**Title: - Test systems in Drug Discovery for hazard identification and risk assessment of human Drug-Induced Liver Injury**

**Subtitle:** Industry-led perspective from EFPIA members of the EU Innovative Medicines Initiative Drug Liver Injury Project, MIP DILI.

Abstract 200 Words

**Key Words:** human, hepatocytes, HepaRG, HepG2, DILI, liver, drug discovery, drug development, hepatobiliary, cytotoxicity, mitochondrial toxicity, cholestasis, immune system, IMI, test systems, endpoints.

**Abbreviations**: EFPIA, European Federation of Pharmaceutical Industries & Associations; FDA, Food and Drug Administration; BSEP, bile salt transport pump; MIE, Molecular Initiating Event; DILI, drug-induced liver injury; MIP-DILI, Mechanism-Based Integrated Systems for the Prediction of Drug-Induced Liver Injury; MMP, mitochondrial membrane potential; PHH, primary human hepatocytes; MoA, mode of action; DME, drug-metabolising enzymes; iDILI, immune DILI; ECAR, extracellular acidification rate; OCR, oxygen consumption rate; CYP, cytochrome P450; ADRs, adverse drug reactions; NPCs, non-parenchymal cells, MPS, microphysiological systems

**Abstract**

*Introduction*

The liver is an important target for drug-induced toxicities. Early detection of hepatotoxic drugs requires use of well-characterized test systems, yet current knowledge, gaps and limitations of tests employed remains an important issue for drug development.

*Areas Covered*

The current state of the science, understanding and application of test systems in use for the detection of drug-induced cytotoxicity, mitochondrial toxicity, cholestasis and inflammation is summarized. The test systems highlighted herein cover mostly *in vitro* and some *in vivo* models and endpoint measurements used in the assessment of small molecule toxic liabilities. Opportunities for research efforts in areas necessitating the development of specific tests and improved mechanistic understanding are highlighted.

*Expert Opinion*

Use of *in vitro* test systems for safety optimization will remain a core activity in drug discovery. Substantial inroads have been made with a number of assays established for human DILI. There nevertheless remain significant gaps with a need for improved *in vitro* tools and novel tests to address specific mechanisms of human DILI. Progress in these areas will necessitate not only models fit for application, but also mechanistic understanding of how chemical insult on the liver occurs in order to identify translational and quantifiable readouts for decision-making.

1. **Introduction**

Identifying chemical safety liabilities during the discovery of new candidate drugs is an important activity to help mitigate the risk of attrition during the later stages of drug development or to avoid market withdrawal due to unwanted adverse drug reactions (ADRs). Among the most common target organ toxicities associated with ADRs during clinical research testing and post-market prescribing are those associated with liver dysfunction and toxicity [1]. Because toxicological evaluation in preclinical species does not detect all drug-associated liver injuries [2], the pharmaceutical industry has looked for well-characterized human *in vitro* hepatocellular-based test systems to better characterize experimental compounds for their potential to induce Drug-Induced Liver injury (DILI) in humans. Simple, two-dimensional (2D) monocultures have been successfully used for the detection and ranking of intrinsic dose-related toxicity, however, much effort is still on-going to improve cell models with an emphasis on maintaining critical cellular functions. In particular, understanding the sensitivity and specificity of these tests, as well as developing approaches to detect potential liabilities that might be associated with idiosyncratic forms of human DILI, still remain important priorities in preclinical toxicology research to ensure the progression of compounds with optimal safety profile in the clinic.

In recent years, multiple review articles have described applications of cellular models in both 2D and 3D configuration in toxicology [3] [4] [5]. Several of these models offer improvements as far as their physiological and pharmacological relevance to the clinical situation, as evidenced by their closer comparability to human liver tissue. Nevertheless, the evaluation of most advanced 2D *in vitro* toxicology models has yet to be thoroughly bench-marked across multiple organizations. Moreover, evaluations often focus on only a few parameters, for example cytochrome P450 (CYP) content, and do not capture an integrated view of the totality of changes that occur when hepatocytes are placed in culture systems. This thorough benchmarking exercise is essential to both demonstrate reproducible performance characteristics in the detection of chemical liabilities and to highlight areas where advances in culture models can improve hepatocellular functionality. Availability of well curated training and test sets of compounds comprising of appropriately annotated DILI and non-DILI drugs should be used [6] to benchmark and compare current practices and endpoints commonly employed by the pharmaceutical industry.

In this review paper, members from the Innovative Medicines Initiative (IMI) Mechanism-based Integrated Prediction of Drug Induced Liver Injury (MIP-DILI) consortium present an industry perspective on the test systems currently employed in drug discovery, and highlight their utility, limitations, gaps and opportunities for hazard identification and risk assessment of human DILI.

