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*Special Section – New Models in Drug Metabolism and Transport—Minireview*

# Do In Vitro Assays Predict Drug Candidate Idiosyncratic Drug-Induced Liver Injury Risk?

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## ABSTRACT

**In vitro assays are commonly used during drug discovery to try to decrease the risk of idiosyncratic drug-induced liver injury (iDILI). But how effective are they at predicting risk? One of the most widely used methods evaluates cell cytotoxicity. Cytotoxicity assays that used cell lines that are very different from normal hepatocytes, and high concentrations of drug, were not very accurate at predicting idiosyncratic drug reaction risk. Even cytotoxicity assays that use more biologically normal cells resulted in many false-positive and false-negative results. Assays that quantify reactive metabolite formation, mitochondrial injury, and bile salt export pump (BSEP) inhibition have also been described. Although evidence suggests that reactive metabolite formation and BSEP inhibition can play a role in the mechanism of iDILI, these assays are not very accurate at predicting risk. In contrast, inhibition of the mitochondrial electron transport chain appears not to play an important role in the mechanism of iDILI, although other types of mitochondrial injury may do so. It is likely that there are many additional mechanisms by which drugs can cause iDILI. However, simply measuring more parameters is unlikely to provide better predictive assays unless those parameters are actually involved in the mechanism of iDILI. Hence, a better mechanistic understanding of iDILI is required; however, mechanistic studies of iDILI are very difficult. There is substantive evidence that most iDILI is immune mediated; therefore, the most accurate assays may involve those that determine immune responses to drugs. New methods to manipulate immune tolerance may greatly facilitate development of more suitable methods.**

## Introduction

The approval and success of a new drug candidate is dependent on the balance between efficacy and safety. Most drug candidates fail; an important reason for failure is inadequate safety. If a compound is going to fail, it is best that it fail early, before harmful effects can occur in animal safety studies or human clinical trials and before a lot of money has been invested in its development. Adverse idiosyncratic drug reactions (IDRs) pose a particular problem because they are often not discovered until after a drug has been released onto the market and millions of patients have been treated. Therefore, IDRs increase the risk and inefficiency of drug development. Animal toxicology studies do not usually detect IDR risk; after all, IDRs are also idiosyncratic in animals. The major IDR that results in drug candidate failure is idiosyncratic drug-induced liver injury (iDILI); therefore, most assays focus on the prediction of iDILI. With the development of combinatorial chemistry to produce many candidates plus the widespread use of in vitro methods in drug discovery to optimize pharmacology and drug exposure, use of in vitro assays for high-throughput toxicity testing is a very attractive option. However, no assay is useful if it has no predictive value; just how predictive of iDILI are current in vitro assays?

Although there are many mechanistic hypotheses, our mechanistic understanding of iDILI is poor and little is known with certainty. One possible hypothesis, which is outlined in Fig. 1, is that iDILI arises via a combination of drug-related adverse biologic effects plus susceptibility factors that arise in individual patients (Ulrich, 2007). There is increasing evidence that immune reactions play key roles in most IDRs, including iDILI; however, drugs and drug metabolites may stimulate immune responses in multiple ways (see Fig. 2), and other types of injury may also influence whether a drug-induced immune response arises. Furthermore, since the mechanisms by which drugs cause iDILI in susceptible patients have not been defined, it is currently unclear whether Fig. 1 provides a useful overall framework or is oversimplified and conceptually flawed.

Here, we describe the most common types of assays that have been proposed for use in toxicity screening of drug candidates, and we also discuss the currently available evidence of their value in predicting iDILI risk.

## Cytotoxicity Assays

**Rationale.** Hepatocytes are the major cell type within the liver and are a common target in iDILI. The most clinically concerning consequence

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**ABBREVIATIONS:** ALT, alanine aminotransferase; BSEP, bile salt export pump; $C_{ss}$, total plasma steady-state drug concentration; CVB, covalent binding; DAMP, danger-associated molecular pattern; DILI, drug-induced liver injury; HLA, human leukocyte antigen; iDILI, idiosyncratic drug-induced liver injury; IDR, idiosyncratic drug reaction; P450, cytochrome P450; RM, reactive metabolite; SCH, sandwich configuration hepatocyte; THLE, T-antigen immortalized human liver epithelial.

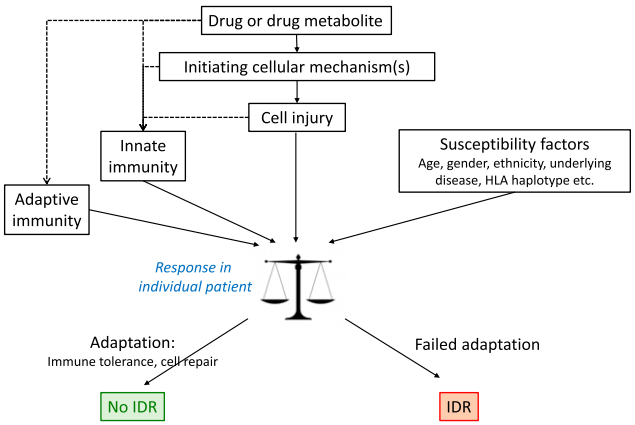


**Fig. 1.** Mechanisms by which iDILI and other IDRs arise. Factors that influence the balance between adaptation and iDILI. Postulated role of immune responses in iDILI.

is acute liver failure, which arises after injury to a substantial proportion of hepatocytes (Yuan and Kaplowitz, 2013). Numerous human hepatocyte-experimental model systems have been described, which are outlined in the next section. It is conceivable that data provided by these models could provide useful insights into iDILI mechanisms. It has also been proposed that the data provided by these studies may help to distinguish between drugs that cause iDILI and safe drugs (i.e., aid in iDILI hazard identification and derisking, if used during drug discovery) (Atienzar et al., 2016). However, in vitro cell toxicity studies cannot be expected to address the factors that explain why iDILI arises only in susceptible patients, which remain largely undefined. A particular challenge is posed by the relatively slow onset of iDILI in susceptible patients. Typically, the first signal of liver injury, increased plasma alanine aminotransferase (ALT), is not evident until the drug has been administered for many weeks or months (Chalasani et al., 2014). In contrast, in vitro toxicity studies typically are undertaken only for a maximum of several days. This is insufficient for development of adaptive immune responses, which appear to play an important role in iDILI caused by numerous drugs. Since the currently available isolated liver cells cannot be expected to reproduce all relevant iDILI mechanisms and susceptibility factors in vitro, even if the most in vivo–like conditions are used, they cannot be expected to provide accurate prediction of whether a test drug will cause clinically concerning liver injury in an individual patient. On the other hand, even if iDILI is immune mediated, cell injury may play an important role in the induction of an immune response as discussed below. Therefore, it is possible that in vitro assays could predict iDILI risk. However, there are major problems with the in vitro toxicity assays that have been traditionally used. One obvious issue is that the cells used for in vitro assays are not normal hepatocytes. In particular, cell lines such as HepG2 cells have very limited capacity to metabolize drugs, and evidence suggests that most toxicity is mediated by reactive metabolites (RMs) of a drug rather than the parent drug as discussed below. Even primary human hepatocytes rapidly lose their drug-metabolizing capacity. Drug-metabolizing activity is only one of the most obvious differences between normal hepatocytes and those used for in vitro assays. Hepatocytes in vitro will never be "normal," but it is possible that more recent platforms such as HepaRG spheroids will provide more predictive ability as discussed below. Another important issue is the drug concentration used for testing. It is typical that no toxicity is observed with therapeutic drug concentrations, and it is common to use concentrations 100 times the clinical $C_{max}$ of a drug. This is based on the rationale that the concentration in the liver is often much higher than in the blood (Xu et al., 2008). Although many drugs are concentrated in the liver, they might also be concentrated in hepatocytes in vitro, but the activity of transporters involved in drug uptake is also likely to be different than their activities in vivo. Therefore, it is impossible to easily determine the appropriate test concentration. It is the dose that makes the poison; therefore, the use of such high concentrations for testing is likely to lead to a high rate of false-positive results.

