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

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**Nonstandard abbreviations used in the paper:**

ALT, Alanine aminotransferase
BSEP, Bile Salt Export Pump
$C_{ss}$, total plasma steady state drug concentration
CTLA-4, cytotoxic T lymphocyte-associated-4
CVB, covalent binding
CYP, cytochromes P450
FMOs, flavin monooxygenases
GST, glutathione-*S*-transferase
IDR, Idiosyncratic Drug Reaction
iDILI, Idiosyncratic Drug Induced Liver Injury
LTKB, Liver Toxicity Knowledge Base
PD-1, programed cell death protein-1
RM, reactive metabolite
SOD, superoxide dismutase
TDI, time dependent inhibition
UGT, UDP-glucuronyltransferase

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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 IDR risk. Even cytotoxicity assays that utilize more biologically normal cells resulted in many false positive and 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. 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.

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## 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. Idiosyncratic adverse 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, they 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 know with certainty. One possible hypothesis, which is outlined in Figure 1, is that iDILI arises via a combination of drug-related adverse biological 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 Figure 1), and other types of injury may also influence whether a drug-induced immune response arises. Furthermore, since the mechanisms

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by which drugs cause iDILI in susceptible patients have not been defined, it is currently unclear whether Figure 1 provides a useful overall framework or is over-simplified and conceptually flawed.

To follow is a list of the most common types of assays that have been proposed for use in toxicity screening of drug candidates, and a discussion of 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 is acute liver failure, which arises following 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 de-risking, 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 (raised plasma 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

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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 utilized 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 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 Cmax 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 are 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.

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**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, 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 cytochromes P450 (CYP) enzymes and other drug metabolizing enzymes than hepatocytes in human liver *in vivo,* and typically they do not exhibit polarized plasma membrane transporter expression (e.g. HepG2, FaO, THLE) (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, CYP 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 of individual different human CYP enzymes that are similar to those present in 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 CYP and other drug metabolizing enzymes, plus loss of polarized plasma membrane protein expression. Improved maintenance of cell viability, drug metabolizing enzyme activities, and polarized plasma

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membrane expression can be improved markedly by use of alternative cell culture conditions. The available options include hepatocyte culture in sandwich configuration (i.e. with rigid collagen substratum, plus overlay with a gelled layer of either collagen or Matrigel$^{TM}$); 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* (Goldring et al., 2017; Hewitt et al., 2007). The viability and CYP activities of isolated hepatocytes have also been improved by co-culture of hepatocytes with macrophage-derived accessory cells in bioengineered devices (Underhill and Khetani, 2018) and by formation of *ex-vivo* 3D aggregates of defined size that contain hepatocytes alone, or hepatocytes plus other liver non-parenchymal cell types ("liver spheroids") (Bell et al., 2016).

Toxicities of test drugs have been investigated by evaluation of cell growth inhibition (MTT assays), membrane lysis (release from cells into media of LDH 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 pre-defined 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

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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 USA 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 non-hepatotoxic (Xu et al., 2008). One approach is the Liver Toxicity Knowledge Base (LTKB), which assigns withdrawn drugs and licensed drugs to different DILI classes (most concern, high concern, low concern and no concern), based primarily on information extracted from US FDA 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. Firstly, 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). Secondly, using liver-derived cell lines (HepG2 or THLE) that have low drug metabolizing activity are less likely to provide accurate estimates of iDILI risk than data obtained using liver cell lines that

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express high CYP activities, or isolated primary hepatocytes (Dambach et al., 2005; Garside et al., 2014; Gustafsson et al., 2014). Thirdly, multi-parametric evaluation of multiple cell endpoints by high content cell imaging of isolated hepatocytes provided only modest improvement in sensitivity, when compared with cell cytotoxicity evaluation (Garside et al., 2014). Fourthly, that more physiologically relevant micro-engineered liver co-cultures (Underhill and Khetani, 2018), or 3D hepatocyte spheroids (Proctor et al., 2017) should 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 Css was considered, while 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 so 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.

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## Covalent Binding Assays

**Rationale.** Reactive metabolite (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 CYP enzymes, although other enzymes (e.g. flavin monooxygenases, FMOs) can be involved, and some RMs are formed via conjugation reactions that are mediated by sulfotransferases, UGTs or GSTs (Kalgutkar et al., 2012). Depending upon the nature and reactivity of the RM, the interaction may be non-covalent (e.g. initiation of redox) or 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 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

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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, for example carboxylic acid derived acyl glucuronides, most RMs are highly chemically reactive and so have very short half lives. Detection of chemically stable metabolites may suggest a metabolic route that proceeds via a reactive intermediate, for example aromatic hydroxylation, which often is due to initial epoxide formation. Furthermore, time dependent inhibition (TDI) of CYP 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 following 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, while 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 radiolabelled 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 radiolabelled test drugs to liver proteins. CVB studies with microsomes or liver S9 enable investigation of co-factor dependence, which is indicative of the likely responsible enzyme (e.g., NADPH for CYPs or FMOs). Hepatocytes express a broader and more

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physiologically relevant variety of enzymes and can be exposed to tests compounds for long time intervals (up to 24 hr 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 non-toxic 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., who evaluated 12 drugs that had been either withdrawn or given FDA black box warning for IDR risk; 18 drugs that had received IDR cautionary labeling; and 12 drugs that had not caused IDRs (Nakayama et al., 2009). Ordinal regression analysis of data on human hepatocyte CVB and their therapeutic doses enabled classification of the 42 drugs into three zones, which for 30 of the drugs (71%) correlated well with their IDR classifications. When a similar analysis was undertaken using human liver microsomes 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 drugs that cause iDILI and other IDRs, and safe drugs (Obach et al., 2008).

The correlation between dose adjusted *in vitro* human hepatocyte CVB results and IDR risk observed by Nakayama et al. (Nakayama et al., 2009) was confirmed and extended by Thompson et al., who evaluated 36 drugs (Thompson et al., 2012). 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 human daily therapeutic drug dose. For 23 of the drugs, CVB

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burden was $\geq$ 0.1 µmol/day, and 21 of these drugs (91%) caused IDRs (predominantly iDILI).

The drugs for which CVB burden was < 0.1 µmol/day comprised 7 safe drugs and 6 drugs which

caused IDRs. In this study, the correlation between *in vitro* signals and IDRs was improved

markedly when *in vitro* cell cytotoxicity and BSEP inhibition data were also analyzed (combined

IDR sensitivity 100%, specificity 78%). It was proposed that these data suggests that the IDRs

caused by many of the tested drug 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, plus *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 to Clinical Characteristics.** Mitochondria are the principle 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

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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 with prolonged treatment it

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 caused 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 HIV 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. Even though the severity varies between patients, these adverse reactions

are not really idiosyncratic. The only idiosyncratic DILI 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,

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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. It 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 utilized. 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 ß-position.

