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Models of Idiosyncratic Drug-Induced Liver Injury

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Abstract

Drug-induced liver injury (DILI) is a leading cause of attrition during the early and late stages of drug development and after a drug is marketed. DILI is generally classified as either intrinsic or idiosyncratic. Intrinsic DILI is dose dependent and predictable (e.g., acetaminophen toxicity). However, predicting the occurrence of idiosyncratic DILI, which has a very low incidence and is associated with severe liver damage, is difficult because of its complex nature and the poor understanding of its mechanism. Considering drug metabolism and pharmacokinetics, we established experimental animal models of DILI for 14 clinical drugs that cause idiosyncratic DILI in humans, which is characterized by the formation of reactive metabolites and the involvement of both innate and adaptive immunity. On the basis of the biomarker data obtained from the animal models, we developed a cell-based assay system that predicts the potential risks of drugs for inducing DILI. These findings increase our understanding of the mechanisms of DILI and may help predict and prevent idiosyncratic DILI due to certain drugs.

1. INTRODUCTION

In drug development research, preclinical studies use in vivo animal models and in vitro cell-based assays to determine the efficacy and safety of a drug and to evaluate in detail its pharmacology, toxicity, and pharmacokinetics. Many of the adverse effects that occur during clinical trials and after marketing are caused by increased pharmacological effects and dose responsiveness; theoretically, it is possible to predict and avoid the occurrence of adverse effects. However, even when toxicity and adverse effects are absent from preclinical and clinical studies of the drug approval process, severe toxicities such as liver damage, cardiotoxicity, bone marrow toxicity, and allergic reactions may still occur after marketing. Such adverse effects are termed idiosyncratic drug toxicity and are extremely rare in occurrence, irrespective of the known pharmacological effects, and are not dose responsive.

Remarkable progress in assessing drug metabolism and transport as well as in developing pharmacokinetic analyses has been made during the past decade. Experimental animal models, in vitro cell-based systems such as metabolic enzymes and transporter expression systems, and patient information such as gene polymorphisms have all been considered. The knowledge acquired from these studies has facilitated the prediction of drug metabolism and kinetics in clinical practice and has allowed the prediction of individual differences. Consequently, the percentage of clinical trials discontinued owing to drug metabolism and kinetics has dropped sharply from 40% in 1991 to 9% and 1% in 2000 and 2011, respectively. However, in the past 10 years, cases of adverse effects or toxicities have been as high as 13% (1), 20% (2), and 19% (3). In particular, drug-induced liver injury (DILI) is a major cause of discontinuation of clinical trials and withdrawal of drug candidates (in 30% of cases) after marketing (4–7). It has been estimated that over 900 drugs have been associated with hepatotoxicity (8). Pemoline, ximelagatran, and lumiracoxib were withdrawn from the global market in 2005, 2006, and 2008, respectively, because of hepatotoxicity. In clinical practice, death or liver transplantation due to liver injury caused by drugs such as diclofenac, erythromycin, flucloxacillin, and halothane has been reported (9). In December 2013, fasiglifam (TAK-875), a treatment for type 2 diabetes, was discontinued late in a phase III study because of liver damage (10). Thus, DILI remains a major public health problem.

Numerous studies have focused on identifying candidate compounds that may cause liver damage at an early stage of development, although attempts are hampered by several problems. The mechanisms of DILI have not been entirely elucidated and may differ across drugs. In addition, DILI is expected to involve several mechanisms that interact in a complex manner. Therefore, establishing a test system for predicting DILI is difficult. This review introduces the role of reactive metabolites and immune- and inflammation-related factors in the pathogenesis of DILI and the establishment and application of in vivo and in vitro evaluation systems focusing on idiosyncratic drug responses, including the animal models and cell-based assay systems developed in our laboratory.

2. INTRINSIC AND IDIOSYNCRATIC DILI

Various drugs can cause liver damage, and individual risk depends on the patient's constitution, genotype, disease state, concomitant medications, diet, lifestyle habits, and environmental factors. Therefore, the pathology and clinical symptoms vary widely across patients. DILI is roughly classified as hepatocellular, cholestasis, both hepatocellular and cholestasis, or steatosis on the basis of pathological findings. Depending on the onset mechanism, DILI can also be classified as intrinsic DILI, characterized by direct cytotoxic action of a drug or its metabolite, or idiosyncratic DILI, characterized by the constitution of an individual. Intrinsic DILI develops in a dose-dependent manner, its pathogenesis has been elucidated, and predicting its occurrence is relatively easy. In

contrast, idiosyncratic DILI does not depend on dose, its frequency of onset is low, and its occurrence cannot be predicted. Even the chemical structure and biochemical properties of drugs are not sufficient to predict its onset (11). Reactive metabolites may play a role in the onset of idiosyncratic DILI (**Supplemental Table 1**); however, confirming their involvement in an animal model is difficult because metabolic reactions vary widely across species. Thus, scientific approaches based on the detailed pathogenic mechanisms cannot be applied.

Supplemental Material >

Idiosyncratic DILI is widely associated with immune- and inflammation-related reactions during the exacerbation of liver damage (12–14). In addition, many drugs that induce idiosyncratic DILI can be established with animals with reduced liver glutathione (GSH) levels, and reactive metabolites have been suggested to essentially be involved in the onset of idiosyncratic DILI (13–15). For these reasons, the cases of DILI for which the mechanism cannot be explained are classified in this review as idiosyncratic DILI.

3. CLASSIFICATION ISSUES FOR DRUGS THAT CAUSE IDIOSYNCRATIC DILI

The US Food and Drug Administration (FDA)-approved prescription drug labels are listed in DailyMed (**http://dailymed.nlm.nih.gov/dailymed/index.cfm**) and the Liver Toxicity Knowledge Base Benchmark Dataset (LTKB-BD; **https://www.fda.gov/science-research/liver-toxicity-knowledge-base-ltkb/ltkb-benchmark-dataset**) developed by the FDA (16). FDA-approved prescription drug labels categorize risk of DILI as withdrawn from the market (WDN), black box warning (BBW), warning and precautions (WP), adverse reactions (AR), or no mention (NM). Drug labeling is regulated by law under the Code of Federal Regulations Title 21 Part 201 (21CFR201.56; **http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?fr=201.57**). Drug labeling implicitly balances the information pertaining to causality, incidence, and severity acquired from controlled trials, published literature reports, and spontaneous reports to adverse event reporting systems (for more information, see 16). The LTKB-BD classifies risk of DILI by considering the clinical severity of DILI and using the FDA-approved labels in order to assign drugs to one of the following three categories: most, less, or no DILI concern.

In a previous study, Oda et al. (17) referred to the FDA-approved label and the LTKB-BD regarding clinical DILI potential (Section 2), although some of the information presented therein is controversial. For example, chlorpromazine is classified as an AR and less-DILI-concern drug and sulindac is classified as a WP and most-DILI-concern drug according to the FDA-approved label and the LTKB-BD, respectively. According to the National Institute of Diabetes and Digestive and Kidney Diseases LiverTox database (**https://www.ncbi.nlm.nih.gov/books/NBK547852/**), chlorpromazine was formerly considered the most common cause of DILI in the United States; however, with its decreased use, chlorpromazine-associated jaundice is now rarely reported. Similarly, sulindac causes clinically apparent but rare acute liver injury (approximately 5 cases in 100,000 prescriptions and in approximately 0.1% of users). Thus, chlorpromazine may not be a true DILI-negative drug, and sulindac may not be a true DILI-positive drug.