**Types of Assay.** The characteristics, advantages, and disadvantages of the most commonly used cell-based assays are summarized in Table 1. Hepatocytes can be isolated with high viability and good yield and maintained in vitro for many days; therefore, they are suitable for use in toxicity testing (Hewitt et al., 2007). However, the availability of human hepatocytes is limited, and these cells do not replicate in vitro. This limits their potential use during early phases of drug discovery when many hundreds or thousands of test compounds must be evaluated. Various human liver-derived cell lines have been described that proliferate in vitro and hence are more suitable for high-volume routine compound testing. As mentioned above, all of these cell lines exhibit much lower activities of many cytochrome P450 (P450) enzymes and other drug-metabolizing enzymes than hepatocytes in the human liver in vivo, and typically they do not exhibit polarized plasma membrane transporter expression [e.g., HepG2, FaO, or T-antigen immortalized human liver epithelial (THLE) cells] (Godoy et al., 2013). Currently, the most impressive "in vivo–like" phenotype is exhibited by HepaRG cells, which can be differentiated in vitro to distinct cell types that exhibit phenotypic characteristics of other hepatocyte or biliary epithelial cells (Guillouzo et al., 2007). The absent, or relatively poor, P450 activities of other liver-derived cell lines can be improved by transfection with plasmids encoding these enzymes. For example, a panel of transfected THLE-derived cell lines has been described, which express activities

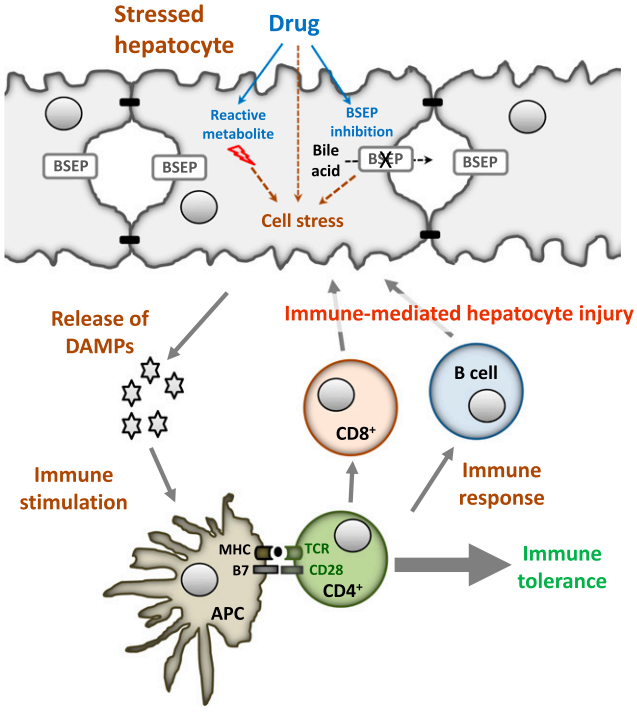


**Fig. 2.** A drug, or more commonly a RM, causes hepatocyte stress. One possible mechanism of cell stress is inhibition of BSEP leading to elevated levels of bile acids. The cell stress leads to the release of DAMP molecules. The DAMPs activate antigen-presenting cells. This can lead to the activation of T or B cells, which in turn can cause liver injury. However, the more common immune response is tolerance. APC, antigen-presenting cell; MHC, major histocompatibility complex; TCR, T-cell receptor.

TABLE 1

Examples of in vitro human liver cell models proposed for use in iDILI prediction

| Cell Model | Characteristics | Advantages | Disadvantages | References |
|---|---|---|---|---|
| HepG2 cells | Human liver-derived cell line | Simple model | Low drug-metabolizing enzyme expression | Godoy et al. (2013), Atienzar et al. (2014, 2016) |
| | | Suitable for short-term and longer-term studies | Poor expression of plasma membrane transporters | |
| | | Multiple endpoints can be evaluated | | |
| | | Suitable for high-volume and multiparametric data generation | | |
| HepaRG cells | Human hepatoma-derived cell line | Can be differentiated into hepatocyte-like and biliary epithelial-like cells | Limited commercial availability | Guillouzo et al. (2007), Ott et al. (2017) |
| | | Better maintenance of "hepatocyte-like" drug-metabolizing enzymes and membrane transporters than HepG2 cells | Complex cell culture conditions are needed | |
| | | Multiple endpoints can be evaluated | Cells exhibit limited metabolizing enzyme phenotype | |
| | | | P450 activities are lower than those in isolated primary hepatocytes | |
| Cell lines expressing human P450s | Human liver-derived cell lines transfected with individual human P450s | Stably transfected cell lines are a simple model, well suited to multiparametric data generation plus acute and long-term toxicity studies | Transfected cell lines do not express hepatocyte-like cell phenotype, or many membrane transporters expression | Dambach et al. (2005), Hosomi et al. (2011), Tolosa et al. (2013), Gustafsson et al. (2014) |
| | | Enable direct comparison between cell toxicity caused by parent compound (to mock-transfected cells) and metabolites (to transfected cells) | Unbalanced metabolism, since only selected P450s are expressed | |
| Isolated hepatocytes | Isolated human hepatocytes | Freshly isolated hepatocytes express "in vivo–like" high drug metabolism activity | Limited availability of human donor livers | Hewitt et al. (2007), Xu et al. (2008); Usui et al. (2009), Ansede et al. (2010), Nakamura et al. (2010), Wolf et al. (2010), Thompson et al. (2012), Barber et al. (2015) |
| | | Good maintenance of viability, for multiple days, when cultured as monolayers | Human liver exhibits marked interindividual variability | |
| | | When cultured appropriately (ideally in sandwich configuration), regain polarized plasma membrane transporter expression and are well suited to multiparametric data generation | Freshly isolated hepatocytes in suspension are viable only for several hours and do not exhibit polarized plasma membrane transporter expression | |
| | | | Longer-term hepatocyte culture is technically challenging and these cells fail to maintain high P450 expression | |
| | | | Interindividual donor variability may influence results | |
| Micropatterned hepatocyte/accessory cell cocultures | Isolated hepatocytes cocultured with macrophages or other accessory cells | Good maintenance of viability, drug-metabolizing enzymatic activity, and membrane transporters, for multiple days | Technically challenging to prepare; commercial supply is required | Underhill and Khetani (2017) |
| | | Numerous endpoints can be evaluated, at a single cell level | Limited variety and physiologic relevance of accessory cells | |
| | | | Limited toxicity characterization | |
| | | | Interindividual donor variability may influence results | |
| Liver microtissues (spheroids) | Liver cell aggregates, which includes hepatocytes and multiple nonparenchymal cell types | Good maintenance of viability, drug-metabolizing enzyme activity, and membrane transporters, for multiple days | Technically challenging to prepare; commercial supply is required | Bell et al. (2016), Proctor et al. (2017) |
| | | | Limited toxicity characterization | |
| | | | Endpoints cannot be quantified at a single cell level | |

(*continued*)

TABLE 1—*Continued*

| Cell Model | Characteristics | Advantages | Disadvantages | References |
|---|---|---|---|---|
| | | | Interindividual donor variability may influence results | |
| Microfluidic devices | Hepatocytes, multiple liver cell types, or liver-derived cell line devices that reproduce in vivo oxygen tension gradients and/or fluid flow | Improved maintenance of viability and drug-metabolizing enzyme activity, and membrane transporters, for multiple days, compared with nonmicrofluidic cell models | Technically challenging to prepare; commercial supply of devices is required | Vernetti et al. (2016) |
| | | | Limited toxicity characterization | |
| | | | Interindividual donor variability may influence results | |
| Stem cell–derived hepatocytes | Hepatocytes or other differentiated human liver cells, generated via stem cell technology | If successful, could provide cells in large amounts that replace a need for cell isolation from human donor livers | Complex cell differentiation protocols are required | Goldring et al. (2017) |
| | | | Currently available conditions provide poorly differentiated cells | |
| | | | Limited toxicity characterization | |

of individual different human P450 enzymes that are similar to those present in the human liver in vivo (Dambach et al., 2005). Another approach being used to develop hepatocyte-like cells that are suitable for in vitro toxicity testing is via use of induced pluripotent stem cell technology (Goldring et al., 2017).