**Evidence.** Virtually all of the studies of mitochondrial injury are performed *in vitro*, often using

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concentrations of the drug much higher (100 times Cmax) 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 CYP 2D6, 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 even though they do not directly cause iDILI, they could increase the risk of co-administered 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 co-administered 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.

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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, i.e. 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, i.e. if inhibition of mitochondrial function is important, and the drug is

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found to inhibit mitochondrial function, then it should be associated with iDILI risk. Yet in one paper gentamicin is listed as causing mitochondrial injury (Porceddu et al., 2012) even though it essentially never causes iDILI. This paper 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 degree of clinical risk, e.g. simvastatin would be predicted to have a higher risk of iDILI than isoniazid. Although as mentioned earlier, determining the exact incidence of severe iDILI (i.e. acute liver failure) is likely impossible, it is important to be able to differentiate drugs in which the incidence is unacceptable (for most classes of drugs 1/1,000-1/10,000), 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 this paper it is unclear which drugs were categorized as negative, but it appears that they are agents such as taurine, saccharin, glucose, folic acid, and caffeine (Porceddu et al., 2012). 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 paper by Aleo et al., 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 $C_{max}$ (Aleo et al., 2014). 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, in general, the clinical characteristics of iDILI are inconsistent with the characteristics of the toxicity caused by agents that cause mitochondrial damage. Virtually all of

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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.

## Bile Salt Export Pump (BSEP) Inhibition

**Rationale with Comparison to 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 in most patients eventually is fatal unless treated by liver transplantation. Less functionally severe *ABCB11* mutations cause non-progressive cholestasis (Benign Recurrent Intrahepatic Cholestasis Type 2, and Intrahepatic Cholestasis of Pregnancy) (Lam et al., 2010). The first studies undertaken *Abcb11* (the rodent orthologue 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

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markedly higher expression than wild type mice of hepatic bile acid enzymes and other

hepatobiliary efflux transporters. 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 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 (Li et al., 2017;

Zhang et al., 2012).

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 (Kenna et al., 2015; Thompson et al., 2012).

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 IL-17 (Li et al.,

2017).

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**Types of Assay.** The most widely used method for investigation for BSEP inhibition by drugs is determination of their effect on ATP-dependent vesicular uptake of a probe substrate (most commonly [$^3$H]-taurorocholic 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 non-competitive 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 (SCH), which exhibit polarized membrane transporter expression that is similar to that observed *in vivo* (Swift et al., 2010). SCH are pre-incubated in buffer with or without calcium, which is required to maintain tight junctions between adjacent hepatocytes, then incubated briefly with a suitable BSEP probe substrate in the absence and presence of test drug. Uptake into SCH is mediated by sinusoidal plasma membrane uptake transporters. Efflux from SCH 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 SCH into calcium containing buffer is due to sinusoidal transport, not BSEP activity, while excretion into calcium-free media is due to both sinusoidal transport plus BSEP

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activity. Therefore studies undertaken in SCH enable investigation of effects of test drugs on multiple hepatic transporters to be explored in a physiologically relevant cell model (Swift et al., 2010). SCH also can 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, SCH have lower throughput and are more technically challenging than BSEP membrane vesicle assays, therefore 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 which cause human idiosyncratic DILI and drugs that do not with only modest sensitivity (~50%), but with moderately high specificity (70-80%) (Dawson et al., 2012; Morgan et al., 2013; Yucha et al., 2017). Calculating the ratio between total steady state plasma drug concentration ($C_{ss,plasma}$) and BSEP $IC_{50}$ further reduced the number of false positives. Drugs that exhibited $C_{ss,plasma}$ / BSEP IC50 > 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 (PBPK) 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, PBPK-based simulations of *in vivo* exposure 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), while 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 non-susceptible individuals can mount adaptive protective responses, which currently are poorly understood, and because idiosyncratic DILI arises via multiple mechanisms. Numerous drugs that inhibit BSEP and cause DILI also exhibit other liabilities, in particular reactive metabolite formation and/or mitochondrial injury (Kenna et al., 2015; Thompson et al., 2012). 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 recent published re-analysis of previously published data on BSEP inhibition by drugs has shown that most BSEP inhibitors can be placed in Biopharmaceutics Drug Disposition Classification System (BDDCS) class 2 (highly metabolized, poorly soluble) (Chan and Benet, 2017). 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 BDDCS classification 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

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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 the paper by Fattinger et al.(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 to 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 evidence 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, histology, positive lymphocyte

transformation tests, anti-drug antibodies, and 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. For example, no one clinical center will have a large

number of cases, and lymphocyte transformation tests require expertise in the method, finding

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anti-drug antibodies requires knowledge of the reactive metabolite 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 Tregs 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, always 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 anti-cytotoxic 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

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(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 Figure 1.

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 cross react to a drug or drug-modified protein and lead to a strong immune response (Cho and Uetrecht, 2017). The cross reactivity 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 Figure 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 utilized should have the enzymes required to generate significant amounts of RM. Although most

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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 anti-penicillin

antibodies, but in most cases they are IgG antibodies and there is no adverse reaction

(Strannegård et al., 1987). Another example is the fact that almost all patients treated with

procainamide for an extended period of time develop autoantibodies, 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 IL-6 and TNF-$\alpha$, which

resolves with continued treatment (Pollmacher 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 where 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 shape 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

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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 TNF-$\alpha$ and IFN-$\gamma$ 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 anti-drug 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

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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 ALT was modest, it was extended over a period of weeks

and led to piecemeal necrosis and decreased liver function (Metushi et al., 2015). The amount of

injury is not simply a function of the level of ALT but rather the level of ALT integrated over a

period of time. Patients who develop iDILI leading to liver failure often have modest ALT

levels, but it is sustained.  In contrast to amodiaquine, liver injury caused by other drugs in this

model was mild and resolved with extended treatment. However, the model was able to

differentiate drugs that cause iDILI in humans from those that do not (Mak et al., 2018). As with

the inflammagen 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 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 CYP in the

hepatocyte culture prevented the response to the supernatant, which is consistent with the

response being caused by a reactive metabolite. This assay was also able to differentiate the

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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, and therefore it is impossible to know how

useful it may be.