Other DILI databases, such as the Drug-Induced Liver Injury Network (DILIN), the Open Toxicogenomics Project-Genomics Assisted Toxicity Evaluation System (TG-GATEs), the Liver Toxicological Map (LTMap), DrugBank, and the Hazardous Substance Data Bank (HSDB), have now been constructed (18, 19). However, none of the databases define a specific drug for idiosyncratic DILI. In the literature, the term idiosyncratic is used only for drugs that cause very rare and severe DILI, but the classification of drugs is ambiguous and inconsistent. This is one of the reasons why conducting research in this field is challenging.

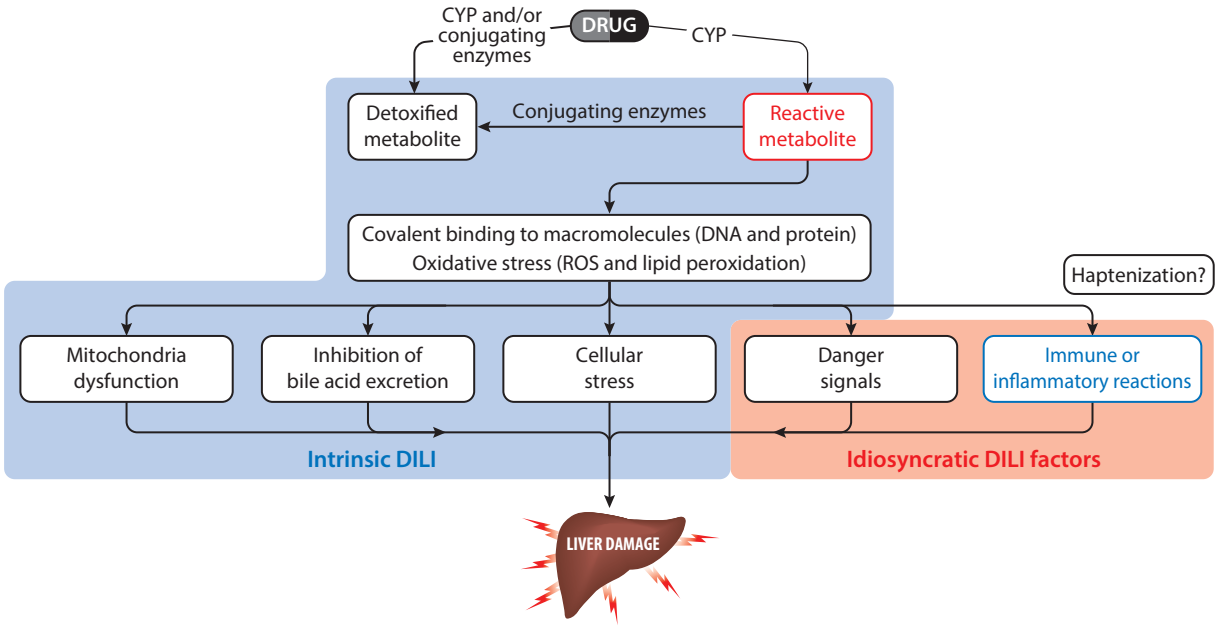

Figure 1

Mechanisms of DILI associated with metabolic activation of drugs. Drug-metabolizing enzymes such as CYPs catalyze not only detoxification reactions but also metabolic activation, which produces a reactive metabolite that is chemically highly reactive. Reactive metabolites covalently bind to in vivo molecules such as nucleic acids and proteins from cell constituents to form adducts, which express cytotoxicity through mitochondrial dysfunction, inhibition of bile acid excretion, and cellular stress. If the amount of reactive metabolites produced exceeds the detoxification capacity, liver damage occurs. As with intrinsic DILI, the production of reactive metabolites is also important in the mechanism of idiosyncratic DILI. Reactive metabolites have been identified from drugs that have been withdrawn from the market because of liver damage. Therefore, studies have focused mainly on the production of reactive metabolites in order to elucidate the pathogenesis and onset of idiosyncratic DILI. Abbreviations: CYP, cytochrome P450; DILI, drug-induced liver injury; ROS, reactive oxygen species.

4. INITIATION OF DILI BY REACTIVE METABOLITES

The liver is primarily responsible for drug metabolism and detoxification. In the liver, most detoxification reactions of drugs are catalyzed by hepatic drug-metabolizing enzymes, particularly the cytochrome P450 (CYP) family enzymes. However, drug-metabolizing enzymes such as CYPs catalyze not only detoxification reactions but also metabolic activation, which produces a reactive metabolite that is chemically highly reactive. Therefore, adverse effects and toxicities of these drugs occur mostly in the liver. Reactive metabolites covalently bind to in vivo molecules such as nucleic acids and cell constituent proteins to form adducts, which express cytotoxicity through mitochondrial dysfunction, inhibition of bile acid excretion, and cellular stress (**Figure 1**). The reactive metabolites formed are captured and detoxified by protective scavengers such as the reduced form of GSH; these are present in high concentrations in the liver (1 μmol/g liver on an empty stomach, 10 μmol/g liver on a full stomach). However, if the amount of reactive metabolites produced exceeds the detoxification capacity of GSH conjugation, liver damage occurs. Intrinsic DILI induced by acetaminophen (APAP) can be explained by such a mechanism.

Supplemental Material >

As with intrinsic DILI, the production of reactive metabolites is also important in the mechanism of idiosyncratic DILI. Drugs that cause idiosyncratic DILI are listed in **Supplemental Table 1**. Reactive metabolites have been identified from drugs that have been withdrawn from

the market because of liver damage, and the insert labels of most of these drugs list liver damage as a possible side effect. Therefore, studies have focused mainly on the production of reactive metabolites in order to elucidate the pathogenesis and onset of idiosyncratic DILI. For example, halothane, which has long been used as a general anesthetic, causes fulminant hepatitis, albeit very rarely (1/35,000 patients). Halothane is metabolized by CYP2E1 to generate a reactive metabolite, acid chloride, and trifluoroacetate proteins, which are thought to cause liver damage (20); halothane is recognized as a drug that causes idiosyncratic DILI. The in vivo metabolic rates of conversion of halothane, isoflurane, and desflurane to the active metabolite acid chloride are 20%, 0.2%, and 0.02%, respectively, and are correlated with the incidence of liver injury in humans (20). The mechanism of halothane-induced liver injury has been well investigated with experimental animal models, and a strain difference exists at the onset of hepatic injury in mice (21). However, no strain difference in the amount of trifluoroacetic acid adducts in the liver was observed, suggesting that the formation of protein adducts was necessary for the development of halothane-induced liver injury but was not related to the severity of injury. The immune- and inflammation-related factors eosinophils (22), thymic stromal lymphopoietin and interleukin-4 (IL-4) (23, 24), natural killer T cells (25), neutrophils (21), and IL-17 (13) play important roles in halothane-induced liver injury. However, the full picture of halothane-induced liver injury has not yet been elucidated. Herein, we describe an animal model of liver injury in which the potential scavenger level is reduced (Section 4.1), GSH synthesis is inhibited by L-buthionine-(*S,R*)-sulfoximine (BSO) to establish animal models of DILI (Section 4.2), and acyl metabolites are involved (Section 4.3).