**2. Test Systems**

Here, an *in vitro* test system is defined as a model, typically hepatocellular-based in 2D or 3D configuration, and the bioanalytical or biochemical endpoint(s) or biomarker of toxicological interest to be interrogated. These test systems range from simple 2D cytotoxicity models to more refined models coupled with specific endpoints to support greater in-depth investigations of specific mechanisms of hepatotoxicity. These currently available test systems are often successful for the detection of compounds that directly injure hepatocytes in a dose-dependent manner without the contribution of external molecular initiating events (MIEs) or additional factors required for chemical insult to occur on the hepatocyte (Table 1). These types of hepatotoxicity are commonly referred to as intrinsic events, are often detected in preclinical toxicology species, and are readily linked to cytotoxicity. By contrast, extrinsic or idiosyncratic toxicities may have a dose-related component, but are typically multifactorial with multiple contributing intra- and extrahepatic factors such that certain individuals may be predisposed to DILI. As such, these cannot be readily detected in conventional toxicology animal species [7] [8].

The test systems described herein point to the value and limitations industry faces when evaluating compounds for cytotoxicity, mitochondrial toxicity, cholestasis and immune-mediated potential for human DILI. A clear understanding of the mechanisms that give rise to chemical insult on the liver is often not available, resulting in the inability to identify the most appropriate test systems for the detection of DILI. The strengths and short-comings of current test systems, as well as their use for mechanistic understanding of certain forms of hepatocellular injuries and for risk assessment of human DILI are reviewed.

**2.1 Cytotoxicity test systems**

*2.1.1 Current understanding & importance*

Drug-induced changes of cell health in simple *in vitro* systems, such as the HepG2 cell line, are commonly adopted as a first-line screening approach in pharmaceutical companies. Although cell death is a crude endpoint, its applicability should not be underestimated as an indicator of intrinsic toxic properties within a chemical series or as a tool for structure-activity relationship studies, especially when general cell health is affected at low compound concentrations. The assessment of cell lethality or the impact on cell health can be measured using one or more endpoints known to correlate with general cell health (Table 2). Therefore, it is not surprising that cytotoxicity shows high concordance with systemic tolerance and general organ toxicity in preclinical species [9]. Although cytotoxicity assays may not provide a mechanistic understanding of toxicity, the fundamental advantages of these assays are the simplicity of interpretation, low costs, and rapid turnaround times. This makes them amenable to a throughput and cycle time appropriate for actively influencing the fast paced “design-make-test” cycle of medicinal chemistry. While test concentrations often used in validation efforts are tailored to multiples of the maximum human plasma concentration at the clinical dose (Cmax), the pharmaceutical industry typically uses fixed concentration ranges in preliminary screens, often aiming at top concentrations (solubility permitting) in excess of 200 µM. This represents a pragmatic approach as the Cmax of newly synthesized compounds are seldom known and screening of compounds needs to be standardized. The approach covers a wide range of concentrations and permits rank-ordering of compounds. Depending on the endpoints used, these data can provide retrospective estimations and a guide towards potentially safe values of Cmax [10] [11].

Ultimately, while lacking sophistication and sensitivity, cytotoxicity assays serve as an early filter for discarding compounds and entire compound series with overt toxicological liabilities too severe to progress in discovery research, and the ranking of remaining compounds for further characterization of potential false negatives.

2.1.2 *Routinely used endpoints*

A number of cytotoxicity measures exist, including simple cell counting using light microscopy or fluorescent markers, biochemical assays for key cellular processes or products (e.g., ATP, tetrazolium and formazan, or resazurin measurements), assessments of cell membrane integrity (Table 1), and more complex imaging in 3D microtissue systems (*see Table 2, Cytotoxicity test systems & routine assays*). It should be noted that many of the commonly used surrogate markers for cytotoxicity are often dependent on other cellular processes, causing differences in sensitivity (e.g. cell counting by high-content imaging identifies more cytotoxic compounds than ATP-based measurements in direct side-by-side comparisons) [12]. High content imaging has emerged as a state-of-the-art technology for assessment of cell health and cytotoxicity, both because of the higher sensitivity compared to traditional biochemical methods, and because of the rich multiplexed information which captures multiple potential mechanisms [13]. Superior detection of hepatotoxicants by high-content imaging has been reported.

The cytotoxicity assay is generally not considered to reflect organ-specific toxicities, such as DILI, but facilitates early hazard identification of compounds likely to produce unacceptable toxicity. Cytotoxicity assessment of close to 300 compounds with reported organ-specific toxicities in cell lines of hepatic, cardiac, or renal origin showed similar sensitivity in all cell lines except for a small subset of compounds [14]. The lack of organ-specific and/or selective toxicities is believed to arise once cells are removed from their native environment, especially the tissue/organ specific environment with its complex 3D cell-cell interactions, para- and endocrine factors, *etc.* [15] . Other limitations for the detection of DILI include changes to the cellular phenotype, as evidenced by downregulation of the constitutive expression of the drug-metabolizing enzymes in cultured primary hepatocytes, and difficulties in replicating the pharmacokinetics of *in vivo* liver exposure to drugs in culture [16].