Another recent assay involves 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

determined. Monocytes were separated and cultured in specialized medium for 7 days to

generate dendritic cells. These cells were then co-cultured 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 reactive metabolite (Pearce et al., 2005), but the

parent drug may be able to induce an immune response. In the case of allopurinol, no reactive

metabolite 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 predict which drugs are likely to be associated with a

significant risk of iDILI. However, there is far too little data to know how accurately 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. Fist, 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 if a drug is metabolized differently in an animal

model than in humans this may lead to a false negative. 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*. Also 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 physiological systems, such as

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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.

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# References

Aleo MD, Luo Y, Swiss R, Bonin PD, Potter DM and Will Y (2014) Human drug-induced liver injury severity is highly associated with dual inhibition of liver mitochondrial function and bile salt export pump. *Hepatology*, **60**: 1015-1022.

Ansede JH, Smith WR, Perry CH, St Claire RL and Brouwer KR (2010) An in vitro assay to assess transporter-based cholestatic hepatotoxicity using sandwich-cultured rat hepatocytes. *Drug Metab Dispos*, **38**: 276-280.

Atienzar FA, Blomme EA, Chen M, Hewitt P, Kenna JG, Labbe G, Moulin F, Pognan F, Roth AB, Suter-Dick L, Ukairo O, Weaver RJ, Will Y and Dambach DM (2016) Key Challenges and Opportunities Associated with the Use of In Vitro Models to Detect Human DILI: Integrated Risk Assessment and Mitigation Plans. *Biomed Res Int*, **2016**: 9737920.

Atienzar FA, Novik EI, Gerets HH, Parekh A, Delatour C, Cardenas A, MacDonald J, Yarmush ML and Dhalluin S (2014) Predictivity of dog co-culture model, primary human hepatocytes and HepG2 cells for the detection of hepatotoxic drugs in humans. *Toxicol Appl Pharmacol*, **275**: 44-61.

Barber JA, Stahl SH, Summers C, Barrett G, Park BK, Foster JR and Kenna JG (2015) Quantification of Drug-Induced Inhibition of Canalicular Cholyl-l-Lysyl-Fluorescein Excretion From Hepatocytes by High Content Cell Imaging. *Toxicol Sci*, **148**: 48-59.

Bell CC, Hendriks DF, Moro SM, Ellis E, Walsh J, Renblom A, Fredriksson Puigvert L, Dankers AC, Jacobs F, Snoeys J, Sison-Young RL, Jenkins RE, Nordling Å, Mkrtchian S, Park BK, Kitteringham NR, Goldring CE, Lauschke VM and Ingelman-Sundberg M (2016) Characterization of primary human hepatocyte spheroids as a model system for drug-induced liver injury, liver function and disease. *Sci Rep*, **6**: 25187.

Bourdi M, Chen W, Peter RM, Martin JL, Buters JT, Nelson SD and Pohl LR (1996) Human cytochrome P450 2E1 is a major autoantigen associated with halothane hepatitis. *Chem Res Toxicol*, **9**: 1159-1166.

Chalasani NP, Hayashi PH, Bonkovsky HL, Navarro VJ, Lee WM, Fontana RJ and Practice PCOTACOG (2014) ACG Clinical Guideline: the diagnosis and management of idiosyncratic drug-induced liver injury. *Am J Gastroenterol*, **109**: 950-66; quiz 967.

Chan R and Benet LZ (2017) Measures of BSEP Inhibition In Vitro Are Not Useful Predictors of DILI. *Toxicol Sci*,

Chen M, Zhang J, Wang Y, Liu Z, Kelly R, Zhou G, Fang H, Borlak J and Tong W (2013) The liver toxicity knowledge base: a systems approach to a complex end point. *Clin Pharmacol Ther*, **93**: 409-412.

Cho T and Uetrecht J (2017) How Reactive Metabolites Induce an Immune Response That Sometimes Leads to an Idiosyncratic Drug Reaction. *Chem Res Toxicol*, **30**: 295-314.

35

Cho TE and Uetrecht J (2018) Testing the mitochondrial dysfunction hypothesis for idiosyncratic drug-induced liver injury (IDILI). *The Toxicologist*, **162**: 23-24.

Chowdhury NI, Remington G and Kennedy JL (2011) Genetics of antipsychotic-induced side effects and agranulocytosis. *Curr Psychiatry Rep*, **13**: 156-165.

Dambach DM, Andrews BA and Moulin F (2005) New technologies and screening strategies for hepatotoxicity: use of in vitro models. *Toxicol Pathol*, **33**: 17-26.

Dawson S, Stahl S, Paul N, Barber J and Kenna JG (2012) In vitro inhibition of the bile salt export pump correlates with risk of cholestatic drug-induced liver injury in humans. *Drug Metab Dispos*, **40**: 130-138.

De Bus L, Depuydt P, Libbrecht L, Vandekerckhove L, Nollet J, Benoit D, Vogelaers D and Van Vlierberghe H (2010) Severe drug-induced liver injury associated with prolonged use of linezolid. *J Med Toxicol*, **6**: 322-326.

Dieckhaus CM, Thompson CD, Roller SG and Macdonald TL (2002) Mechanisms of idiosyncratic drug reactions: the case of felbamate. *Chem Biol Interact*, **142**: 99-117.

Dykens JA, Jamieson J, Marroquin L, Nadanaciva S, Billis PA and Will Y (2008) Biguanide-induced mitochondrial dysfunction yields increased lactate production and cytotoxicity of aerobically-poised HepG2 cells and human hepatocytes in vitro. *Toxicol Appl Pharmacol*, **233**: 203-210.

Fattinger K, Funk C, Pantze M, Weber C, Reichen J, Stieger B and Meier PJ (2001) The endothelin antagonist bosentan inhibits the canalicular bile salt export pump: a potential mechanism for hepatic adverse reactions. *Clin Pharmacol Ther*, **69**: 223-231.

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

Garside H, Marcoe KF, Chesnut-Speelman J, Foster AJ, Muthas D, Kenna JG, Warrior U, Bowes J and Baumgartner J (2014) Evaluation of the use of imaging parameters for the detection of compound-induced hepatotoxicity in 384-well cultures of HepG2 cells and cryopreserved primary human hepatocytes. *Toxicol In Vitro*, **28**: 171-181.

