, severe hepatocellular lesions were observed (Waring et al., 2006; Shaw et al., 2007). Peptidoglycan-lipoteichoic acid, an inflammatory agent extracted from gram-positive bacteria, also potentiates TVX-induced liver injury (Shaw et al., 2009). These data indicate that inflammation is a risk factor for TVX hepatotoxicity.

In addition to TVX, animal studies have revealed that LPS-induced inflammation potentiated hepatotoxicity of many other drugs including ranitidine (Luyendyk et al., 2003), sulindac (Zou et al., 2009b), chlorpromazine (Buchweitz et al., 2002), halothane (Dugan et al., 2010), amiodarone (Lu et al., 2012) and diclofenac (Deng et al., 2006). LPS interacts with inflammasomes, the
multiprotein complexes responsible for the activation of inflammatory responses (Schroder et al., 2012). NLR family pyrin domain containing 3 (NLRP3) is a component of the inflammasome, and activation of NLRP3 triggers proinflammatory signals and hepatocyte pyroptosis (Wree et al., 2014). Treatment with LPS potentiated carbamazepine hepatotoxicity in WT mice, but not in Nlrp3-null mice, indicating the pivotal role of NLRP3 and inflammasomes in carbamazepine-induced liver injury (Wang et al., 2019).

In humans under physiological condition, the serum LPS level is relatively low because the epithelial membranes in the intestines retain LPS inside the gut and prevent them entering bloodstream (Saldeen and Bas, 1994; Wassenaar et al., 2018). Unfortunately, many factors can destruct intestinal barrier, leading to an increase of LPS level in bloodstream and increase the risk of DILI (Ghosh et al., 2020). Alcohol consumption, obesity, and high fat diet have been considered as risk factors of DILI because they increase intestinal permeability and blood levels of LPS (Bala et al., 2014; Boutagy et al., 2016). However, convincing data from the clinic are unavailable to ascertain whether LPS should be considered as a bona fide risk factor for DILI. Given the fact that many factors may increase LPS levels in the blood, more clinical studies are needed to determine the impact of LPS on DILI.

In addition to LPS produced from bacteria, virus particles also induce inflammation and exacerbate DILI. Cheng and colleagues employed viral RNA mimetic polyI:C to mimic viral infection-induced inflammation and found that polyI:C significantly potentiated halothane-induced hepatocellular injury (Cheng et al., 2009b). In addition, patients with human
immunodeficiency virus (HIV) have a high incidence of DILI when receiving antiretroviral therapy, especially in HIV patients co-infected with hepatitis B or C (Aceti et al., 2002; Pignataro et al., 2004; Hunt, 2012). One possible explanation is that inflammation triggered by hepatitis B or C sensitizes HIV patients to DILI (Ganey et al., 2004).

The exact mechanism by which inflammatory stress potentiates DILI is not fully understood. Nevertheless, it has been speculated that tumor necrosis factor-α (TNF-α) plays a critical role in the development of DILI (Shaw et al., 2007). TNF-α is a proinflammatory cytokine and the serum concentration of TNF-α is significantly increased after LPS exposure (Shaw et al., 2007; Zou et al., 2009a; Shaw et al., 2010). TNF-α potentiates apoptotic cell death of hepatocytes especially under the condition of GSH depletion (Colell et al., 1998; Matsumaru et al., 2003; Yan et al., 2016). In addition, TNF-α has a positive correlation with other cytokines, such as IL-18 and interferon γ, which may team up amplifying the impact of inflammatory stress on liver damage (Shaw et al., 2009). Furthermore, TNF-α enhances the activation of neutrophils and contributes to the development of DILI (Shaw et al., 2009). Moreover, TNF-α increases the level of plasminogen activator inhibitor-1, which inhibits fibrinolysis, leading to fibrin accumulation, hypoxia, and liver damage (Watkins and Seeff, 2006; Shaw et al., 2009; Shaw et al., 2010).

5.2. Age and DILI

Age, either young or old, can be a risk factor for DILI for certain medications. Compared to patients between 25 to 34 years old, the rate of INH-induced liver injury increased two-fold in patients between 35 to 49 years old, and five-fold in patients above 50 years old (Fountain et al.,
The underlying mechanisms for the age-related INH-induced liver injury include a decline in liver regeneration capacity, concurrent diseases, and age-related changes of INH pharmacokinetics in the elderly (Schmucker et al., 2011; Lin et al., 2016; Mach et al., 2016). Opposite to INH-induced liver injury, the young age is a risk factor for valproic acid hepatotoxicity, especially for children < 2 years old, which is partially due to the differences of valproic acid metabolism and disposition between adults and children (Bryant and Dreifuss, 1996; Andrade et al., 2019).