When maintained in monolayer cell culture for more than a few days, isolated hepatocytes also exhibit progressive loss of cell viability, reduced expression of many P450 and other drug-metabolizing enzymes, and loss of polarized plasma membrane protein expression. Maintenance of cell viability, drug-metabolizing enzyme activities, and polarized plasma membrane expression can be improved markedly by use of alternative cell culture conditions. The available options include hepatocyte culture in a sandwich configuration (i.e., with rigid collagen substratum, plus overlay with a gelled layer of either collagen or Matrigel), use of culture media supplemented with hormones and hepatocyte growth factors (Maurel model), and hepatocyte culture in devices that provide fluid shear stress and oxygen tension gradients that are similar to those that arise in the liver in vivo (Hewitt et al., 2007; Goldring et al., 2017). The viability and P450 activities of isolated hepatocytes have also been improved by coculture of hepatocytes with macrophage-derived accessory cells in bioengineered devices (Underhill and Khetani, 2017) and by formation of ex vivo three-dimensional aggregates of defined size that contain either hepatocytes alone or hepatocytes plus other liver nonparenchymal cell types ("liver spheroids") (Bell et al., 2016).

Toxicities of test drugs have been investigated by evaluation of cell growth inhibition (MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays), membrane lysis (release from cells into media of lactate dehydrogenase or other enzymes), and depletion of cellular ATP. In addition, some investigators have used high-content cell imaging methods to evaluate additional parameters such as cell size and shape, nuclear and mitochondrial morphology and number, cytoskeleton, and markers of oxidative stress. Others have used microarrays to explore effects of drugs on transcription of predefined genes, or global gene transcription (Atienzar et al., 2016).

**Evidence.** Many published studies have attempted to evaluate whether the in vitro liver cell models summarized in the previous section are able to discriminate between drugs that cause iDILI and safe drugs. However, crucially important parameters, which include test drug concentration range, the assay duration and the drugs evaluated, the range and variety of assay endpoints, and the data analysis method, typically vary markedly between different studies. In addition, many studies have tested small numbers of test drugs (<50) and often these have been classified differently in different studies. For example, although clinical use of tacrine in the United States was discontinued in 2013 due to iDILI concern, the drug has been classified in different studies as either highly hepatotoxic (Atienzar et al., 2014), moderately hepatotoxic (O'Brien et al., 2006), or nonhepatotoxic (Xu et al., 2008). One approach is to use the Liver Toxicity Knowledge Base, which assigns withdrawn drugs and licensed drugs to different drug-induced liver injury (DILI) classes (most concern, high concern, low concern, and no concern) based primarily on information extracted from U.S. Food and Drug Administration drug labels (Chen et al., 2013). However, drug labels are not developed in a uniform manner, and they are generally developed early in the history of a drug when information is limited; therefore, they are often not very accurate. The LiverTox website (https://livertox.nih.gov) provides a more comprehensive evaluation of risk; however, its evaluation is not quantitative. The fact is that, with few exceptions, we simply do not have accurate incidence and iDILI risk data for drugs. Most in vitro studies involve a collection of drugs that sometimes cause iDILI and can arbitrarily be classed as high or less iDILI concern, and many of the drugs associated with the highest risk are not studied. By use of high concentrations of drug, it can appear that an assay has high predictive value because it classifies a drug with a relatively low iDILI risk as a true positive for the assay. This puts into question attempts to correlate cytotoxicity with risk.

Although evidence-based systematic comparison between data obtained by different investigators is not possible, the following generalizations can be made. First, it is important to consider drug exposure in patients in vivo to evaluate in vitro assay signals. The most commonly used parameter is total plasma steady-state drug concentration ($C_{ss}$) (Xu et al., 2008). Second, using liver-derived cell lines (HepG2 or THLE) that have low drug-metabolizing activity is less likely to provide accurate estimates of iDILI risk than data obtained using either liver cell lines that express more physiologically relevant P450 activities, or isolated primary hepatocytes (Dambach et al., 2005; Garside et al., 2014; Gustafsson et al., 2014). Third, multiparametric evaluation of multiple cell endpoints by high-content cell imaging of isolated hepatocytes provided only modest improvement in sensitivity compared with cell cytotoxicity evaluation (Garside et al., 2014). Fourth, that more physiologically relevant microengineered liver cocultures (Underhill and Khetani, 2017), or three-dimensional hepatocyte spheroids (Proctor

et al., 2017), provide improved sensitivity of iDILI detection than isolated hepatocytes cultured as monolayers. The iDILI sensitivity of cytotoxicity values obtained when hepatocyte spheroids were incubated for 14 days with 110 drugs (of which, 63% caused DILI) was < 60% when in vivo total plasma $C_{ss}$ was considered, whereas iDILI specificity was > 80% (Proctor et al., 2017). However, there were still many false-positive and false-negative results.

The fact is that cytotoxicity assays (i.e., those that measure cell death) do not accurately reproduce the mechanisms by which iDILI arises in susceptible patients and thus cannot be expected to predict iDILI risk with high accuracy. Ultimately, no in vitro system will be as relevant as results from in vivo studies and, with few exceptions, most animals and humans treated with pharmacologically relevant doses of drug do not have increases in serum ALT or other simple cytotoxicity measurements when treated with a drug that can cause iDILI. Therefore, how can we expect in vitro cytotoxicity assays to accurately predict iDILI risk? Other in vitro biomarkers may predict iDILI risk, but without a better understanding of the early steps in the mechanism of iDILI, it is not possible to design better predictive assays. Basing in vitro assays on unproven hypotheses is very problematic and, given the uncertainties of the quantitative iDILI risk associated with most drugs, correlational studies with high concentrations of drug are not a valid test of a hypothesis.

## Covalent Binding Assays

**Rationale.** RM formation is a well documented mechanism by which numerous chemicals, which include the drug acetaminophen, can initiate liver toxicity. Most RMs are formed via oxidative or reductive reactions that are catalyzed by P450 enzymes, although other enzymes (e.g., flavin monooxygenases) can be involved and some RMs are formed via conjugation reactions that are mediated by sulfotransferases, UDP-glucuronyltransferases, or glutathione *S*-transferases (Kalgutkar et al., 2012). Depending on the nature and reactivity of the RM, the interaction may be noncovalent (e.g., initiation of redox) or may involve covalent modification of macromolecules (proteins, lipids, or DNA). Soft electrophiles (e.g., the quinone imine RM of paracetamol) react predominantly with sulfhydryl groups on reduced glutathione and with cysteine residues on proteins. Highly reactive "hard electrophiles," such as aldehydes and imines, bind preferentially to hard nucleophile sites on DNA and proteins but not to glutathione and other soft nucleophiles. Both hard and soft electrophile RMs can cause cytotoxicity, and the underlying mechanisms are complex. These include redox reactions, organelle injury that arises due to protein adduct formation, and activation of innate immune responses (Kalgutkar et al., 2012).