Godoy P, Hewitt NJ, Albrecht U, Andersen ME, Ansari N, Bhattacharya S, Bode JG, Bolleyn J, Borner C, Böttger J, Braeuning A, Budinsky RA, Burkhardt B, Cameron NR, Camussi G, Cho CS, Choi YJ, Craig Rowlands J, Dahmen U, Damm G, Dirsch O, Donato MT, Dong J, Dooley S, Drasdo D, Eakins R, Ferreira KS, Fonsato V, Fraczek J, Gebhardt R, Gibson A, Glanemann M, Goldring CE, Gómez-Lechón MJ, Groothuis GM, Gustavsson L, Guyot C, Hallifax D, Hammad S, Hayward A, Häussinger D, Hellerbrand C, Hewitt P, Hoehme S, Holzhütter HG, Houston JB, Hrach J, Ito K, Jaeschke H, Keitel V, Kelm JM, Kevin Park B, Kordes C, Kullak-Ublick GA, LeCluyse EL, Lu P, Luebke-Wheeler J, Lutz A, Maltman DJ, Matz-Soja M, McMullen P, Merfort I, Messner S, Meyer C, Mwinyi J, Naisbitt DJ, Nussler AK, Olinga P, Pampaloni F, Pi J,

Pluta L, Przyborski SA, Ramachandran A, Rogiers V, Rowe C, Schelcher C, Schmich K, Schwarz M, Singh B, Stelzer EH, Stieger B, Stöber R, Sugiyama Y, Tetta C, Thasler WE, Vanhaecke T, Vinken M, Weiss TS, Widera A, Woods CG, Xu JJ, Yarborough KM and Hengstler JG (2013) Recent advances in 2D and 3D in vitro systems using primary hepatocytes, alternative hepatocyte sources and non-parenchymal liver cells and their use in investigating mechanisms of hepatotoxicity, cell signaling and ADME. *Arch Toxicol*, **87**: 1315-1530.

Goldring C, Antoine DJ, Bonner F, Crozier J, Denning C, Fontana RJ, Hanley NA, Hay DC, Ingelman-Sundberg M, Juhila S, Kitteringham N, Silva-Lima B, Norris A, Pridgeon C, Ross JA, Young RS, Tagle D, Tornesi B, van de Water B, Weaver RJ, Zhang F and Park BK (2017) Stem cell-derived models to improve mechanistic understanding and prediction of human drug-induced liver injury. *Hepatology*, **65**: 710-721.

Guillouzo A, Corlu A, Aninat C, Glaise D, Morel F and Guguen-Guillouzo C (2007) The human hepatoma HepaRG cells: a highly differentiated model for studies of liver metabolism and toxicity of xenobiotics. *Chem Biol Interact*, **168**: 66-73.

Gustafsson F, Foster AJ, Sarda S, Bridgland-Taylor MH and Kenna JG (2014) A correlation between the in vitro drug toxicity of drugs to cell lines that express human P450s and their propensity to cause liver injury in humans. *Toxicol Sci*, **137**: 189-211.

Hewitt NJ, Lechón MJ, Houston JB, Hallifax D, Brown HS, Maurel P, Kenna JG, Gustavsson L, Lohmann C, Skonberg C, Guillouzo A, Tuschl G, Li AP, LeCluyse E, Groothuis GM and Hengstler JG (2007) Primary hepatocytes: current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. *Drug Metab Rev*, **39**: 159-234.

Hinson JA, Roberts DW and James LP (2010) Mechanisms of acetaminophen-induced liver necrosis. *Handb Exp Pharmacol*, 369-405.

Hosomi H, Fukami T, Iwamura A, Nakajima M and Yokoi T (2011) Development of a highly sensitive cytotoxicity assay system for CYP3A4-mediated metabolic activation. *Drug Metab Dispos*, **39**: 1388-1395.

Kalgutkar AS, Dalvie D, Obach RS and Smith DA. (2012) *Reactive Drug Metabolites*. Wiley-VCH, Weinheim.

Kalgutkar AS, Gardner I, Obach RS, Shaffer CL, Callegari E, Henne KR, Mutlib AE, Dalvie DK, Lee JS, Nakai Y, O'Donnell JP, Boer J and Harriman SP (2005) A comprehensive listing of bioactivation pathways of organic functional groups. *Curr Drug Metab*, **6**: 161-225.

Kato R and Uetrecht J (2017) Supernatant from Hepatocyte Cultures with Drugs That Cause Idiosyncratic Liver Injury Activates Macrophage Inflammasomes. *Chem Res Toxicol*, **30**: 1327-1332.

Kenna JG and Thompson RA (2016) Integrated Reactive Metabolite Strategies. In *Metabolite Safety in Drug Development,* (Eds, Iverson SL and Smith DA) Wiley, pp. 111-140.

Kenna JG, Stahl SH, Eakins JA, Foster AJ, Andersson LC, Bergare J, Billger M, Elebring M, Elmore CS and Thompson RA (2015) Multiple compound-related adverse properties contribute to liver injury caused by endothelin receptor antagonists. *J Pharmacol Exp Ther*, **352**: 281-290.

Kim SH and Naisbitt DJ (2016) Update on Advances in Research on Idiosyncratic Drug-Induced Liver Injury. *Allergy Asthma Immunol Res*, **8**: 3-11.

Kis E, Ioja E, Nagy T, Szente L, Herédi-Szabó K and Krajcsi P (2009) Effect of membrane cholesterol on BSEP/Bsep activity: species specificity studies for substrates and inhibitors. *Drug Metab Dispos*, **37**: 1878-1886.

Lam P, Soroka CJ and Boyer JL (2010) The bile salt export pump: clinical and experimental aspects of genetic and acquired cholestatic liver disease. *Semin Liver Dis*, **30**: 125-133.

Lee KK, Fujimoto K, Zhang C, Schwall CT, Alder NN, Pinkert CA, Krueger W, Rasmussen T and Boelsterli UA (2013) Isoniazid-induced cell death is precipitated by underlying mitochondrial complex I dysfunction in mouse hepatocytes. *Free Radic Biol Med*, **65**: 584-594.

Li M, Cai SY and Boyer JL (2017) Mechanisms of bile acid mediated inflammation in the liver. *Mol Aspects Med*, **56**: 45-53.

Luyendyk JP, Maddox JF, Cosma GN, Ganey PE, Cockerell GL and Roth RA (2003) Ranitidine treatment during a modest inflammatory response precipitates idiosyncrasy-like liver injury in rats. *J Pharmacol Exp Ther*, **307**: 9-16.

Maiuri AR, Wassink B, Turkus JD, Breier AB, Lansdell T, Kaur G, Hession SL, Ganey PE and Roth RA (2017) Synergistic Cytotoxicity from Drugs and Cytokines In Vitro as an Approach to Classify Drugs According to Their Potential to Cause Idiosyncratic Hepatotoxicity: A Proof-of-Concept Study. *J Pharmacol Exp Ther*, **362**: 459-473.