5.3. Gender and DILI

It is commonly believed that women are at a higher risk of developing more severe DILI than men (Russo et al., 2004; Lucena et al., 2009). Indeed, the risk of immune-mediated DILI is significantly higher in women than men. Distinct hormone composition between males and females may contribute to different susceptibility to DILI. Culture of spleen cells from female mice showed fewer regulatory T cells and higher pro-inflammatory cytokines than males, which is partially due to the gender difference of 17β-estradiol (Cho et al., 2013). In addition, pre-treatment with progesterone potentiates halothane-induced liver injury because progesterone increases the level of hepatic proinflammatory cytokines and chemokines (Toyoda et al., 2011). Moreover, gender differences in drug metabolizing enzymes and transporters may also contribute to DILI susceptibility, as CYP2E1 and P-glycoprotein have higher activities in men than in women; while women have a higher CYP3A4 activity than men (Meibohm et al., 2002; Schwartz, 2003).
5.4. Comorbidity and DILI

Hepatitis B and C are well-known risk factors for DILI, which create a pro-inflammatory environment and potentiate DILI (Schenker et al., 1999; Burdette et al., 2012; Kim et al., 2016). HCV patients with anti-TB treatment had a 31.7% incidence of DILI, and the incidence was even higher at 75% in those co-infected with HBV and HCV, while the DILI incidence for control group (infected with TB only) was 10% (Kim et al., 2016). In addition to hepatitis B and C, obesity, diabetes, and nonalcoholic fatty liver disease (NAFLD) are also risk factors for DILI. Both obesity and diabetes increase hepatic oxidative stress, which can deteriorate mitochondrial function and increase susceptibility to hepatotoxic drugs (Koliaki and Roden, 2013; Pais et al., 2014). Patients with NAFLD have increased CYP2E1 activity and dysfunctional mitochondrial respiratory chain in hepatocytes, leading to an aggressive production of ROS and a high risk of DILI (Aubert et al., 2012; Fromenty, 2017). Furthermore, concurrent diseases with the disorder of bile acid homeostasis can potentiate DILI (Gomez-Ospina et al., 2016). It has been found that deficiency of farnesoid X receptor causes deoxycholic acid accumulation, which increases the risk of APAP-induced liver injury (Yan et al., 2021).

5.5. Gut microbiota and DILI

The bacteria in the gut have their own metabolic system and contribute to drug metabolism and disposition in the host (Sousa et al., 2008; Enright et al., 2016; Yip et al., 2018). Tacrine-induced liver injury is associated with the increases of gut bacteria including *Lactobacillus, Bacteroides* and *Enterobacteriaceae* (Yip et al., 2018). These three strains of bacteria have deglucuronidation capacity, which slow down tacrine elimination, increase tacrine plasma concentration, and
therefore potentiate tacrine hepatotoxicity (Yip et al., 2018). In addition, the metabolites from gut microbiota can enter systemic circulation and disturb liver functions. P-cresol is a bacterial metabolite produced by *Clostridium difficile*, which competes with APAP for the detoxification process mediated by sulfotransferase 1A1, leading to APAP accumulation and liver injury (Clayton et al., 2009). Another bacterial metabolite PPD, produced by *Escherichia coli* and *Citrobacter freundii*, depletes GSH in hepatocytes and exacerbates APAP-induced liver injury (Gong et al., 2018; Niu and Chen, 2020). Overall, gut bacteria as well as their metabolites can alter drug metabolism and host responses and increase the risk of DILI.