There is a large amount of circumstantial evidence that most iDILI is also caused by RMs. However, testing hypotheses is much more difficult for iDILI than for direct cytotoxicity; therefore, their role is unproven. The liver is the predominant site of metabolism of most xenobiotics; hence, it is the organ most commonly responsible for RM formation. Furthermore, many drugs that cause IDRs have been shown to be metabolized to RMs (Kalgutkar et al., 2005). This could be an important reason why the liver is the target organ affected by many IDRs. Perhaps the most compelling evidence that RMs may play an important mechanistic role has been provided by the demonstration of drug-related adaptive immune responses in patients with iDILI caused by numerous drugs, but not in drug-treated patients who do not exhibit iDILI. The immune responses include serum autoantibodies to P450s that metabolize the drugs and to other liver proteins, antibodies to drug adduct-modified liver proteins, and drug-specific T-cell responses (Kim and Naisbitt, 2016).

**Types of Assay.** Although some RMs are sufficiently stable to be detectable in standard drug metabolism studies (e.g., carboxylic acid–derived acyl glucuronides), most RMs are highly chemically reactive and thus have very short half-lives. Detection of chemically stable metabolites may suggest a metabolic route that proceeds via a reactive intermediate (e.g., aromatic hydroxylation, which often is due to initial epoxide formation). Furthermore, time-dependent inhibition of P450 activity is most frequently caused by RM formation (Riley et al., 2007). The method used most commonly to detect RMs is via detection of adducts formed after their reactivity with nucleophiles. A variety of suitable analytical methods have been described (Kenna and Thompson, 2016). Typically, these use liver microsomes (which contain most drug-metabolizing enzymes) or liver S9 (which contains microsomes plus cytosolic enzymes) to generate the RM. Soft electrophile RMs can be trapped using glutathione, cysteine, or *N*-acetyl cysteine, whereas hard nucleophile RMs can be trapped with methoxylamine or cyanide. The data are not quantitative unless suitable authentic reference standards are available. Quantification can be undertaken by use of radiolabeled or fluorescently labeled trapping chemicals; however, this requires a large amount of resources and is not suitable for high-throughput screening.

The most robust method for quantification of RM formation is to study covalent binding (CVB) of radiolabeled test drugs to liver proteins. CVB studies with microsomes or liver S9 enable investigation of cofactor dependence, which is indicative of the likely responsible enzyme (e.g., NADPH for P450s or flavin monooxygenases). Hepatocytes express a broader and more physiologically relevant variety of enzymes and can be exposed to test compounds for long time intervals (up to 24 hours or more), enabling evaluation of drugs that exhibit poor in vitro metabolic turnover.

**Evidence.** Numerous studies have shown that quantitative in vitro CVB data obtained using liver microsomes and hepatocytes cannot differentiate between drugs that cause iDILI, or other IDRs, and nontoxic drugs. However, when daily drug dose was also considered, a correlation between the amount of in vitro CVB to human hepatocyte proteins and IDR risk (predominantly due to iDILI) was observed. This was first described by Nakayama et al. (2009), who evaluated 12 drugs that had been either withdrawn or given a U.S. Food and Drug Administration black box warning for IDR risk, 18 drugs that had received IDR cautionary labeling, and 12 drugs that had not caused IDRs. Ordinal regression analysis of data on human hepatocyte CVB and their therapeutic doses enabled classification of the 42 drugs into three zones; for 30 of the drugs (71%), these correlated well with their IDR classifications. When a similar analysis was undertaken using human liver microsome CVB data, a good correlation with IDR risk was not observed (Nakayama et al., 2009). Another study also found that human liver microsome CVB studies provided a less clear differentiation between safe drugs and drugs that cause iDILI and other IDRs (Obach et al., 2008).

The correlation between dose-adjusted in vitro human hepatocyte CVB results and IDR risk observed by Nakayama et al. (2009) was confirmed and extended by Thompson et al. (2012), who evaluated 36 drugs. These investigators calculated CVB burden values for each drug, which took account of CVB, the fraction of the drug that underwent metabolic turnover in vitro, and the human daily therapeutic drug dose. For 23 of the drugs, CVB burden was ≥0.1 $\mu$mol/day; 21 of these drugs (91%) caused IDRs (predominantly iDILI). The drugs for which CVB burden was <0.1 $\mu$mol/d comprised seven safe drugs and six drugs which caused IDRs. In this study, the correlation between in vitro signals and IDRs was improved markedly when in vitro cell cytotoxicity and bile salt export pump (BSEP) inhibition data were also analyzed (combined IDR sensitivity, 100%; specificity, 78%). Thompson et al. (2012) proposed that these data suggest that the IDRs caused by many of the

tested drugs can arise via multiple contributory mechanisms. Further support for this hypothesis was provided subsequently when the same investigators analyzed CVB burdens, in vitro cell cytotoxicity, BSEP inhibition, and mitochondrial injury caused by three endothelin receptor antagonists (Kenna et al., 2015). Sitaxentan was withdrawn during drug development due to severe iDILI and exhibited strong signals in all of the assays. Bosentan has a DILI black box warning and exhibited modest CVB burden as well as in vitro BSEP inhibition. Ambrisentan does not cause iDILI, although its systemic exposure is higher than that of bosentan, but it exhibited no signals in any of the other assays. Overall, it appears that the formation of a RM is a liability in a drug candidate. However, some drugs such as ximelagatran, which is associated with a high incidence of iDILI (i.e., quite frequent ALT elevations in patients), do not appear to form a RM, and other drugs that form a significant amount of RM are relatively safe. Therefore, additional drug-associated factors are likely important determinants of risk.

## Mitochondrial Injury

**Rationale with Comparison with Clinical Characteristics.** Mitochondria are the principal energy source for cells. In addition, they are also to a large degree responsible for controlling cell death. Therefore, damage to mitochondria is likely to have serious consequences. It has been proposed that mitochondrial injury is a common mechanism of iDILI (Pessayre et al., 2012). There is convincing evidence that mitochondria are a major target of acetaminophen-induced liver injury (Hinson et al., 2010). Presumably the reason that the liver is the major organ damaged by acetaminophen is because the toxicity is mediated by the reactive imidoquinone metabolite, which is formed in the liver. Other drugs also cause liver injury involving mitochondrial injury. Specifically, linezolid inhibits mitochondrial protein synthesis and prolonged treatment with linezolid leads to liver injury with lactic acidosis (De Bus et al., 2010). However, the injury is not limited to the liver, and linezolid more commonly causes anemias and neuropathies (Vazquez et al., 2016). Fialuridine inhibits mitochondrial DNA synthesis and causes liver failure with lactic acidosis and steatosis (McKenzie et al., 1995). In addition, patients treated with fialuridine developed peripheral neuropathies, muscle pain, pancreatitis, and/or thrombocytopenia. Other nucleoside-type agents used to treat human immunodeficiency virus infections also can cause mitochondrial injury because they inhibit mitochondrial DNA synthesis; however, these drugs do not cause the same severity of injury as fialuridine. Although the severity varies between patients, these adverse reactions are not really idiosyncratic. The only iDILI for which there is substantive evidence that mitochondria are the target is valproate-induced liver injury. Valproate-induced liver injury is usually associated with microvesicular steatosis and lactic acidosis (Zimmerman, 1999). In addition, a major risk factor is a polymorphism in mitochondrial DNA polymerase (Stewart et al., 2010). Valproate-induced liver injury is also unique in that the incidence is much higher in infants than in adults. Another possible example of iDILI involving mitochondria is etomoxir, the therapeutic mechanism of which is to irreversibly inhibit the enzyme that is required for transfer of fatty acids from the cytosol to the intramembrane space of the mitochondria. However, it is not clear that it is idiosyncratic. Specifically, one dose of etomoxir caused an increase in ALT in rats, although it appeared to resolve with continued treatment (Vickers, 2009). Clinical development of this agent was terminated because of hepatotoxicity.