Mak A, Kato R, Weston K, Hayes A and Uetrecht J (2018) Editor's Highlight: An Impaired Immune Tolerance Animal Model Distinguishes the Potential of Troglitazone/Pioglitazone and Tolcapone/Entacapone to Cause IDILI. *Toxicol Sci*, **161**: 412-420.

Mak A and Uetrecht J (2015a) Immunization with amodiaquine-modified hepatic proteins prevents amodiaquine-induced liver injury. *J Immunotoxicol*, **12**: 361-367.

Mak A and Uetrecht J (2015b) The Role of CD8 T Cells in Amodiaquine-Induced Liver Injury in PD1-/- Mice Cotreated with Anti-CTLA-4. *Chem Res Toxicol*, **28**: 1567-1573.

Mak A and Uetrecht J (2015c) The Combination of Anti-CTLA-4 and PD1-/- Mice Unmasks the Potential of Isoniazid and Nevirapine To Cause Liver Injury. *Chem Res Toxicol*, **28**: 2287-2291.

Marroquin LD, Hynes J, Dykens JA, Jamieson JD and Will Y (2007) Circumventing the Crabtree effect: replacing media glucose with galactose increases susceptibility of HepG2 cells to mitochondrial toxicants. *Toxicol Sci*, **97**: 539-547.

38

McKenzie R, Fried MW, Sallie R, Conjeevaram H, Di Bisceglie AM, Park Y, Savarese B, Kleiner D, Tsokos M and Luciano C (1995) Hepatic failure and lactic acidosis due to fialuridine (FIAU), an investigational nucleoside analogue for chronic hepatitis B. *N Engl J Med*, **333**: 1099-1105.

Metushi IG, Cai P, Zhu X, Nakagawa T and Uetrecht JP (2011) A fresh look at the mechanism of isoniazid-induced hepatotoxicity. *Clin Pharmacol Ther*, **89**: 911-914.

Metushi IG, Hayes MA and Uetrecht J (2015) Treatment of PD-1(-/-) mice with amodiaquine and anti-CTLA4 leads to liver injury similar to idiosyncratic liver injury in patients. *Hepatology*, **61**: 1332-1342.

Metushi IG, Sanders C, Lee WM and Uetrecht J (2014) Detection of anti-isoniazid and anti-cytochrome P450 antibodies in patients with isoniazid-induced liver failure. *Hepatology*, **59**: 1084-1093.

Morgan RE, van Staden CJ, Chen Y, Kalyanaraman N, Kalanzi J, Dunn RT, Afshari CA and Hamadeh HK (2013) A multifactorial approach to hepatobiliary transporter assessment enables improved therapeutic compound development. *Toxicol Sci*, **136**: 216-241.

Nakamura H, Okada A, Kawakami A, Yamasaki S, Ida H, Motomura M, Imanishi D and Eguchi K (2009) Isoniazid-triggered pure red cell aplasia in systemic lupus erythematosus complicated with myasthenia gravis. *Rheumatol Int*,

Nakayama S, Atsumi R, Takakusa H, Kobayashi Y, Kurihara A, Nagai Y, Nakai D and Okazaki O (2009) A zone classification system for risk assessment of idiosyncratic drug toxicity using daily dose and covalent binding. *Drug Metab Dispos*, **37**: 1970-1977.

Ng W, Lobach AR, Zhu X, Chen X, Liu F, Metushi IG, Sharma A, Li J, Cai P, Ip J, Novalen M, Popovic M, Zhang X, Tanino T, Nakagawa T, Li Y and Uetrecht J (2012) Animal models of idiosyncratic drug reactions. *Adv Pharmacol*, **63**: 81-135.

O'Brien PJ, Irwin W, Diaz D, Howard-Cofield E, Krejsa CM, Slaughter MR, Gao B, Kaludercic N, Angeline A, Bernardi P, Brain P and Hougham C (2006) High concordance of drug-induced human hepatotoxicity with in vitro cytotoxicity measured in a novel cell-based model using high content screening. *Arch Toxicol*, **80**: 580-604.

Oates NS, Shah RR, Idle JR and Smith RL (1983) Influence of oxidation polymorphism on phenformin kinetics and dynamics. *Clin Pharmacol Ther*, **34**: 827-834.

Obach RS, Kalgutkar AS, Soglia JR and Zhao SX (2008) Can in vitro metabolism-dependent covalent binding data in liver microsomes distinguish hepatotoxic from nonhepatotoxic drugs? An analysis of 18 drugs with consideration of intrinsic clearance and daily dose. *Chem Res Toxicol*, **21**: 1814-1822.

Ong MM, Latchoumycandane C and Boelsterli UA (2007) Troglitazone-induced hepatic necrosis in an animal model of silent genetic mitochondrial abnormalities. *Toxicol Sci*, **97**: 205-213.

Ott LM, Ramachandran K and Stehno-Bittel L (2017) An Automated Multiplexed Hepatotoxicity and CYP Induction Assay Using HepaRG Cells in 2D and 3D. *SLAS Discov*, **22**: 614-625.

Pachkoria K, Lucena MI, Crespo E, Ruiz-Cabello F, Lopez-Ortega S, Fernandez MA, Romero-Gomez M, Madrazo A, Duran JA, de Dios AM, Borraz Y, Navarro JM and Andrade RJ (2008) Analysis of IL-10, IL-4 and TNF-alpha polymorphisms in drug-induced liver injury (DILI) and its outcome. *J Hepatol*, **49**: 107-114.

Pearce RE, Uetrecht JP and Leeder JS (2005) Pathways of carbamazepine bioactivation in vitro II. The role of human cytochrome P450 enzymes in the formation of 2-hydroxyiminostilbene. *Drug Metab Dispos*, **33**: 1819-1826.

Pessayre D, Fromenty B, Berson A, Robin MA, Letteron P, Moreau R and Mansouri A (2012) Central role of mitochondria in drug-induced liver injury. *Drug Metab Rev*, **44**: 34-87.

Pollmacher T, Hinze-Selch D and Mullington J (1996) Effects of clozapine on plasma cytokine and soluble cytokine receptor levels. *J Clin Psychopharmacol*, **16**: 403-409.

Porceddu M, Buron N, Roussel C, Labbe G, Fromenty B and Borgne-Sanchez A (2012) Prediction of liver injury induced by chemicals in human with a multiparametric assay on isolated mouse liver mitochondria. *Toxicol Sci*, **129**: 332-345.