5.6. Lifestyle and DILI

Both malnutrition and overnutrition are risk factors for DILI. Malnutrition is significantly associated with anti-TB drug-induced liver injury (Mehta and therapeutics, 1990; Makhlouf et al., 2008; Ali et al., 2020). Malnutrition-mediated alteration of drug metabolism, antioxidant defense, and tissue repair may contribute to the hepatotoxicity of anti-TB drugs, but more mechanistic studies are needed (Speerhas, 1995; Makhlouf et al., 2008; Villanueva-Paz et al., 2021). In addition, individuals who have a high fat diet are likely to develop liver inflammation because the fat-abundant diet contributes to the overgrowth of intestinal bacteria, which can damage intestinal integrity and increase the exposure of inflammatory agents to the liver (Utzeri and Usai, 2017). Moreover, a high fat diet and overnutrition increase the possibility to develop obesity and NAFLD, leading to an increase in hepatocellular oxidative stress and the risk of DILI (Koliaki and Roden, 2013).
Alcoholic drinks including beers, wines, and liquors are very common in daily life, which can increase the risk of DILI by CYP2E1 induction (Zimmerman and Maddrey, 1995), GSH suppression (Lauterburg and Velez, 1988), disruption of gut microbiota (Mutlu et al., 2009), and promotion of liver inflammation (Fujimoto et al., 2000; Thakur et al., 2007). Alcohol-APAP syndrome has been reported since 1980s, in which alcohol increases APAP hepatotoxicity by inducing the expression of CYP2E1 as well as suppressing hepatic GSH synthesis (Zimmerman and Maddrey, 1995). The induction of CYP2E1 by alcohol increases the production of reactive metabolite of APAP, while GSH depletion results in the failure of antioxidant defense (Zimmerman and Maddrey, 1995). In addition, chronic alcohol consumption has been identified as an independent predictor for mortality of APAP-induced liver injury (Schmidt et al., 2002). Chronic alcohol abuse also increases the risk of anti-TB drug-induced liver injury as 42.2% of DILI cases showed significant alcohol consumption (Gaude et al., 2015).

Cigarette may also potentiate DILI. A retrospective study found that tobacco is an independent risk factor for APAP-induced liver injury (Schmidt and Dalhoff, 2003). Benzothiazole in cigarette induces CYP1A2 (Xiao Dong et al., 1998), and pre-treatment with benzothiazole increased APAP bioactivation and hepatotoxicity (Seo et al., 2000). However, compared to alcohol consumption, the relationship between smoking and hepatotoxicity is less prevalent (Stine and Chalasani, 2017). Indeed, a decreased risk for anti-TB drug-induced liver injury was observed in active smokers when compared to non-smokers (Zaverucha-do-Valle et al., 2014), and a low association was identified between smoking and hepatotoxicity of flutamide, an antiandrogenic drug (Wada et al., 1999).
Illicit substances including cocaine and amphetamine-type stimulants also boost DILI. Clinical studies have observed mild to severe liver injury in cocaine users, which in part is due to norcocaine, a metabolite of cocaine that causes hepatic oxidative stress (Silva et al., 1991). In addition, 3,4-methylenedioxymethamphetamine, an amphetamine-type stimulant, can cause serious liver damage and even acute liver failure (Greene et al., 2003). Since drug abusers usually consume more than one addictive substance, the concurrent use of illicit drugs make the liver condition even worse (Pateria et al., 2013). Therefore, when a hepatotoxic medicine is prescribed to drug abusers, the vulnerable liver may not be able to tolerate the prescription and result in liver damage.

6. Summary and perspectives

The detail mechanisms of DILI are unknown for most drugs, but it is generally accepted that three steps are needed in the development of DILI: (1) exposure of drug and its metabolites to the liver; (2) interactions of drug and its metabolites with molecular targets in the liver, leading to cellular dysfunction and injury; and (3) cellular dysfunction and injury trigger cell defense, adaptation, and tissue repair, and inappropriate cell responses result in liver injury (Figure 2). Many co-factors that potentiate DILI have been identified, and the current review categorized them into two types, the specific and the general co-factors. Specific co-factors increase the risk of DILI by directly targeting a specific molecule that is involved in the three steps of liver injury, such as an inhibitor of drug metabolizing enzyme or transporter (Figure 2). General co-factors,
including inflammation, age, gender, comorbidity, gut microbiota, nutrition, and lifestyle, can potentiate DILI by affecting multiple molecules in the process of liver injury (Figure 2).