Another mechanism by which a drug could cause liver injury involving mitochondria is uncoupling oxidative phosphorylation. At one time, dinitrophenol (the classic oxidative phosphorylation uncoupling agent) was sold for weight loss. Dinitrophenol led to the death of several people, but there did not seem to be any cases in which it led to significant liver injury. There is no substantive evidence that drugs can cause iDILI via uncoupling oxidative phosphorylation.

**Types of Assay.** The most common assay is the glucose-galactose assay, in which toxicity to cells is compared between cells cultured in glucose to those cultured in galactose (Marroquin et al., 2007). Cells cultured in galactose are dependent on mitochondria for ATP production, but in glucose, energy can be produced by glycolysis and therefore the cells are less sensitive to mitochondrial damage. The cells commonly used for this assay are HepG2 cells, which have very limited drug-metabolizing capacity. The common endpoint used in the assay is the mitochondrial membrane potential as determined by a fluorescent probe. Another common assay is the oxygen uptake assay. If a drug candidate inhibits the mitochondrial electron transport chain, it will decrease the rate of oxygen uptake. The production of lactic acid can also be measured. Again, HepG2 cells are the most commonly used cells for the assay.

Although it is possible to measure mitochondrial DNA and mitochondrial protein synthesis, or inhibition of fatty acid metabolism, such assays are not commonly used. It is sometimes possible to guess from the structural features of a drug whether it would inhibit mitochondrial DNA synthesis (nucleoside analogs) or fatty acid metabolism (carboxylic acids branched in the $\beta$-position).

**Evidence.** Virtually all of the studies of mitochondrial injury are performed in vitro, often using concentrations of the drug much higher (100 times $C_{max}$) than therapeutic concentrations as discussed above, and this is likely to lead to a high risk of false-positive results. There is one in vivo study, which involved the use of mitochondrial $SOD^{+/-}$ mice (Ong et al., 2007); however, others have not been able to reproduce the results that were reported in this study. If mitochondrial injury is a common primary iDILI mechanism, it is not clear why it never appears to occur in vivo in animals, even at very high doses. As mentioned above, with the exception of valproate-induced DILI, the few examples of liver injury where there is evidence of mitochondrial involvement are not idiosyncratic. The characteristics of typical iDILI are inconsistent with the characteristics of mitochondrial injury. Specifically, typical iDILI is not associated with lactic acidosis or microvesicular steatosis. Phenformin and metformin do inhibit complex I of the mitochondrial electron transport chain and often cause lactic acidosis (Dykens et al., 2008), but they rarely, if ever, cause iDILI. A major reason that phenformin is less safe than metformin and was withdrawn is that it is metabolized by CYP2D6, which is polymorphic; therefore, poor metabolizers are at increased risk (Oates et al., 1983). In contrast, the major mode of clearance of metformin is renal; therefore, it is easier to predict who is at increased risk of metformin-induced lactic acidosis, and its use is contraindicated in patients with renal failure. It is possible that these drugs could cause cell stress and although they do not directly cause iDILI, they could increase the risk of coadministered drugs. However, metformin is a very commonly used drug for the treatment of type II diabetes, a population that takes a large number of drugs, and there is no evidence that metformin increases the iDILI risk of coadministered drugs.

A combination of rotenone and isoniazid was found to be toxic to hepatocytes in vitro at concentrations that individually did not cause toxicity (Lee et al., 2013). Rotenone is the classic agent associated with the inhibition of complex I, although it has very low toxicity in mammals. The inhibition of complex I by rotenone is somewhat different from the inhibition by metformin, because it is essentially irreversible and causes an increase in reactive oxygen species (Siddiqui et al., 2013). Therefore, rotenone and related agents could be more liable to cause iDILI than reversible inhibitors such as metformin. Isoniazid appears to inhibit complex II of the mitochondrial electron transport chain, possibly through its hydrazine metabolite. Isoniazid is associated

with a relatively high incidence of iDILI. There is evidence that isoniazid iDILI is immune mediated (Metushi et al., 2011), but mitochondrial injury could also play a role. We tested this hypothesis in vivo. The combination of rotenone and isoniazid was fatal when administered at doses of the two compounds that did not cause significant toxicity when they were administered individually (Cho and Uetrecht, 2018). Even though the combination was fatal at about 8 days, death appeared to be due to general metabolic failure, and there was no evidence of significant liver injury. We can unmask the ability of isoniazid to cause liver injury by inhibition of immune tolerance as discussed below. However, in this model, addition of rotenone at slightly lower doses that were not fatal did not significantly increase the severity of the isoniazid-induced liver injury. This strongly suggests that inhibition of the electron transport chain does not play a significant role in the mechanism of liver injury caused by isoniazid in vivo.

Therefore, the most common types of mitochondrial injury assays, which measure effects on the mitochondrial electron transport chain, are unlikely to be useful for predicting the risk that a drug candidate is likely to cause iDILI. The published results that claim predictive ability have important limitations. It is understandable that there would be false negatives—that is, the drug is classified as safe when tested for mitochondrial injury, yet it is associated with an unacceptable iDILI risk because iDILI is caused by some other mechanism(s). However, there should not be many false positives—that is, if inhibition of mitochondrial function is important, and the drug is found to inhibit mitochondrial function, then it should be associated with iDILI risk. Yet one study lists gentamicin as causing mitochondrial injury (Porceddu et al., 2012) even though it essentially never causes iDILI. This article claims that in vitro mitochondrial injury has a very high iDILI predictive value, yet the drug concentrations tested were very high (100 times $C_{max}$). Some of the drugs tested in this study are administered topically in humans, which further complicates risk assessment. In addition, there is little correlation between the potency of mitochondrial injury and the degree of clinical risk (e.g., simvastatin would be predicted to have a higher risk of iDILI than isoniazid). As mentioned earlier, although it is likely impossible to determine the exact incidence of severe iDILI (i.e., acute liver failure), it is important to be able to differentiate drugs in which the incidence is unacceptable (for most classes of drugs, 1/1000–1/10,000) from drugs for which there are a few isolated reports but the severe liver injury risk is acceptable (<1/100,000). Many of the drugs that tested positive in these assays are cytotoxic anticancer drugs such as chlorambucil, but, they rarely cause liver injury. In the study by Porceddu et al. (2012), it is unclear which of the tested compounds were categorized as negative but it appears that they are agents such as taurine, saccharin, glucose, folic acid, and caffeine. Since few safe drugs were tested, it is possible that the compound concentrations were so high that almost all drugs were positive, which might simply correlate with the physical properties of the drug. In another highly quoted article by Aleo et al. (2014), in which both BSEP inhibition and "Mitotox" were evaluated, few of the tested drugs were associated with the highest IDILI risk, and none of the tested drugs had an Mitotox $IC_{50}$ lower than the $C_{max}$. Data which demonstrate that mitochondrial injury assays can discriminate between pairs of similar drugs which differ markedly in IDILI risk, yet do not exhibit other iDILI liabilities, have not been reported.

In summary, the clinical characteristics of iDILI are generally inconsistent with the characteristics of the toxicity caused by agents that cause mitochondrial damage. Virtually all of the studies that suggest that mitochondrial injury is important for the mechanism of iDILI are in vitro, and it does not appear to be possible to reproduce iDILI in animals with drugs that inhibit mitochondrial function. Furthermore, the currently available data do not provide compelling evidence that mitochondrial injury provides useful discrimination between drugs that cause iDILI and safe drugs, because many of the tested drugs exhibit additional liabilities. Although mitochondrial injury may be involved in the mechanism of some iDILI, it seems very unlikely that simple inhibition of the mitochondrial electron transport chain is a primary mechanism; therefore, the usual mitochondrial assays are considered unlikely to predict clinical iDILI risk.