Proctor WR, Foster AJ, Vogt J, Summers C, Middleton B, Pilling MA, Shienson D, Kijanska M, Ströbel S, Kelm JM, Morgan P, Messner S and Williams D (2017) Utility of spherical human liver microtissues for prediction of clinical drug-induced liver injury. *Arch Toxicol*, **91**: 2849-2863.

Riley RJ, Grime K and Weaver R (2007) Time-dependent CYP inhibition. *Expert Opin Drug Metab Toxicol*, **3**: 51-66.

Siddiqui MA, Ahmad J, Farshori NN, Saquib Q, Jahan S, Kashyap MP, Ahamed M, Musarrat J and Al-Khedhairy AA (2013) Rotenone-induced oxidative stress and apoptosis in human liver HepG2 cells. *Mol Cell Biochem*, **384**: 59-69.

Stewart JD, Horvath R, Baruffini E, Ferrero I, Bulst S, Watkins PB, Fontana RJ, Day CP and Chinnery PF (2010) Polymerase gamma gene POLG determines the risk of sodium valproate-induced liver toxicity. *Hepatology*, **52**: 1791-1796.

Strannegård IL, Majeed HA and Ahlstedt S (1987) Antibodies to penicillin in children receiving long-term secondary prophylaxis for rheumatic fever. *Allergy*, **42**: 502-506.

Strautnieks SS, Bull LN, Knisely AS, Kocoshis SA, Dahl N, Arnell H, Sokal E, Dahan K, Childs S, Ling V, Tanner MS, Kagalwalla AF, Németh A, Pawlowska J, Baker A, Mieli-Vergani G, Freimer NB, Gardiner RM and Thompson RJ (1998) A gene encoding a liver-specific ABC transporter is mutated in progressive familial intrahepatic cholestasis. *Nat Genet*, **20**: 233-238.

Swift B, Pfeifer ND and Brouwer KL (2010) Sandwich-cultured hepatocytes: an in vitro model to evaluate hepatobiliary transporter-based drug interactions and hepatotoxicity. *Drug Metab Rev*, **42**: 446-471.

Thompson RA, Isin EM, Li Y, Weidolf L, Page K, Wilson I, Swallow S, Middleton B, Stahl S, Foster AJ, Dolgos H, Weaver R and Kenna JG (2012) In vitro approach to assess the potential for risk of idiosyncratic adverse reactions caused by candidate drugs. *Chem Res Toxicol*, **25**: 1616-1632.

Tolosa L, Gómez-Lechón MJ, Pérez-Cataldo G, Castell JV and Donato MT (2013) HepG2 cells simultaneously expressing five P450 enzymes for the screening of hepatotoxicity: identification of bioactivable drugs and the potential mechanism of toxicity involved. *Arch Toxicol*, **87**: 1115-1127.

Ulrich RG (2007) Idiosyncratic toxicity: a convergence of risk factors. *Annu Rev Med*, **58**: 17-34.

Underhill GH and Khetani SR (2018) Bioengineered Liver Models for Drug Testing and Cell Differentiation Studies. *Cell Mol Gastroenterol Hepatol*, **5**: 426-439.e1.

Usui T, Faulkner L, Farrell J, French NS, Alfirevic A, Pirmohamed M, Park BK and Naisbitt DJ (2018) Application of in Vitro T Cell Assay Using Human Leukocyte Antigen-Typed Healthy Donors for the Assessment of Drug Immunogenicity. *Chem Res Toxicol*, **31**: 165-167.

Usui T, Mise M, Hashizume T, Yabuki M and Komuro S (2009) Evaluation of the potential for drug-induced liver injury based on in vitro covalent binding to human liver proteins. *Drug Metab Dispos*, **37**: 2383-2392.

Vazquez JA, Arnold AC, Swanson RN, Biswas P and Bassetti M (2016) Safety of long-term use of linezolid: results of an open-label study. *Ther Clin Risk Manag*, **12**: 1347-1354.

Vernetti LA, Senutovitch N, Boltz R, DeBiasio R, Shun TY, Gough A and Taylor DL (2016) A human liver microphysiology platform for investigating physiology, drug safety, and disease models. *Exp Biol Med (Maywood)*, **241**: 101-114.

Vickers AE (2009) Characterization of hepatic mitochondrial injury induced by fatty acid oxidation inhibitors. *Toxicol Pathol*, **37**: 78-88.

Wang R, Chen HL, Liu L, Sheps JA, Phillips MJ and Ling V (2009) Compensatory role of P-glycoproteins in knockout mice lacking the bile salt export pump. *Hepatology*, **50**: 948-956.

Watkins PB, Desai M, Berkowitz SD, Peters G, Horsmans Y, Larrey D and Maddrey W (2011) Evaluation of drug-induced serious hepatotoxicity (eDISH): application of this data organization approach to phase III clinical trials of rivaroxaban after total hip or knee replacement surgery. *Drug Saf*, **34**: 243-252.

Welsh RM and Selin LK (2002) No one is naive: the significance of heterologous T-cell immunity. *Nat Rev Immunol*, **2**: 417-426.

Whitington PF, Freese DK, Alonso EM, Schwarzenberg SJ and Sharp HL (1994) Clinical and biochemical findings in progressive familial intrahepatic cholestasis. *J Pediatr Gastroenterol Nutr*, **18**: 134-141.

Wolf KK, Vora S, Webster LO, Generaux GT, Polli JW and Brouwer KL (2010) Use of cassette dosing in sandwich-cultured rat and human hepatocytes to identify drugs that inhibit bile acid transport. *Toxicol In Vitro*, **24**: 297-309.

Woodhead JL, Brock WJ, Roth SE, Shoaf SE, Brouwer KL, Church R, Grammatopoulos TN, Stiles L, Siler SQ, Howell BA, Mosedale M, Watkins PB and Shoda LK (2017a) Application of a Mechanistic Model to Evaluate Putative Mechanisms of Tolvaptan Drug-Induced Liver Injury and Identify Patient Susceptibility Factors. *Toxicol Sci*, **155**: 61-74.

Woodhead JL, Pellegrini L, Siler SQ, Shoda LK, Watkins PB and Howell BA (2017b) Prospective liver safety comparison of two treatments for autosomal-dominant polycystic kidney disease (ADPKD) using quantitative systems toxicology modeling. *J Pharmacokinet Pharmacodyn*, **44**: S70-S71.

Woosley RL, Drayer DE, Reidenberg MM, Nies AS, Carr K and Oates JA (1978) Effect of acetylator phenotype on the rate at which procainamide induces antinuclear antibodies and the lupus syndrome. *N Engl J Med*, **298**: 1157-1159.