4.1. Knockdown of GSH or SOD Synthesis to Establish a High-Sensitivity Animal Model of DILI

In general, the scavenger enzyme activity of rodent GSH *S*-transferase is 10 to 20 times higher than that of humans; thus, predicting DILI in humans is more difficult. The de novo synthesis of GSH in mammalian cells is regulated mainly by γ-glutamylcysteine synthetase (γ-GCS), which plays a central role in the antioxidative stress capacity of cells. Thus, a GSH-depleted rat model was investigated for the prediction of human DILI. An adenovirus vector with a short hairpin RNA (AdGCSh-shRNA) against rat γ-GCS heavy-chain subunit (GCSh) was constructed and used to knock down GCSh (26). In the in vivo study of rats, the hepatic GSH level decreased by 80% 14 days after a single administration of AdGCSh-shRNA, and this depletion persisted for at least 2 weeks. Unlike in normal rats, APAP-induced hepatotoxicity was significantly potentiated in the GSH knockdown rat model (26). By using the same GSH knockdown rat model, Morita et al. (27) reported highly sensitive detection of the hepatotoxicity of diclofenac and flutamide, which are considered idiosyncratic hepatotoxic drugs in acute and subacute toxicity tests.

Oxidative stress is a cause of DILI. Superoxide dismutases (SODs) are important antioxidant enzymes that defend against reactive oxygen species (ROS). Mitochondria are the major source of superoxide production, and SOD2 is localized mainly in the mitochondria-scavenging superoxide radicals. An adenovirus vector with a short hairpin RNA against rat SOD2 (AdSOD2-shRNA) was applied to the rat model to evaluate APAP-induced hepatotoxicity with high sensitivity (28). When the conventional knockout method was adapted at the genomic level, homozygous knockout of GCSh or SOD2 was lethal in rodents (29).

Hosomi et al. (30) applied a cell-based assay system overexpressing CYP3A4 with GCSh knockdown to evaluate CYP3A4-mediated cytotoxicity by using an adenovirus vector expressing CYP3A4 (AdCYP3A4) and an AdGCSh-shRNA system. The cytotoxicity of reactive metabolite(s) produced by CYP3A4 and subsequent GSH conjugation were detected with high sensitivity in albendazole, carbamazepine (CBZ), dapsone, flutamide, trazodone, and troglitazone (TGZ)

(30–32). Therefore, this can be a highly sensitive animal model based on the mechanism of developing DILI. However, due to the complexity of the experimental system and the difficulty in ensuring quantitative reproducibility, the system has not been widely used.

4.2. Inhibition of GSH Synthesis by BSO to Establish a High-Sensitivity Animal Model of DILI

GSH plays important roles in redox signaling, xenobiotic detoxification, antioxidant defense, regulation of cell proliferation, and apoptosis (33). GSH depletion contributes to oxidative stress, which plays a key role in the pathogenesis of many diseases, including cystic fibrosis, hypertension, diabetes, and liver injury (33). The pathogenic mechanism of GSH at the molecular level has been well investigated. BSO is a representative inhibitor of GSH synthesis; it also inhibits γ-GCS, a rate-limiting enzyme of GSH synthesis (34). BSO showed similar changes in gene expression in vivo, naturally decreasing the GSH content in mice (35). Therefore, BSO is considered appropriate for investigating the mechanism and function of GSH detoxification. Under normal conditions, hepatic GSH content was significantly decreased in rodents at 6–9 h after a single administration of BSO and returned to a normal level at 24 h (36). Several models of DILI developed by means of coadministering BSO are shown in **Table 1**.

In rats with CBZ-induced liver injury, a single administration of CBZ did not affect the level of alanine aminotransferase (ALT) in plasma, even when rats were cotreated with BSO. However, the repeated administration of CBZ for 5 days in combination with BSO resulted in prominent increases in plasma ALT in rats (37). Interestingly, in mice, BSO was not required under the same CBZ dosing for developing DILI (14). The content of GSH in rat liver (approximately 7 μmol/g of tissue) is similar to that in mouse liver (approximately 8 μmol/g of tissue) (38). Therefore, rats might have a lower ability to form reactive metabolites than mice, or rats might have relatively high GSH conjugation activity. These species differences in the formation and detoxification of reactive metabolites provide valuable information for assessing the risk of DILI. BSO is an inhibitor that can be easily used in vitro and in vivo, it can be quantitatively evaluated in an assay system, and it is being used in various evaluations of toxicity.

4.3. Role of Acyl Glucuronide Metabolites in DILI

Acyl glucuronidation is one of the major metabolic routes of carboxylic acid–containing drugs. Glucuronidation is generally considered a detoxification pathway. However, acyl glucuronides (AGs) are unstable under physiological conditions and consequently undergo hydrolysis or intramolecular rearrangement through migration of the drug moiety from the 1-*O*-position to the 2-, 3-, or 4-position on the glucuronic acid ring (39). Because of their electrophilic nature and ability to cause substitution reactions with nucleophilic groups in proteins or other macromolecules, AGs can covalently modify endogenous proteins, leading to adverse toxicity associated with carboxylic acid–containing drugs (40).

Several in vitro assay methods to assess the toxicity of AGs have been proposed. First, the half-lives of AGs can be evaluated in potassium phosphate buffer. The half-life of AGs in WDN drugs is shorter than that in safe drugs (41–43). Second, a Lys-Phe peptide adduct assay can be conducted, wherein Lys-Phe is used as a novel trapping agent that forms glycation adducts via a Schiff base. The use of this assay demonstrated a correlation between the formation of a peptide adduct and the formation of primary AG (44). Third, an immunostimulation assay can be performed with human peripheral blood mononuclear cells (hPBMCs), wherein cytokines and chemokines such as IL-6 and IL-8 are induced in hPBMCs by treatment with AGs (45). These methods have been used to determine the role of lumiracoxib acyl-β-D-glucuronide (lumiracoxib-AG) (a carboxylic

Table 1 Animal models of DILI and pathogenic mechanisms

Method of administration	Drug	Animal	Main pathogenic factor(s)	Reference
Coadministration with LPS	Chlorpromazine	SD rat	Neutrophil	65
	Diclofenac	SD rat	Neutrophil	51
	Ranitidine	SD rat	TNF-α	52
	Sulindac	SD rat	TNF-α	53
	Trovafloxacin	BALB/c mouse	TNF-α	54
Single administration	Amiodarone	BALB/c mouse	Mitochondrial stress	126
	ANIT[a]	BALB/c mouse	Th17	55
	Diclofenac	BALB/c mouse	IL-1β/Th17	56
	Dicloxacillin	BALB/c mouse	Th2	58
	Enalapril[b]	BALB/c mouse	Oxidative stress/neutrophil	127
	Fasiglifam (TAK-875)	ICR mouse	ER stress/TLR	128
	Flucloxacillin	BALB/c mouse	Th17/TLR4	57
	Flutamide	BALB/c mouse	Th2	59
	Halothane	BALB/c mouse	Th17	13
	Lamotrigine[b]	C57BL/6 and BALB/c	DAMPs/ROS	129
	Methimazole[b]	BALB/c mouse	Th2	60
	Troglitazone	BALB/c mouse	JAK/STAT3	121
Repetitive administration	Azathioprine	BALB/c mouse	ROS/XO	130
	Carbamazepine	BALB/c mouse	Th17	14
	Phenytoin[b]	C57BL/6 mouse	DAMPs/Th17	15
	Carbamazepine[b]	F344 rat	DAMPs	37
				122

[a]ANIT is not a clinically used drug.