## BSEP Inhibition

**Rationale with Comparison with Clinical Characteristics.** BSEP, which is an ATP-dependent membrane transport protein expressed on the apical plasma membrane domain, excretes bile acids from hepatocytes into bile (Lam et al., 2010). Several genetically inherited human liver diseases arise due to genetically inherited mutations in the gene that encodes BSEP (*ABCB11*), which result in reduced transporter expression or activity. The most severe is primary familial intrahepatic cholestasis type 2 (PFIC2), where complete loss of BSEP function causes severe cholestatic liver damage (Strautnieks et al., 1998). PFIC2 is first evident in young children, develops progressively over many years, and eventually is fatal in most patients unless treated by liver transplantation. Less functionally severe *ABCB11* mutations cause nonprogressive cholestasis (benign recurrent intrahepatic cholestasis type 2, and intrahepatic cholestasis of pregnancy) (Lam et al., 2010). The first studies undertaken in *Abcb11*$^{-/-}$ (the rodent ortholog of the human gene) knockout mice revealed that liver injury was not observed unless the animals were fed a diet enriched in bile acids (Wang et al., 2009). The *Abcb11*$^{-/-}$ mice expressed markedly higher expression of hepatic bile acid enzymes and other hepatobiliary efflux transporters than wild-type mice. Subsequently, a mouse strain that does not exhibit upregulation of bile acid–metabolizing enzymes or other haptic transporters was developed by backcrossing the original *Abcb11*$^{-/-}$ strain with wild-type mice for 10 generations (Zhang et al., 2012). These *Abcb11*$^{-/-}$ mice developed progressive cholestatic liver injury, and they exhibited plasma chemistry and liver histopathology abnormalities that were similar to human PFIC2, even when fed a normal diet (Zhang et al., 2012). The mechanisms by which defective BSEP function causes liver injury in humans and rodents are complex, and involve impaired fatty acid oxidation and inflammatory responses that are triggered by elevated bile acid concentrations (Zhang et al., 2012; Li et al., 2017).

Most of the drugs that have been implicated as causing iDILI by inhibiting BSEP do not cause liver injury similar to the liver disease caused by a BSEP genetic deficiency. Specifically, the liver injury associated with a genetic deficiency in BSEP is characterized by a cholestatic pattern with an increase in alkaline phosphatase, but the γ-glutamyl transpeptidase is normal in most cases (Whitington et al., 1994). In contrast, most drugs that have been implicated in BSEP-mediated iDILI (e.g., bosentan, troglitazone, ketoconazole, and tolcapone) cause predominantly hepatocellular liver injury rather than cholestatic injury. Many of these drugs are metabolized to RMs and/or exhibit other iDILI liabilities (Thompson et al., 2012; Kenna et al., 2015). Therefore, it seems unlikely that such drugs cause iDILI primarily through BSEP inhibition. However, the increased levels of bile salts caused by these drugs could cause cell injury that helps to induce an immune response. In fact, increased levels of bile salts do cause inflammation with activation of inflammasomes and release of inflammatory cytokines such as interleukin-17 (Li et al., 2017).

**Types of Assay.** The most widely used method for investigation for BSEP inhibition by drugs is to determine their effect on ATP-dependent vesicular uptake of a probe substrate (most commonly, [$^3$H]-taurocholic acid) into inside-out membrane vesicles that express the transporter. Membranes prepared from insect-derived *Spodoptera frugiperda* 9 or

21 (sf9 or sf21) cell lines transfected with a baculovirus vector that contains the *ABCB11* gene sequence are used most frequently, because they can be produced in large amounts and are commercially available. The different cholesterol content of insect and mammalian cell membranes has not been found to affect the inhibitory potencies or affinities of a variety of test drugs (Kis et al., 2009). Testing of a range of substrate concentrations enables determination of the $IC_{50}$. Typically, the assay is undertaken only at a single substrate concentration, which is suitable for determination of competitive BSEP inhibition. However, some drugs and their metabolites exhibit noncompetitive BSEP inhibition, which can be quantified only when a range of substrate concentrations are used (Yang et al., 2014).

BSEP inhibition can also be quantified using isolated hepatocytes cultured in vitro in sandwich configuration hepatocytes (SCHs), which exhibit polarized membrane transporter expression that is similar to that observed in vivo (Swift et al., 2010). SCHs are preincubated in buffer with or without calcium, which is required to maintain tight junctions between adjacent hepatocytes, and then incubated briefly with a suitable BSEP probe substrate in the absence and presence of test drug. Uptake into SCHs is mediated by sinusoidal plasma membrane uptake transporters. Efflux from SCHs into media is mediated by BSEP, which is expressed on the apical plasma membrane domain, and by efflux transporters expressed on the sinusoidal domain. Excretion from SCHs into calcium-containing buffer is due to sinusoidal transport, not BSEP activity, whereas excretion into calcium-free media is due to both sinusoidal transport plus BSEP activity. Therefore, studies undertaken in SCHs enable investigation of effects of test drugs on multiple hepatic transporters to be explored in a physiologically relevant cell model (Swift et al., 2010). SCHs can also be used in investigatory studies to explore potential transporter-mediated regulatory mechanisms and possible inhibitory effects of drug metabolites that are formed in the cells. However, SCHs have lower throughput and are more technically challenging than BSEP membrane vesicle assays; therefore, SCHs are less suitable for high-volume compound screening.

**Evidence.** Several studies have shown that in vitro BSEP $IC_{50}$ values determined using vesicle assays can distinguish between drugs that cause human iDILI and drugs that do not, with only modest sensitivity (approximately 50%) but with moderately high specificity (70%–80%) (Dawson et al., 2012; Morgan et al., 2013; Yucha et al., 2017). Calculating the ratio between the total steady-state plasma drug concentration ($C_{ss,plasma}$) and BSEP $IC_{50}$ further reduced the number of false positives. Drugs that exhibited a $C_{ss,plasma}$/BSEP $IC_{50} > 0.1$ and were administered systemically for prolonged durations caused human iDILI (Morgan et al., 2013; Yucha et al., 2017). A more biologically relevant in vivo exposure parameter is the unbound drug (or inhibitory metabolite) concentration in hepatocytes at the site of interaction with BSEP in patients. However, unbound plasma drug concentrations have not been found to be suitable for exposure adjustment of BSEP $IC_{50}$ data. This could be because drug concentrations in plasma do not accurately reflect drug concentrations within hepatocytes in vivo, which for many drugs are many fold higher, and/or because the unbound drug concentrations were not determined and could have been lower than expected, perhaps due to binding the membrane vesicles or to the wells of the assay plates.

Physiologically based pharmacokinetic modeling has provided additional support for the proposal that BSEP inhibition is an iDILI risk factor. When combined with in vitro BSEP inhibition data and experimentally determined bile acid cytotoxicity potency values, physiologically based pharmacokinetic modeling–based simulations of in vivo exposure to troglitazone and its sulfated metabolite provided good predictions of the frequency and time of onset of liver injury (plasma elevation) observed in clinical trials (Yang et al., 2014). Similar modeling studies indicated that BSEP inhibition is a plausible explanation for iDILI caused by tolvaptan (Woodhead et al., 2017a), whereas lixivaptan was correctly predicted to be less likely to cause DILI than tolvaptan (Woodhead et al., 2017b).

Currently, it is not possible to predict whether BSEP inhibition in an individual patient will cause iDILI or whether acute liver failure may arise. This is likely to be because nonsusceptible individuals can mount adaptive protective responses, which currently are poorly understood, and because iDILI arises via multiple mechanisms. Numerous drugs that inhibit BSEP and cause DILI also exhibit other liabilities, particularly RM formation and/or mitochondrial injury (Thompson et al., 2012; Kenna et al., 2015). It is important to take into account the complexity of DILI when translating in vitro BSEP inhibition data to the design and selection of safe drugs.