Xu JJ, Henstock PV, Dunn MC, Smith AR, Chabot JR and de Graaf D (2008) Cellular imaging predictions of clinical drug-induced liver injury. *Toxicol Sci*, **105**: 97-105.

Yang K, Woodhead JL, Watkins PB, Howell BA and Brouwer KL (2014) Systems pharmacology modeling predicts delayed presentation and species differences in bile acid-mediated troglitazone hepatotoxicity. *Clin Pharmacol Ther*, **96**: 589-598.

Yuan L and Kaplowitz N (2013) Mechanisms of drug-induced liver injury. *Clin Liver Dis*, **17**: 507-18, vii.

Yucha RW, He K, Shi Q, Cai L, Nakashita Y, Xia CQ and Liao M (2017) In Vitro Drug-Induced Liver Injury Prediction: Criteria Optimization of Efflux Transporter IC50 and Physicochemical Properties. *Toxicol Sci*, **157**: 487-499.

Zhang Y, Li F, Patterson AD, Wang Y, Krausz KW, Neale G, Thomas S, Nachagari D, Vogel P, Vore M, Gonzalez FJ and Schuetz JD (2012) Abcb11 deficiency induces cholestasis coupled to impaired β-fatty acid oxidation in mice. *J Biol Chem*, **287**: 24784-24794.

Zimmerman H. (1999) *Hepatotoxicity: The adverse effects of drugs and other chemicals on the liver*. Lippincott Williams & Wilkins, Philadelphia.

# Figure Legends

Figure 1. Postulated mechanism of iDILI. A drug, or more commonly a reactive metabolite, 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 danger associated molecular pattern molecules (DAMPS). The DAMPS active antigen presenting cells (APCs). 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.

Figure 2. Factors that influence the balance between adaptation and iDILI

**Table 1. Examples of *in vitro* human liver cell models proposed for use in iDILI prediction.**

| Cell model | Characteristics | Advantages | Disadvantanges | References |
|---|---|---|---|---|
| HepG2 cells | Human liver-derived cell line | • Simple model<br>• Suitable for short term and longer term studies<br>• Multiple endpoints can be evaluated<br>• Suitable for high volume and multi-parametric data generation | • Low drug metabolizing enzyme expression<br>• Poor expression of plasma membrane transporters | (Atienzar et al., 2014; Atienzar et al., 2016); (Godoy et al., 2013) |
| HepaRG cells | Human hepatoma-derived cell line | • Can be differentiated into hepatocyte-like and biliary epithelial-like cells<br>• Better maintenance of "hepatocyte-like" drug metabolizing enzymes and membrane transporters than HepG2 cells<br>• Multiple endpoints can be evaluated | • Limited commercial availability<br>• Complex cell culture conditions are needed<br>• Cells exhibit limited metabolizing enzyme phenotype<br>• CYPs activities are lower than those in isolated primary hepatocytes | (Guillouzo et al., 2007) (Ott et al., 2017) |
| Cell lines expressing human CYPs | Human liver-derived cell lines transfected with individual human CYPs | • Stably transfected cell lines are a simple model, well suited to multi-parametric data generation plus acute and long term toxicity studies<br>• Enable direct comparison between cell toxicity caused by parent compound (to mock-transfected cells) and metabolites (to transfected cells) | • Transfected cell lines do not express hepatocyte-like cell phenotype, or many membrane transporters expression<br>• Unbalanced metabolism, since only selected CYPs are expressed | (Dambach et al., 2005) (Hosomi et al., 2011); (Tolosa et al., 2013); (Gustafsson et al., 2014) |
| Isolated hepatocytes | Isolated human hepatocytes | • Freshly isolated hepatocytes express "*in vivo*-like" high drug metabolism activity<br>• Good maintenance of viability, for multiple days, when cultured as monolayers<br>• When cultured appropriately (ideally in sandwich configuration), regain polarized plasma membrane transporter expression and are well suited to multi-parametric data generation | • Limited availability of human donor livers<br>• Human liver exhibit marked inter-individual variability<br>• Freshly isolated hepatocytes in suspension are viable only for several hours and do not exhibit polarized plasma membrane transporter expression<br>• Longer term hepatocyte culture is technically challenging and these cells fail to maintain high CYP expression<br>• Inter-individual donor variability may influence results | (Hewitt et al., 2007); (Xu et al., 2008); (Ansede et al., 2010); (Wolf et al., 2010); (Barber et al., 2015); (Nakamura et al., 2009); (Usui et al., 2009); (Thompson et al., 2012) |