[b]BSO was used to establish the animal model.

Abbreviations: ANIT, α-naphthyl isothiocyanate; BSO, L-buthionine-(*S*,*R*)-sulfoximine; DAMP, damage-associated molecular pattern; DILI, drug-induced liver injury; ER, endoplasmic reticulum; IL-1β, interleukin 1β; JAK/STAT3, Janus kinase 3/signal transducer and activator of transcription 3; LPS, lipopolysaccharide; ROS, reactive oxygen species; SD, Sprague Dawley; Th, T helper; TLR, Toll-like receptor; TNF-α, tumor necrosis factor α; XO, xanthine oxidase.

group–containing molecule) in lumiracoxib-induced liver injury (46). Furthermore, trovafloxacin-induced liver toxicity caused by an AG metabolite was detected by chemokine (C-X-C motif) ligand 2 (47). The involvement of diclofenac-AG in an in vivo model of DILI was investigated; diclofenac-AG was indicated to be partly involved in the pathogenesis of diclofenac-induced acute liver injury in mice by activating innate immunity and neutrophils (48). When assessing the toxicity of AG, it is necessary to carefully consider kidney damage as well as liver damage. The advantages and disadvantages of these assays from the perspective of preclinical drug development have been discussed in detail by Iwamura et al. (49).

5. ESTABLISHMENT OF AN ANIMAL MODEL OF IDIOSYNCRATIC DILI AND THE INVOLVEMENT OF IMMUNE- AND INFLAMMATION-RELATED RESPONSES

Lipopolysaccharide (LPS) was coadministered in all rodent models of idiosyncratic DILI to evaluate the clinically used over-the-counter drugs chlorpromazine (50), diclofenac (51), ranitidine

(52), sulindac (53), and trovafloxacin (54) (**Table 1**). LPS disrupts normal immune status in vivo; however, neutrophils or tumor necrosis factor-α (TNF-α) is involved in the development of DILI regardless of the drug tested. Therefore, the usefulness of the LPS-DILI model as a research tool remains controversial.

Kobayashi et al. (13) administered the clinical drug halothane (**Table 1**), which causes idiosyncratic DILI in humans, to wild-type mice. After in vivo single administration at many doses and conditions was examined, models of DILI for multiple drugs were created, and their mechanisms could be analyzed. T helper 17 (Th17) cells were involved mainly in halothane-induced liver injury, which was accompanied by a marked increase in the expression of macrophage inflammatory protein-2 (MIP-2) and an infiltration of the liver by neutrophils (13) (**Table 1**). Furthermore, Th17 cells were involved primarily in liver injury caused by α-naphthyl isothiocyanate (ANIT) (55), diclofenac (56), and flucloxacillin (57). Conversely, Th2 cells were involved mainly in liver injury induced by dicloxacillin (58), flutamide (59), and methimazole (MTZ) (60). Th1 cells were not involved for any of the drugs tested, probably because Th1-mediated cytotoxicity was avoided during the drug development process.

Higuchi et al. (14) and Sasaki et al. (15) developed the first models of DILI through continuous administration of CBZ (14), phenytoin (15), and azathioprine. CBZ is a representative drug that causes idiosyncratic DILI. There was no evidence of hepatotoxicity after repeated administration of CBZ (200 mg/kg, orally) once daily for 24 weeks to mice (61). A slight increase in ALT and histological changes in the liver were reported after repeated administration of CBZ (400 mg/kg, orally) once daily for 1 year in rats (61). After different dosing conditions were investigated, Higuchi et al.'s (14) study of wild-type mice showed that serious liver damage was induced only by the continuous oral administration of a dose of 400 mg/kg for 4 days and 800 mg/kg on the fifth day (**Supplemental Figure 1*a***) but not by a dose of 400 mg/kg for 5 days. Blood levels of CBZ and its three main metabolites peaked at 1.5–3 h (**Supplemental Figure 1*b***), and liver damage worsened when the mice were cotreated with the CYP inhibitors troleandomycin (TAO) or ketoconazole (KTZ). The measurement of CBZ and its metabolite concentrations strongly suggested the involvement of CBZ 3-hydroxylase in liver injury (**Supplemental Figure 1*c*,*d***).

Supplemental Material >

Significant differences in the response of DILI to CBZ between rats and mice have been noted. When both the Cyp3a-mediated toxicity and detoxification pathways were inhibited by KTZ or TAO (**Supplemental Figure 1*b***), liver injury was exacerbated in mice, but detoxification progressed predominantly in rats (37). The in vivo genomic level response to inflammation in mice is more similar to that in humans than in rats (62). Thus, in addition to individual differences and the effects of concomitant medications, species differences need to be considered. Establishing a model of DILI through long-term continuous administration of drugs, as in clinical cases, is difficult because, unlike humans, experimental animals are more likely to develop drug tolerance. However, drug resistance may be a valuable gateway for understanding the mechanism of DILI in clinical practice.

Analysis of the onset mechanism of DILI in mice revealed the involvement of high-mobility group box 1 (HMGB1), which is a damage-associated molecular pattern (DAMP); S100 calcium-binding protein A8 (S100A8) and S100A9, which are calcium-binding proteins; and Toll-like receptor 4 (TLR4), which is an innate immune receptor. TLR4 and its receptor were involved in the formation of receptor for advanced glycation end-products (RAGEs), and administration of various antibodies or inhibitors significantly suppressed liver damage. The overall flow of developing inflammation-associated reactions for DILI is shown in **Figure 2**. In the cases in which DILI was caused by CBZ, IL-17 was detected in the plasma, the production of the chemokine MIP-2 with neutrophil chemotactic activity was promoted, and neutrophils infiltrated the liver (14, 15, 63). These studies suggest that an adduct of a reactive metabolite generated by CYPs in hepatocytes