A recently published reanalysis of previously published data on BSEP inhibition by drugs showed that most BSEP inhibitors can be placed in Biopharmaceutics Drug Disposition Classification System class 2 (highly metabolized, poorly soluble) (Chan and Benet, 2018). In this publication, the proposed relevance of BSEP inhibition as a DILI risk factor was criticized due to the poor iDILI sensitivity of the approach. Instead, it was proposed that the Biopharmaceutics Drug Disposition Classification System could be a more useful approach to use when designing new drugs. This publication has highlighted an important limitation of BSEP inhibition, or indeed any individual in vitro assay approach, if used and interpreted in isolation. Other factors that would not be reflected in common BSEP inhibition assays are the contribution of metabolites to BSEP inhibition (e.g., troglitazone sulfate) (Funk et al., 2001), and the ability of a drug to inhibit compensatory mechanisms that mitigate inhibition of BSEP.

Possibly the best evidence that BSEP inhibition can play a role in the mechanism of iDILI is provided by Fattinger et al. (2001). They found that when glyburide, a drug that inhibits BSEP but rarely causes iDILI, was combined with bosentan, it significantly increased the level of serum bile salts and the incidence of liver injury. Given the safety of drugs such as glyburide and pioglitazone and the different characteristics of the liver injury caused by drugs and the characteristics of liver injury associated with a genetic deficiency of BSEP, it is unlikely that BSEP inhibition alone is a common mechanism of iDILI. However, it is quite plausible that inhibition of BSEP contributes to the risk that a drug candidate will cause iDILI. This also emphasizes the need for improved understanding of iDILI mechanisms and risk factors.

### Immune Response Assays

**Rationale with Comparison with Clinical Characteristics.** It seems obvious that the development of predictive assays should be based on knowledge of the mechanism of iDILI. Although our understanding of the mechanisms of iDILI is superficial, there is a large amount of evidence that iDILI caused by many drugs is immune mediated. This includes the general characteristics of iDILI, which are similar to the characteristics of other types of IDRs for which the evidence of an immune mechanism is even clearer, as well as histology, positive lymphocyte transformation tests, antidrug antibodies, and human leukocyte antigen (HLA) associations. Such evidence is hard to acquire so it is not available for most iDILI cases; therefore, we have to extrapolate from cases in which we do have such data to other cases. This is because no one clinical center will have a large number of iDILI cases, lymphocyte transformation tests require expertise in the method, finding antidrug antibodies requires knowledge of the RM involved and making the appropriate antigen, and HLA associations requires a large number of cases and the appropriate controls. Even if iDILI is ultimately immune mediated, other factors such as cell stress caused by BSEP inhibition

could play a role in the induction of an immune response as mentioned above. In general, attempts to develop animal models of iDILI with characteristics similar to iDILI in humans by stimulating the immune system along with treatment of animals with drugs that cause iDILI in humans have failed (Ng et al., 2012). Although this might seem surprising, it fits with the clinical picture in which patients with inflammatory conditions such as preexisting liver disease or inflammatory bowel disease do not have a marked increase in iDILI risk (Zimmerman, 1999). Even immunization with drug-modified proteins and adjuvant prior to drug treatment, which led to an increase in regulatory T cells and myeloid-derived suppressor cells, paradoxically decreased liver injury (Mak and Uetrecht, 2015a).

In humans treated with drugs that cause iDILI, mild liver injury that resolves with continued drug treatment (referred to as adaptation) occurs much more frequently than symptomatic liver injury. This observation is the basis for Temple's corollary (i.e., if a drug does not cause a detectable incidence of mild liver injury, it is very unlikely to cause severe liver injury) (Watkins et al., 2011). If the liver injury is immune mediated, then the adaptation must involve immune tolerance. Tolerance is the dominant immune response of the liver. Consistent with this hypothesis, impairment of immune tolerance by blocking immune checkpoints led to the first animal model of iDILI with characteristics very similar to those of human iDILI. Specifically, treatment of programed cell death protein-1 (PD-1) knockout mice with anticytotoxic T lymphocyte–associated protein-4 (CTLA-4) antibodies along with amodiaquine, which is associated with a relatively high incidence of iDILI in humans, led to a delayed-onset liver injury with liver histology consisting of a mononuclear leukocyte infiltrate and piecemeal necrosis (Metushi et al., 2015). The injury in this model was mediated by CD8 T cells (Mak and Uetrecht, 2015b). This model was able to unmask the ability of other drugs to cause liver injury, although the injury was milder (Mak and Uetrecht, 2015c). It was also able to differentiate drugs associated with a relatively high incidence of iDILI from those that are relatively safe (Mak et al., 2018). This impaired immune tolerance model supports the hypothesis that iDILI is immune mediated, provides a model to test mechanistic hypotheses, and has the potential to predict which drug candidates are likely to cause a relatively high incidence of iDILI. A summary of the postulated mechanism of iDILI is presented in Fig. 2.

Although polymorphisms in molecules involved in immune tolerance have been associated with an increased risk of iDILI (Pachkoria et al., 2008), clearly most patients who have IDRs including iDILI do not have the degree of impaired immune tolerance as in the PD-$1^{-/-}$ animal model. However, the immune response is always a balance between a strong immune response, which can clear pathogens but also cause tissue damage, and immune tolerance, which decreases tissue damage but may prevent pathogen clearance. Presumably, patients who have severe IDRs have a stronger immune response because of genetic differences and, probably more important, exposure to pathogens that crossreact to a drug or drug-modified protein and lead to a strong immune response (Cho and Uetrecht, 2017). The crossreactivity between two structurally different antigens is referred to as heterologous immunity (Welsh and Selin, 2002). Factors that influence the balance between adaptation and iDILI are illustrated in Figs. 1 and 2.

If the basic mechanism of most iDILI is an immune response stimulated by the drug, or drug metabolites, then it follows that the best test to predict the risk that a drug will cause iDILI would be to test the immune response to the drug and relevant metabolites (most notably, RMs). Although most RMs cannot be tested directly because of their reactivity, the system used should have the enzymes required to generate significant amounts of RMs. Although most patients do not have a clinically evident IDR when treated with a drug, there may be an immune response. For example, most patients who are treated with penicillin develop antipenicillin antibodies but in most cases they are IgG antibodies and there is no adverse reaction (Strannegård et al., 1987). Furthermore, almost all patients treated with procainamide for an extended period of time develop autoantibodies, but most do not develop a clinical autoimmune syndrome (Woosley et al., 1978). Likewise, although clozapine causes agranulocytosis with an incidence of $<1\%$ (and less frequently iDILI), most patients treated with clozapine have an immune response to the drug with an increase in interleukin-6 and tumor necrosis factor-$\alpha$, which resolves with continued treatment (Pollmächer et al., 1996). This subclinical immune response is likely a precursor to the development of agranulocytosis and iDILI, but in most patients it resolves with immune tolerance. This is similar to the adaptation that occurs much more commonly that serious iDILI, as discussed above. It is quite plausible that most patients have a subclinical immune response to most drugs that cause iDILI, or other IDRs. A subclinical immune response that is limited to the liver may be hard to detect in humans, but it may be possible to detect in animals in which the liver can be sampled. It could be argued that a specific HLA gene is required for an immune response; however, strong HLA associations have not been identified for most IDRs. Furthermore, although HLA associations have been identified for clozapine-induced agranulocytosis (Chowdhury et al., 2011), most patients have an immune response to the drug as mentioned above. That suggests that a specific HLA association is not required for a milder immune response, but rather is associated with a stronger immune response that does not resolve with immune tolerance and results in agranulocytosis. Exposure to other immunogens and pathogens also shapes the immune response to a drug as discussed above, and this is likely an important factor that influences what immune responses will lead to a significant adverse reaction.