The risk factors for DILI can potentially be used to predict and prevent DILI, therefore improving the safety profile of medications. However, more studies are needed to define the mechanisms by which co-factors potentiate DILI. In addition, more co-factors for DILI are expected to be discovered based upon the identification of target molecules of drugs in the liver. Unfortunately, the molecular targets of most DILI drugs in the liver remain elusive. The applications of genomics, transcriptomics, proteomics, metabolomics, together with genetically engineered cellular and animal models facilitate the identification of target molecules of drugs in the liver (Chang et al., 2010; Strähle and Grabher, 2010; Gonzalez et al., 2015; Wang et al., 2017; Huang et al., 2019; Paananen and Fortino, 2020), which will provide mechanistic understanding of DILI and expedite the discovery of novel risk factors for DILI. Additionally, the artificial intelligence approaches also show a great potential for predicting DILI (Minerali et al., 2020; Vall et al., 2021).

In summary, co-factors for DILI are the attributes that increase DILI susceptibility and/or potentiate DILI progression. Co-factors affect DILI from various aspects including drug metabolism and disposition, drug-target interactions, and cellular responses. A better understanding of co-factors for DILI provides mechanistic understanding of DILI and allows for improved prediction and prevention of DILI.
Authorship Contributions

Writing the manuscript: R.G., A.L., G.L., I.T., A.S., X.M.
References


Aithal GP, Day CP, for the Ds, and International SAEC (2009) HLA-B*5701 genotype is a major determinant of drug-induced liver injury due to flucloxacillin. *Nature Genetics* **41:**816-819.


associated with decreased expression of ARE-regulated drug metabolizing enzymes and antioxidant genes. 59:169-177.


Funk C, Ponelle C, Scheuermann G, and Pantze MJMp (2001) Cholestatic potential of troglitazone as a possible factor contributing to troglitazone-induced hepatotoxicity: in
vivo and in vitro interaction at the canalicular bile salt export pump (Bsep) in the rat.  
59:627-635.


Kaitin KJTCftSoDDIR (2005) Drug safety withdrawals in the US not linked to speed of FDA approval. 7.


Lilja JJ, Kivistö KT, Neuvonen PJJCP, and Therapeutics (1999) Grapefruit juice increases serum concentrations of atorvastatin and has no effect on pravastatin. 66:118-127.


22:931-935.


Salden HJ and Bas BM (1994) Endotoxin binding to platelets in blood from patients with a sepsis syndrome. *Clinical Chemistry* **40:**1575-1579.


Utzeri E and Usai PJW (2017) Role of non-steroidal anti-inflammatory drugs on intestinal permeability and nonalcoholic fatty liver disease. **23**:3954.


Footnotes

The authors have declared that no conflict of interest exists. This work was supported in part by the National Institute of Allergy and Infectious Diseases (R01AI131983), the National Institute of Diabetes and Digestive and Kidney Diseases (R01DK126875), and the National Center for Complementary and Integrative Health (R21AT011088).
Figure legends

Figure 1. Schematic representation for the off-targets of drugs in hepatocytes. For most drugs, the therapeutic targets are well-defined, whereas the off-targets are understudied and largely unknown. We predict that a victim drug delivers the first hit to hepatocytes through off-targets including enzymes, receptors, and transporters and co-factors potentiate DILI by providing the second hit to the pathways that are related to the off-targets of the victim drug.

Figure 2. Schematic representation for the roles of co-factors in DILI. Three steps are needed in DILI: (1) exposure of drug and its metabolites to the liver; (2) interactions of drug and its metabolites with molecular targets in the liver; and (3) inappropriate cell responses including cell defense, adaptation, and tissue repair. Specific co-factors can potentiate DILI by directly targeting a molecule that is involved in the three steps of liver injury. General co-factors can potentiate DILI by affecting multiple molecules in the process of liver injury.
Figure 1

DRUG → Target → Efficacy

Hepatocyte

Off-target (1st hit):
- Enzyme?
- Receptor?
- Transporter?
- Others?

Co-factors (2nd hit)

LIVER INJURY
Figure 2

General co-factors
- Inflammation
- Age (young & old)
- Gender (male & female)
- Comorbidity (HBV & HCV)
- Gut microbiota
- Nutrition (malnutrition & overnutrition)
- Lifestyle (cigarette, alcohol, & cocaine)

Specific co-factors
(Inhibition, induction, or polymorphisms)
- Drug metabolizing enzymes
- Transporters

Exposure of drug and its metabolites to the liver

Targets in the liver and cellular dysfunction and injury

Inappropriate cell defense, adaptation, and tissue repair

Liver injury

Hepatocyte

Drug