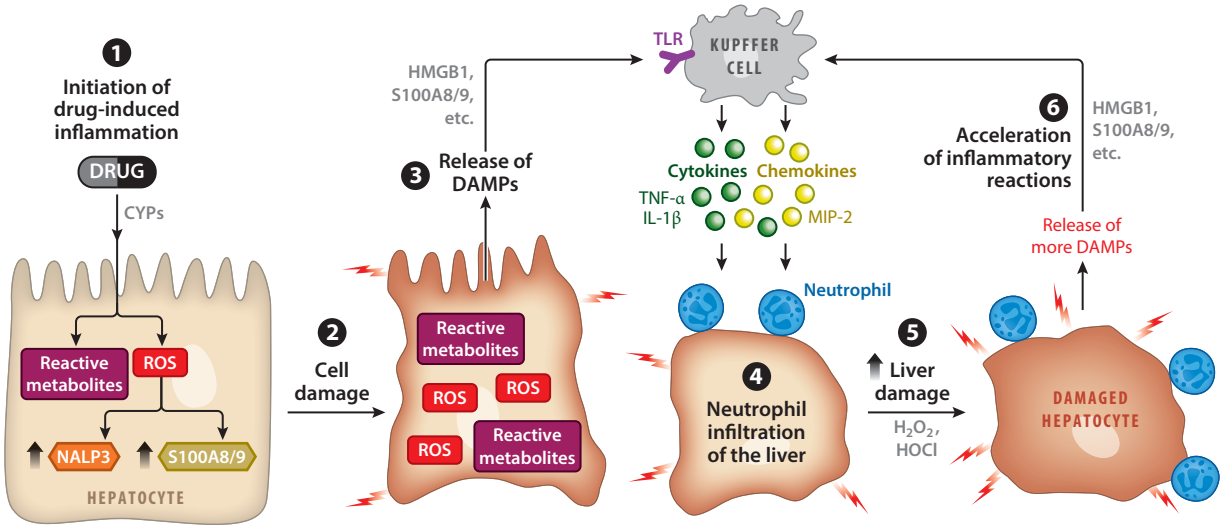

Figure 2

Proposed mechanism of reactive metabolite-mediated inflammatory reactions in the liver for idiosyncratic DILI. The overall flow of the inflammatory response is as follows: (❶) Formation of reactive metabolites and ROS occurs in hepatocytes. ROS activate innate immune system–related factors such as NALP3 and S100A8/9. (❷) The accumulation of reactive metabolite(s) and ROS leads to cell damage. (❸) Damaged cells release DAMPs, which activate Kupffer cells via TLRs and the NALP3 inflammasome. (❹) Activated Kupffer cells release cytokines (e.g., TNF-α and IL-1β) and chemokines (e.g., MIP-2), which lead to neutrophil infiltration of the liver. (❺) Neutrophil infiltration exacerbates liver damage and causes inflammation-mediated liver injury. (❻) This inflammation-mediated liver damage also accelerates inflammatory reactions via further DAMP release from damaged hepatocytes. Such a loop of DAMP-mediated reactions exacerbates liver damage, and reactive metabolites act to first trigger this inflammatory response loop via primary hepatocyte damage. Figure adapted with permission from Reference 63. Abbreviations: CYP, cytochrome P450; DAMP, damage-associated molecular pattern; DILI, drug-induced liver injury; HMGB1, high-mobility group box 1; IL-1β, interleukin 1β; MIP-2, macrophage inflammatory protein-2; NALP3, NACHT, LRR, and PYD domain-containing protein 3; ROS, reactive oxygen species; S100A8/9, S100 calcium-binding protein A8/9; TLR, Toll-like receptor; TNF-α, tumor necrosis factor-α.

is presented as an antigen to macrophages and Kupffer cells, promoting differentiation into Th17 cells and the release of cytokines and chemokines. This was presumed to be the mechanism for the exacerbation of DILI. IL-17 was detected in the plasma of approximately 60% of patients who developed DILI (64); studies of the establishment of animal models of DILI and clinical studies of the elucidation of the pathogenesis mechanism have yielded similar results. Hence, animal models of DILI might provide information that could prevent the development of similar DILI in clinical settings.

6. INVOLVEMENT OF IMMUNE- AND INFLAMMATION-RELATED FACTORS IN THE DEVELOPMENT OF IDIOSYNCRATIC DILI

In halothane-induced liver injury in humans, antibodies to CYP2E1 and to proteins such as calreticulin and protein disulfide isomerase, which are present in liver microsomes, were observed in the serum of patients (65). Similarly, antibodies to CYP2C8/9, CYP1A2, and aldolase B in patients with liver injury caused by tienilic acid, dihydralazine, and TGZ, respectively, have been reported (5, 66). Although such antibody production is not found in all DILI patients and is not considered a direct cause of liver damage, we speculate that some immune- and inflammation-related factors might play a role in idiosyncratic DILI, and the mechanism of injury could be as

follows (**Figure 2**). A reactive metabolite is produced mainly by hepatic drug-metabolizing enzymes. Reactive metabolites or ROS damage cellular components through covalent binding to and lipid peroxidation of proteins in the organs. Reactive metabolites simultaneously activate the innate immune system, such as Kupffer cells and macrophages, and may cause inflammation and allergies. Activated Kupffer cells and macrophages release cytokines (e.g., TNF-α and IL-1β) and chemokines (e.g., MIP-2), which lead to neutrophil infiltration of the liver. Neutrophil infiltration exacerbates liver damage and causes inflammation-mediated liver injury, which also further accelerates inflammatory reactions by releasing DAMP from damaged hepatocytes. This loop of DAMP-mediated reactions exacerbates liver damage, and reactive metabolites act to first trigger this inflammatory response loop via primary hepatocyte damage (**Figure 2**).

In the acquired immune response, T cells that have received antigen information and cytokine signals from antigen-presenting cells differentiate into Th cells and killer T cells, which leads to further development of the immune response. Th cells are divided into Th1, Th2, Th17, and regulatory T cells (Tregs) (**Supplemental Figure 2**). Th1 cells activate cellular immunity via effector cells such as natural killer cells by expressing the transcription factor T-bet and producing interferon-γ (IFN-γ) and IL-2. Conversely, Th2 cells express GATA-binding factor 3 (GATA3), produce IL-4 and IL-5, promote antibody production by B cells, and participate in innate immunity. Th17 cells express retinoid-related orphan receptor γt (RORγt), IL-17, and IL-22 and promote the local migration of neutrophils. Tregs produce IL-10 and suppress each T cell. Forkhead box protein 3 (FOXP3) and transforming growth factor-β (TGF-β) are involved in the differentiation of Tregs (67–69). The incidence of DILI increases with a specific human leukocyte antigen type, suggesting the involvement of the immune system in the mechanism (70–74). Taken together, this information indicates the involvement of immune- and inflammation-related factors in the pathogenesis of DILI can be elucidated by in vivo animal models of DILI.