**Types of Assay.** It has been reported that treatment of Sprague-Dawley rats with a combination of lipopolysaccharide and ranitidine caused liver injury at doses that individually produced no injury (Luyendyk et al., 2003). This is referred to as the inflammagen model. However, the injury occurred within 6 hours, and liver histology was characterized by an infiltration of neutrophils. In contrast, iDILI is characterized by a delay in onset and a mononuclear leukocyte infiltrate. In addition, although there have been rare reports of liver injury associated with the use of ranitidine, it is a safe over-the-counter drug. Furthermore, as mentioned earlier, inflammation such as ulcerative colitis is not associated with an increased risk of iDILI, and we have not been able to develop an animal model by stimulation of the immune system. Therefore, this animal model is not likely to represent a true model of iDILI. This is not an in vitro assay, but it is the basis for the cytokine/drug cytotoxicity synergy assay described below.

It has been suggested that inflammatory cytokines such as tumor necrosis factor-$\alpha$ and interferon-$\gamma$ increase the cytotoxicity of drugs, and this is responsible for iDILI. This led to the cytokine/drug cytotoxicity synergy assay (Maiuri et al., 2017). Mechanistically, this is not plausible. In most cases, the induction of an immune response appears to be due to drug-modified proteins, and antibodies against such proteins have been described (Bourdi et al., 1996; Metushi et al., 2014). Although it is more likely that cytotoxic T cells mediate most of the injury, the antidrug antibodies are a clue to how the immune response was initiated. The synergy assay utilizes HepG2 cells, which do not have significant drug-metabolizing capacity. It is unlikely that direct drug toxicity is responsible for hepatocyte death in iDILI, and there are no T or B cells in the assay that could duplicate the mechanism of injury that occurs in patients. Certainly, high levels of cytokines can be toxic and are likely involved in the mechanism of iDILI but without any other parts of the immune system in the assay (in particular, T or B cells specific for the drug or more likely drug-modified proteins as observed in a lymphocyte transformation test), it is very unlikely that this relates to the mechanism of iDILI.

As mentioned above, treatment of PD-1$^{-/-}$ mice with anti–CTLA-4 antibodies unmasked the ability of drugs to cause liver injury (Mak and Uetrecht, 2015c). With amodiaquine the injury was severe; specifically, although the serum ALT elevation was modest, this was extended was modest, it was extended over a period of weeks and was accompanied by piecemeal necrosis and decreased liver function (Metushi et al., 2015). The amount of injury was not simply a function of the level of serum ALT elevation but rather the level of ALT elevation integrated over a period of time. Patients who develop iDILI leading to liver failure often initially exhibit modest but sustained serum ALT elevations. In contrast to amodiaquine, liver injury caused by other drugs in the PD-1$^{-/-}$ mouse model was mild and resolved with extended treatment. Nonetheless, the model was able to differentiate drugs that cause iDILI in humans from those that do not (Mak et al., 2018). As with the lipopolysccharide-treated rat imflammagen model, this is not an in vitro model and in this case requires several weeks to carry on the test. However, this model can provide a good basis for testing mechanistic hypotheses as described above and with a better mechanistic understanding, it should be possible to develop better in vitro assays to predict iDILI risk.

It is likely that one mechanism by which drugs induce an immune response that can lead to iDILI is if the immune response overcomes immune tolerance by causing the release of danger-associated molecular patterns (DAMPs) that activate antigen-presenting cells. We found that the supernatant from a human hepatocyte spheroid culture with nevirapine was able to activate inflammasomes in THP-1 cells (a human monocyte cell line) (Kato and Uetrecht, 2017). Adding aminobenzotriazole to inhibit P450 in the hepatocyte culture prevented the response to the supernatant, which is consistent with the response being caused by a RM. This assay was also able to differentiate the iDILI potential of troglitazone and pioglitazone (Mak et al., 2018). If the molecules released by hepatocytes that activate antigen-presenting cells (presumably DAMPs) can be identified, it would simplify the assay; however, different drugs may cause the release of different types of DAMPs. The experience with this test is very limited; therefore, it is currently impossible to know how useful it may be.

Another recent assay involved the activation of T cells from a panel of human donors (Usui et al., 2018). Specifically, blood was drawn from multiple normal donors and their HLA genotype was determined. Monocytes were separated and cultured in specialized medium for 7 days to generate dendritic cells. These cells were then cocultured with autologous T cells and various drugs, and the release of various cytokines was determined. Several of the drugs caused activation of T cells only from donors with a specific HLA genotype, and most of the drugs that were studied are chemically reactive so they do not require bioactivation. Exceptions were carbamazepine and oxypurinol, a metabolite of allopurinol. It has been proposed that carbamazepine-induced iDILI is caused by a RM (Pearce et al., 2005), but the parent drug may be able to induce an immune response. In the case of allopurinol, no RM has been identified. This is a very interesting mechanistic study; but given the limitations that many drugs require a specific HLA genotype and probably also bioactivation, the method does not readily lend itself to drug candidate screening.

**Evidence.** It is logical that if iDILI is immune mediated, a study of the immune response to drugs would provide the best way to predict which drugs are likely to be associated with a significant risk of iDILI. However, there are far too little data to know how accurate assays to characterize immune responses to drugs might be for the prediction of iDILI risk. There are many reasons why such assays might provide false-negative predictions. First, such assays are based on the hypothesis that iDILI is immune mediated. Although this seems likely, it is unproven, and we certainly do not know what fraction of iDILI might not be immune mediated. Second, animals are different from humans, and this may lead to a false negative if a drug is metabolized differently in an animal model than in humans. The advantage of the in vitro model based on the release of DAMPs from hepatocytes is that human hepatocytes can be used. However, if the mechanism for DAMP release involves inhibition of transporters involved in bile salt transport, accumulation of bile salts would not occur in vitro. In addition, with a drug such as felbamate in which the first step appears to involve hydrolysis of the carbamate, which is a minor pathway in rodents and occurs outside the liver, neither an animal model nor the in vitro assay is likely to provide an accurate prediction (Dieckhaus et al., 2002). Despite such limitations, more research into the immune response to drugs is likely to be the best strategy for developing better methods to predict iDILI risk.

## Summary and Conclusions

It is likely that a drug, or drug metabolite, can cause iDILI via a large number of different mechanisms. This could explain why although substantial progress has been made in developing better in vitro cell culture systems, we do not have good biomarkers that address the mechanisms involved in iDILI and other IDRs, and no single assay may predict iDILI risk with high accuracy (i.e., specificity and sensitivity). Some endpoints, such as RM formation and BSEP inhibition, appear likely to reflect important parts of the mechanism of some iDILI. Use of assays that evaluate these endpoints during drug discovery should aid in the selection of safer drug candidates. The predictive value of other endpoints, such as inhibition of the mitochondrial electron transport chain, is questionable. The use of more physiologic systems, such as HepaRG spheroids rather than HepG2 cells is likely to improve the predictive value of in vitro assays. However, simply measuring additional biomarkers is unlikely to improve accuracy unless the biomarkers actually reflect a mechanism involved in iDILI, and the in vitro assays adequately reproduce relevant in vivo biology. It is also essential that any mechanistic hypothesis or model be consistent with the characteristics and mechanisms of IDRs in humans. Mechanistic studies of iDILI have been a challenge because of the absence of valid animal models that reflect the mechanisms of IDRs in humans. The use of checkpoint inhibition of immune tolerance appears to be capable of unmasking the ability of a drug to cause immune-mediated liver injury. Animal models based on this strategy are not practical for high-throughput screening; however, they should make it possible to perform mechanistic studies that were not possible in the past. A promising finding is the ability of a supernatant from the incubation of a drug with hepatocytes to activate macrophages as described above; this has the potential to permit in vitro screening of drug candidates. In the future, a better understanding of mechanisms of iDILI, especially the role of immune responses, will likely lead to in vitro assays that more accurately predict iDILI risk. Since many mechanisms are likely to be involved, it may be easier to decrease the risk of false-positive results than false-negative results.

### Authorship Contributions

*Wrote or contributed to the writing of the manuscript:* Kenna, Uetrecht.

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