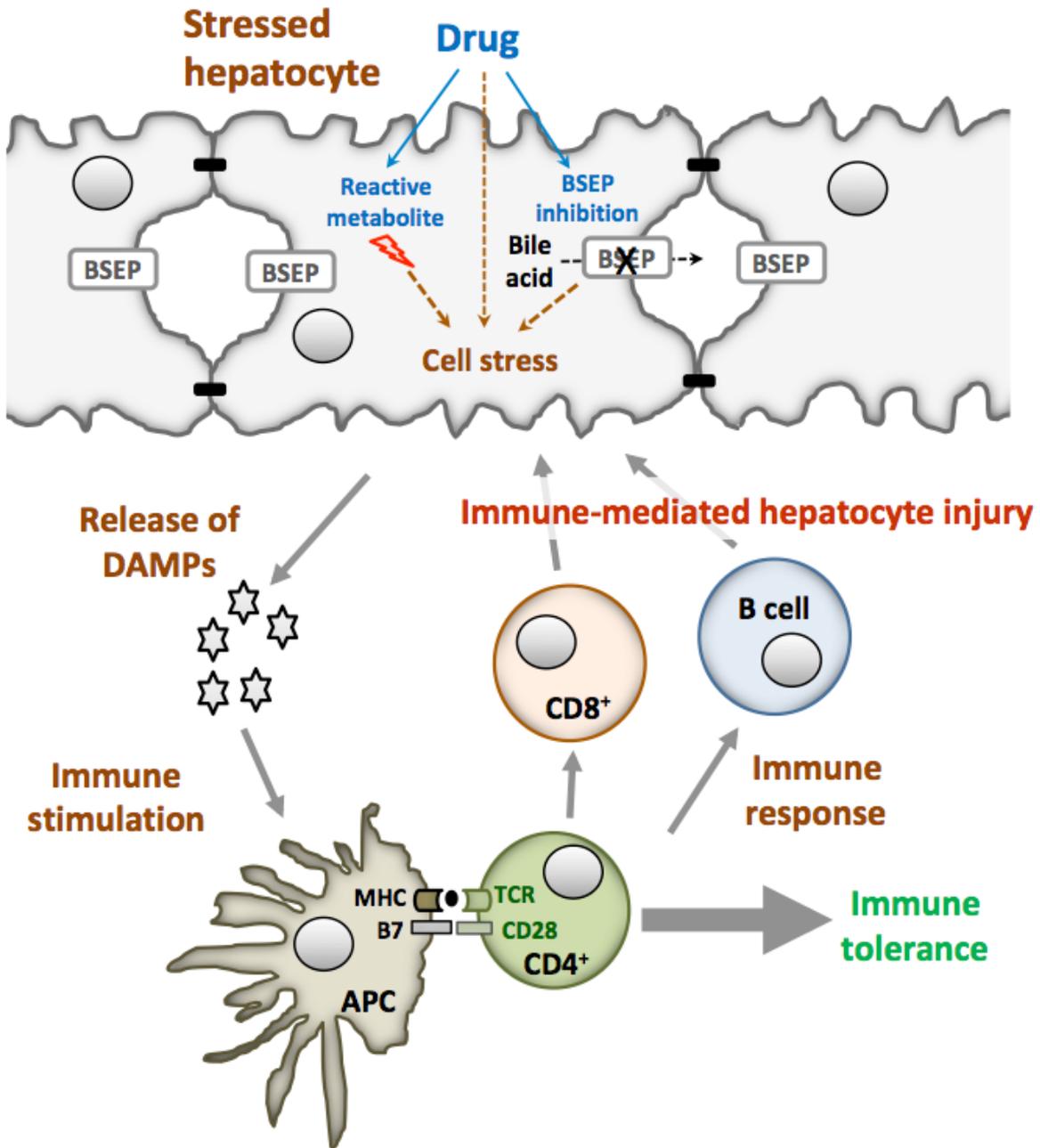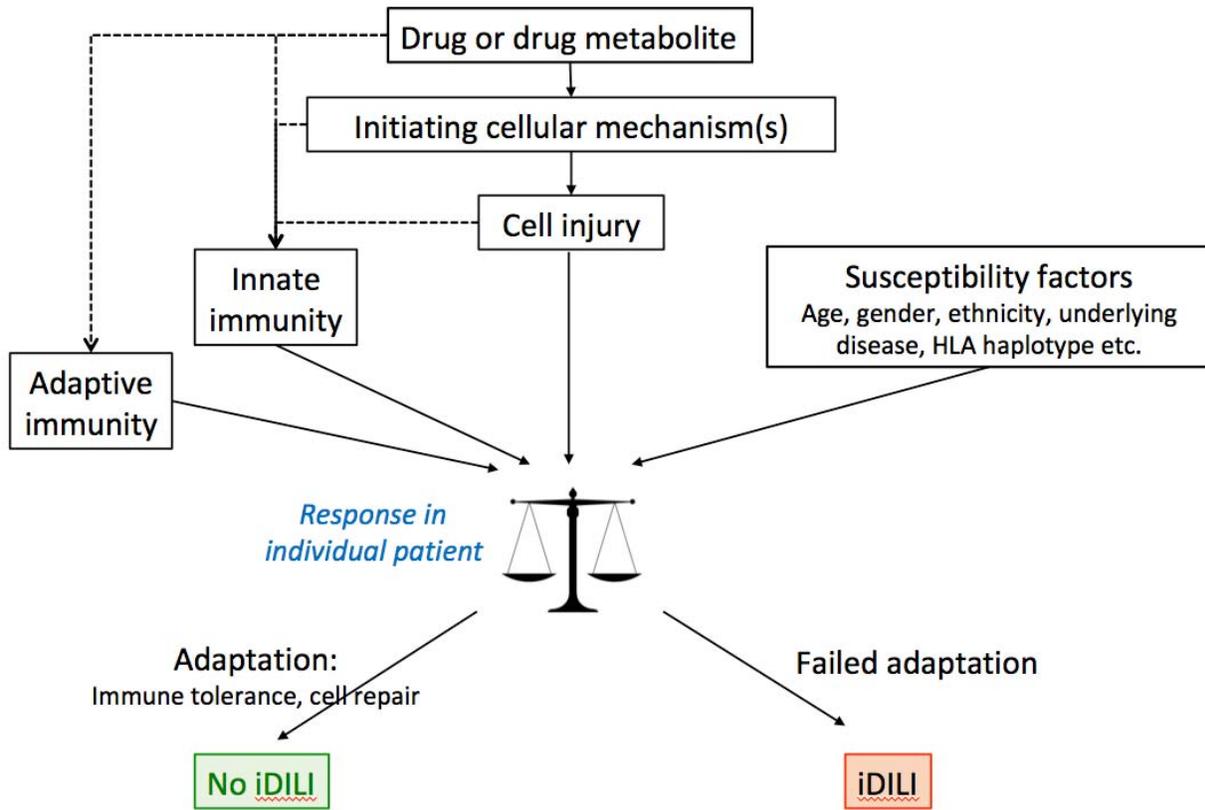| | | | | |
|---|---|---|---|---|
| **Micropatterned hepatocyte / accessory cell co-cultures** | **Isolated hepatocytes co-cultured with macrophages or other accessory cell** | • **Good maintenance of viability, drug metabolizing enzymatic activity and membrane transporters, for multiple days**<br>• **Numerous endpoints can be evaluated, at a single cell level** | • **Technically challenging to prepare, commercial supply is required**<br>• **Limited variety and physiological relevance of accessory cells**<br>• **Limited toxicity characterization**<br>• **Inter-individual donor variability may influence results** | (Underhill and Khetani, 2018) |
| **Liver microtissues (spheroids)** | **Liver cell aggregates which includes hepatocytes and multiple non-parenchymal cell types** | • **Good maintenance of viability, drug metabolizing enzyme activity and membrane transporters, for multiple days** | • **Technically challenging to prepare, commercial supply is required**<br>• **Limited toxicity characterization**<br>• **Endpoints cannot be quantified at a single cell level**<br>• **Inter-individual donor variability may influence results** | (Bell et al., 2016); (Proctor et al., 2017) |
| **Microfluidic devices** | **Hepatocytes, multiple liver cell types or liver-derived cell lines devices which 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, when compared with non-microfluidic cell models** | • **Technically challenging to prepare, commercial supply of devices is required**<br>• **Limited toxicity characterization**<br>• **Inter-individual donor variability may influence results** | (Vernetti et al., 2016) |
| **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**<br>• **Currently available conditions provide poorly differentiated cells**<br>• **Limited toxicity characterization** | (Goldring et al., 2017) |

Figure 1.

Figure 2.