Supplemental Material >

7. AN IN VITRO CELL-BASED EVALUATION SYSTEM FOR PREDICTING RISK OF DILI THAT CONSIDERS REACTIVE METABOLITE FORMATION

In the early stages of preclinical DILI testing in the drug development process, an in vitro cell-based assay system is required for the predictive evaluation of reactive metabolites in humans. As described in Section 4, the production of reactive metabolites by drug-metabolizing enzymes is involved in the onset of not only intrinsic DILI but also idiosyncratic DILI (75). Comprehensively analyzing the structures of various metabolites at the early stage of drug development is difficult. The formation of quinone, quinone imine, quinone methide, nitrenium, and epoxide structures appears to be particularly problematic. In addition, many unidentified traces of metabolites could be assumed to covalently bind to intracellular components such as proteins and nucleic acids. Therefore, researchers developed an in vitro quantitative screening method for reactive metabolites, with human liver microsome and GSH in the presence of NADPH, and used liquid chromatography–tandem mass spectrometry to comprehensively detect GSH adducts formed after the test drug was metabolized (76, 77). If the sum of the test drug and its metabolites is less than 50 pmol/mg liver protein, the possibility of liver damage is low, and this threshold is used as a guideline for drug development (78). However, there are exceptions.

Although the HepG2 cell line is frequently used to study the general cytotoxic potential of drugs in high-content screening (79, 80), these cells do not express significant amounts of phase I drug-metabolizing enzymes such as CYPs; this limits the ability to detect the metabolism-dependent toxicity of drugs (29). Even in fresh human hepatocytes, the enzyme activity of

CYP rapidly decreases to 1% or less after approximately 3 days of culture. This drawback was overcome by developing a test system that uses HepaRG cells. HepaRG cells, an established hepatoma cell line, can differentiate into hepatocyte-like and biliary epithelium-like cells and highly express drug-metabolizing enzymes; these functions are comparable to those of primary human hepatocytes (81, 82). However, even when human hepatocytes are cultured by three-dimensional spheroid systems (83) or when HepaRG cells are used to detect reactive metabolite formation, the prediction accuracy of idiosyncratic DILI has been only slightly improved (82, 84). These tests mostly evaluate events occurring within haptic parenchymal cells without considering the immune- and inflammation-mediated mechanisms. Therefore, improving the ability to predict idiosyncratic DILI requires the development of a new in vitro test system that can evaluate the relationship between idiosyncratic DILI and the expression levels of immune- and inflammation-related factors.

8. AN IN VITRO CELL-BASED EVALUATION SYSTEM FOR PREDICTING RISK OF DILI THAT CONSIDERS IMMUNE- AND INFLAMMATION-RELATED FACTORS

In an in vivo mouse model of APAP-induced DILI, damaged hepatocytes release DAMP molecules, which trigger the activation of resident innate immune cells in the liver, leading to the release of inflammatory mediators and the recruitment of inflammatory cells (7, 85). Inflammatory mediators such as cytokines, chemokines, ROS, and reactive nitrogen species released from innate immune cells participate in the progression of DILI (85, 86). Findings from the in vitro cell-based assay suggested that hepatocytes treated with DILI-inducing drugs (such as amodiaquine, diclofenac, nevirapine, tolcapone, and TGZ) release DAMPs to activate immune cells (87–89).

Oda et al. (17) have attempted to develop a novel cell-based assay to assess the risk of DILI that considers drug metabolism as well as immune- and inflammation-related gene expression. The authors treated human hepatoma HepaRG or HepG2 cells with 96 drugs that have different clinical risks of DILI. The conditioned media, the supernatant of the incubation medium of the test drugs and cells, were used to expose human promyelocytic leukemia HL-60 cells, and the messenger RNA (mRNA) expression levels of immune- and inflammation-related genes in the HL-60 cells were measured (17). Among the immune cell lines, HL-60 cells responded well to treatment with hepatotoxic drugs in terms of inducing the aforementioned immune and inflammatory genes. As revealed previously (90), the levels of monocyte chemoattractant protein-1 (MCP-1), S100A9, IL-1β, IL-8, and TNF-α were measured. The area under the receiver operating characteristic curve (ROC-AUC) was calculated to evaluate the predictive performance of the levels of various mRNAs as markers to discriminate the risk of DILI. The expression of IL-8 in HL-60 cells treated with conditioned media from differentiated HepaRG cells (HL-60/HepaRG) showed the highest ROC-AUC value, 0.758. Notably, the ROC-AUC values of these genes were higher in HL-60/HepaRG cells than in HL-60/HepG2 cells, suggesting that HL-60/HepaRG has greater potential for detecting the metabolic activation of drugs. This study reported a superior overall performance (ROC-AUC = 0.819) with 96% sensitivity and 51% specificity (17). However, the high content of GSH in HepaRG cells (approximately 140 nmol/mg protein) explains why the conditioned media from HepaRG cells did not always yield greater induction of immune-related genes than HepG2 cells did (91–94). GSH depletion or inhibition of other detoxification pathways could have increased the sensitivity of the assay system.

O'Brien et al. (95) reported that conventional markers showed low predictive power (GSH depletion: 19% sensitivity and 85% specificity; cell viability: 10% sensitivity and 92% specificity)

in HepG2 cells. Accumulating in vivo experimental evidence suggests that innate and adaptive immunity and their interaction with drugs are important for idiosyncratic DILI (96, 97). A few such studies using coculture or media transfer methods have been conducted with primary cells or cell lines of human hepatocytes and monocytic cells (87, 98, 99). Therefore, the conditioned medium method could address the problem of low sensitivity.

Furthermore, Oda et al. (100) aimed to establish an assay that was more similar to the in vivo response. hPBMCs were used as the source of immune cells and were cocultured with HepG2 cells to predict the drugs' potential to induce DILI (100). The biomarkers were selected by transcriptome-wide analysis in PBMCs. The combination of the markers by stepwise logistic regression showed the highest ROC-AUC value of 0.94, with a high sensitivity and specificity (93% and 86%) for 77 drugs (100). However, a few drugs were predicted by this model to be false negatives or false positives because of the idiosyncratic nature of the drug or the donor of PBMCs (101). Our established coculture model currently offers the best sensitivity and specificity. Studies with extrapolation to humans and studies evaluating idiosyncratic DILI are needed in the future.

9. USING IN VIVO ANIMAL MODELS TO INVESTIGATE THE ROLE OF microRNA IN THE PATHOGENESIS OF IDIOSYNCRATIC DILI

The dynamic changes in circulating microRNAs (miRNAs) in human plasma resulting from drug administration were first revealed by Wang et al. (102). In mice, APAP increased miR-122 and miR-192 in the serum in a dose-dependent and exposure-duration-dependent manner that paralleled serum ALT and histopathological changes in the liver. The changes in miRNA levels exhibited higher dynamic ranges than did the changes in levels of serum ALT. Many studies have evaluated the relationship between circulating miRNAs and the prognosis of cancer and other diseases (103, 104); however, few studies have investigated the potential use of circulating miRNA levels for predicting DILI. Kagawa et al. (105) reported miRNA biomarkers in serum in the early stages of hepatocellular injury, cholestasis, and steatosis in rats by using drugs that cause hepatocellular injury, cholestasis, and steatosis. In addition, various hepatic diseases in humans have been diagnosed with serum miRNAs (106). miRNAs play a critical role in the differentiation and function of the adaptive and innate immune systems (107). In consideration of this finding, miRNAs can be remarkably helpful in studying the mechanism of DILI, and the role of miRNAs in the pathogenesis of idiosyncratic DILI has been investigated.

Using miRNA microarray analyses, Endo et al. (108) investigated the possible involvement of miRNAs of the Th17-type immune response in DILI caused by the hepatotoxic drug halothane. The use of isoflurane as a low hepatotoxic drug excluded any pharmacological effects on miRNA expression because it is structurally similar to halothane. Consequently, the downregulation of miR-106b 1 h after administration of halothane was associated primarily with inflammation, immune responses, and liver injury (**Figure 3**). Consequently, the suppressed expression of miR-106b, as well as the subsequent upregulation of signal transducer and activator of transcription 3 (STAT3), was involved in the pathogenesis of halothane-induced liver injury (**Figure 3**).

Uematsu et al. (109) used miRNA microarray analyses to investigate the possible involvement of miRNAs of the Th2-type immune response in DILI caused by the hepatotoxic drug MTZ. The authors found that the expression of miR-29b-1-5p and miR-449a-5p was upregulated (**Figure 3**). Among the targets of these miRNAs, Th2-suppressing transcription factors such as SRY-related HMG-box 4 (SOX4) and lymphoid enhancer-binding factor 1 (LEF1) were downregulated from the early phase of liver injury. Thus, the negative regulation of the expression of SOX4 by miR-29b-1-5p and that of LEF1 by miR-449a-5p are thought to be involved in the development of Th2 bias in MTZ-induced liver injury (**Figure 3**).

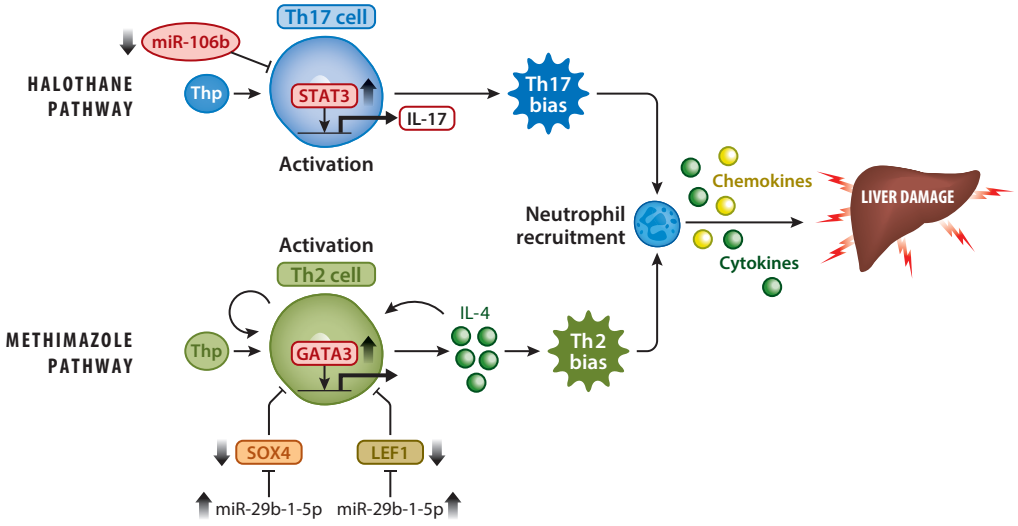

Figure 3

The involvement of miRNAs in halothane- and methimazole-induced liver injury. In halothane-induced liver injury, miR-106b is differentially downregulated. Among the targeted genes of miR-106b, STAT3, a Th17-promoting transcriptional factor, was activated before plasma ALT was elevated in a mouse model of halothane-induced DILI (108). In methimazole-induced liver injury, the decrease in the expression of SOX4 and LEF1 before the onset of liver injury is considered to be important (109). Similarly, time-dependent analyses revealed that a pivotal Th2-related response was induced prior to necrotic events. Both SOX4 and LEF1, which are high-mobility group proteins, directly associate with the GATA3 zinc-finger motif and suppress Th2 (131, 132). The downregulation of SOX4 and LEF1 may stimulate GATA3 activity and facilitate the Th2-type immune response. Abbreviations: ALT, alanine aminotransferase; DILI, drug-induced liver injury; GATA3, GATA-binding factor 3; IL, interleukin; miRNA, microRNA; LEF1, lymphoid enhancer-binding factor 1; SOX4, SRY-related HMG-box 4; STAT3, signal transducer and activator of transcription 3; Th, T helper; Thp, precursor helper T cell.

Similar miRNA studies have elucidated the mechanism of DILI by using in vivo animal models (110, 111). The use of animal models of DILI provides a comprehensive understanding of the involvement of miRNAs, especially in the early phase of DILI progression and the subsequent response of downstream affected genes.

10. REACTIVE METABOLITE-INDEPENDENT MECHANISM FOR TROGLITAZONE-INDUCED LIVER INJURY: DISCREPANCIES BETWEEN IN VIVO AND IN VITRO RESULTS

TGZ, a thiazolidinedione antidiabetic drug used to treat type II diabetes mellitus, induces idiosyncratic DILI in patients, which led to its withdrawal in 2000. TGZ has been generally recognized as a typical drug that induces idiosyncratic DILI. It is metabolized to sulfate (M-1), glucuronide (M-2), and a quinone-type metabolite (M-3) in both humans and experimental animals (112), showing similar metabolic profiles in humans and rodents. The CYP-dependent reactive metabolites of TGZ become trapped as conjugates of GSH, as shown in both in vitro and in vivo experiments. The biotransformation of these conjugates has been assumed to involve quinone methide formation, epoxide formation, and thiazolidinedione ring scission (113–115). CYP3A4 catalyzed these reactive metabolite-producing reactions (116). An in vitro cell-based DILI screening system indicated that reactive metabolites produced by CYP3A4 and subsequently conjugated by GSH

are involved in hepatotoxicity. Many studies employing various in vitro screening systems have investigated TGZ cytotoxicity by using hepatocytes and liver microsomes and have concluded that the active metabolites of TGZ are hepatotoxic.

TGZ has never been reported to induce liver injury in animal experiments performed in vivo, even when BSO was coadministered (117). In reported toxicology studies, TGZ was administered orally at a dose of 800 mg(kg·day) for 24 months to mice, 1,200 mg(kg·day) for 12 months to rats, and 1,200 mg(kg·day) for 12 months to monkeys, but no signs of liver dysfunction were confirmed. Thus, species differences in the pharmacokinetics or toxicokinetics of TGZ between humans and experimental animals are assumed to be associated with observed discrepancies. Even the use of SOD2 hetero-knockdown mice could not reproduce the findings from the mouse model of TGZ-induced DILI (118).

In 2004, Tateno et al. (119) investigated TGZ-induced liver injury in a chimeric mouse with a humanized liver whose replacement index with human hepatocytes was up to 92%. When the chimeric mice were orally administered TGZ at a dose of 1,000 mg(kg·day) for 14 or 23 days, serum ALT was significantly increased by 2.1- or 3.6-fold, respectively. Coadministration of BSO (10 mM in drinking water) unexpectedly prevented a TGZ-dependent increase in ALT, suggesting that the GSH-scavenging pathway might not be functional in TGZ-induced liver injury (120).

In 2019, Jia et al. (121) established a novel mouse model of TGZ-induced liver injury in normal mice. The administration of a single intraperitoneal dose of TGZ (300 mg/kg) to BALB/c female mice significantly elevated ALT (up to 950 IU/L) and aspartate aminotransferase levels 6 h after treatment. Hepatic transcriptome profiles of TGZ-exposed liver were compared with those of nonhepatotoxic rosiglitazone (RGZ), which has the same pharmaceutical effect and a similar chemical structure. The JAK/STAT signaling pathway was activated in TGZ-induced liver injury, leading to the promotion of STAT3 phosphorylation in TGZ-treated mice. These pathways are not TGZ specific but they have been suggested to be activated by many idiosyncratic DILI-related drugs, such as halothane and phenytoin (15, 108). Therefore, it remains unclear whether these pathways may explain idiosyncrasy (121).

Moreover, 300 mg/kg of nonhepatotoxic RGZ did not induce liver injury. Although intraperitoneal administration of TGZ may be controversial, liver injury did not develop for either single or continuous oral administration (up to 1,000 mg/kg for up to 4 weeks) in the present study. Furthermore, the appropriate solvent could affect the success of a mouse model of TGZ-induced hepatotoxicity (121). In general, in vivo studies have shown that pretreatment with BSO can enhance DILI caused by CBZ (37, 122), MTZ (123), and phenytoin (15). Conversely, TGZ-type hepatoprotection by cotreatment with BSO was observed. Research into the mechanism is ongoing.

Enhanced GSH oxidation increases Ca^{2+} release in the sarcoplasmic reticulum, which is regulated by RyR2, an isoform of the ryanodine receptor (RyR) (124), and massive Ca^{2+} release induces apoptosis (125). Importantly, oxidized GSH (GSH disulfide) content, ALT level, and RyR2 mRNA expression were significantly decreased in the BSO+TGZ group compared with the TGZ and RGZ groups (121) (**Figure 4**). Pretreatment with the RyR inhibitor dantrolene (DAN) potently decreased ALT levels and RyR2 mRNA expression in the DAN+TGZ group compared with the TGZ group. DAN has been used clinically to treat malignant hyperthermia and is now suggested as a potential treatment for idiosyncratic DILI. These novel results may provide new insights into RyR activity in the regulation of idiosyncratic DILI and may suggest mechanisms of idiosyncrasy (**Figure 4**). Many mechanisms have been postulated to be involved in the effects of drugs that cause idiosyncratic DILI. We believe that further extrapolating clinical cases in predictive studies will aid in exploring and identifying the key factors.

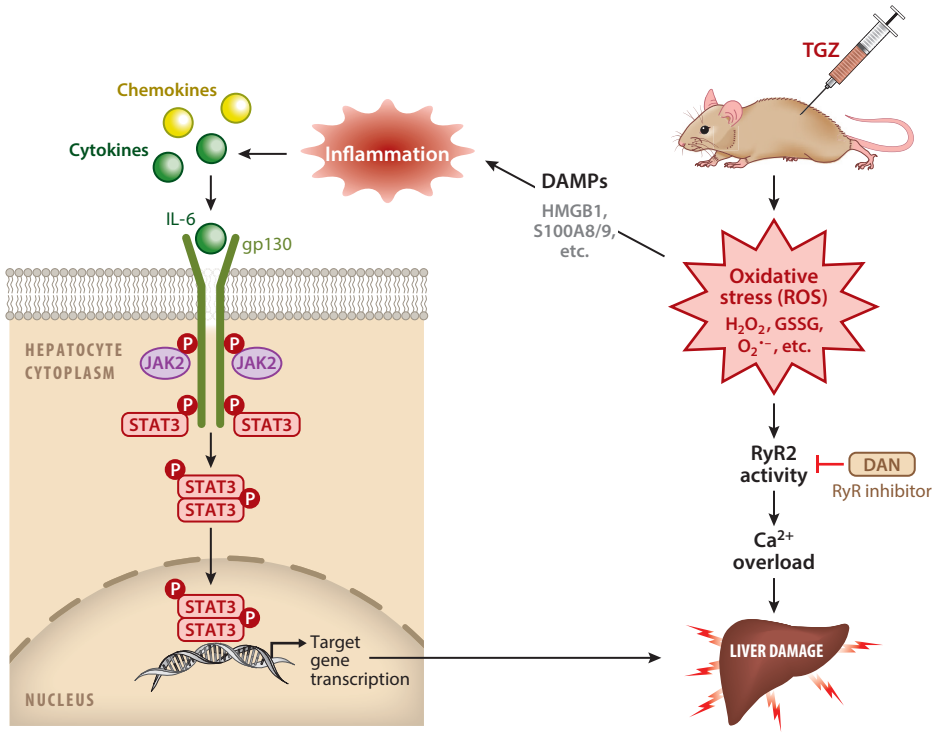

Figure 4

The mechanisms of TGZ-induced liver injury. The involvement of oxidative stress in TGZ-induced liver injury leads to the production of ROS, which promotes the release of DAMPs to secrete inflammatory cytokines and chemokines, thereby activating the JAK/STAT3 signaling pathway, and stimulates RyR2 activity, resulting in an overload of intracellular Ca^{2+}, ultimately leading to liver injury. Figure adapted with permission from Reference 121. Abbreviations: DAMP, damage-associated molecular pattern; DAN, dantrolene; gp130, membrane glycoprotein 130; GSSG, glutathione disulfide; HMGB1, high-mobility group box 1; IL-6, interleukin-6; JAK2, Janus kinase 2; ROS, reactive oxygen species; RyR, ryanodine receptor; S100A8/9, S100 calcium-binding protein A8/9; STAT3, signal transducer and activator of transcription 3; TGZ, troglitazone.

11. CONCLUSIONS

The production of reactive metabolites by drug-metabolizing enzymes is considered to be important for the onset of not only intrinsic DILI but also idiosyncratic DILI. Immune and inflammatory factors are thought to exacerbate or suppress liver injury. As reported in recent preclinical drug development studies, at present, the onset of intrinsic DILI in both in vitro assay systems and in vivo animal models can be predicted with relatively high sensitivity. However, the occurrence of idiosyncratic DILI, which has very low incidence, and cases of severe liver damage are difficult to predict because of our poor mechanistic understanding and the lack of appropriate predictive animal models. Therefore, a sensitive and reliable in vivo animal model and an in vitro or in silico screening system need to be developed.

Over the past decade, despite the efforts of many researchers, no tangible improvements in the prediction and prevention of idiosyncratic DILI have been made. Herein, we describe the roles of reactive metabolites and immune and inflammatory mechanisms in idiosyncratic DILI and present our experience with in vivo experimental animal models of idiosyncratic DILI and the

development of cell-based test systems based on the in vivo information obtained. Although the predictive cell-based test system introduced in this review is at a near-practical level, the quantitative evaluation needs to be repeated for many drugs. Ultimately, in vivo animal models are essential for extrapolation to humans. However, the use of animal models remains challenging due to the lack of an overall understanding of idiosyncratic DILI. In the future, the term idiosyncratic may ultimately become obsolete once the mechanism and development of idiosyncratic DILI are better understood.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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