Both primary hepatocytes and cell lines are used in cytotoxicity tests. Whilst primary human and animal hepatocytes, which retained some level of metabolic capacity, are viewed as the gold standard in cytotoxicity screening [17], cell lines such as HepG2 or THLE cells are widely used due to their low cost, consistent biological phenotype and ease of culturing [18] [19] [20]. Low, as well as variableexpression levels of drug-metabolizing enzymes , , especially of the Phase II enzymes, in different batches of HepG2 cells are a contributing factor for inter-laboratory differences in the detection and classification of known hepatocellular toxicants [21]. Furthermore, in the absence of clear performance bench-marking, approaches for the assessment of cytotoxicity are often found to be company specific, and an agreed set of reference compounds to calibrate test systems would greatly facilitate bench-marking of cell models.

**2.2 Mitochondrial toxicity test systems**

*2.2.1 Current understanding & importance*

The primary function of mitochondria is the production of more than 90% of the cell’s energy in the form of ATP and the regulation of major cellular functions including necrosis and apoptosis signalling pathways [22]. In organs with a high demand for energy, such as the liver, mitochondria are more abundant rendering the liver more sensitive to mitochondrial toxicants. Over the last 10 years mitochondrial dysfunction has become increasingly implicated in the aetiology of drug-induced toxicities [23], and many hepatotoxic drugs have been shown to impair mitochondrial function *in vitro* [24] [25].

Diverse drug classes impact mitochondrial function. Among the most potent are drugs that have been withdrawn from the market or have received Black Box warnings from the Food and Drug Administration (FDA) [26]. Many drugs, such as the thiazolidinedione class of drugs, acutely undermine mitochondrial function by directly inhibiting the electron transport chain, while others can uncouple electron transport from ATP synthesis, as seen with some Non-Steroidal Anti-Inflammatory Drugs (NSAIDs). In both cases, the mitochondrial membrane potential (MMP, ΔѰ) is dissipated and ATP production is diminished or abolished depending on the severity. Other drugs, such as acetaminophen, doxorubicin and ethanol induce, directly or through reactive metabolites, oxidative stress via redox cycling or reactive oxygen species (ROS) formation. In addition, inhibition of metabolic pathways, such as fatty acid β-oxidation (FAO) and the Kreb’s cycle, that fuel oxidative phosphorylation, can impair ATP production, and inhibit the mitochondrial membrane transporters that exchange metabolites across the impermeable inner membrane [23]. A severe inhibition of FAO, either direct or indirect, may also induce accumulation of lipid droplets within hepatocytes (microvesicular steatosis) [27]. Drug-induced mitochondrial toxicity can equally arise due to the inhibition of replication of mitochondrial DNA (e.g. nucleoside analogues) or the inhibition of mitochondrial protein synthesis [28] (e.g.linezolid) [29].

2.2.2 *Routinely used endpoints*

To avoid mitochondrial liabilities, routine screens can be positioned within the drug discovery process. Several assays and cell models for mitochondrial function evaluation have been proposed for use in drug discovery to reduce late-stage attrition of drug candidates [23]. The endpoints in use for the assessment of drug-induced mitochondrial toxicity are broad (Table 3; Figure 2). Each of the assays and endpoint measurements offer complementary information from the global assessment of direct and indirect drug effects on mitochondria to more specific assessment of mechanisms related to the putative MIE or mode of action (MoA). The glucose-galactose-assay (glu-gal) [30] [31] and MMP assays form perhaps the most commonly applied endpoints in combination with cell lines, which offer the greatest simplicity and reproducible results. The tumor-derived HepG2 cell line is more widely used in mitochondrial assays. HepG2 cells form ATP through glycolysis alongside oxidative phosphorylation to maintain growth in anaerobic conditions, whereas primary hepatocytes lack such metabolic plasticity [32].In the glu-gal assay, ATP production is typically measured in HepG2 cells cultivated in galactose-rich medium. This promotes the oxidative phosphorylation pathway and is compared to ATP production measured in cells cultivated in glucose-rich medium, where essentially glycolysis predominates. The MMP assay is an indirect measure of mitochondrial function. In a multiparameter high-content imaging set-up, other mitochondrial toxicity endpoints together with MMP can be measured, e.g. reactive oxygen species and steatosis. Neither the glu-gal assay, nor the MMP endpoint however are able to distinguish whether mitochondrial dysfunction is due to inhibition or uncoupling of the ETC, or mitochondrial permeability transition. As several test systems for mitochondrial toxicity can be employed and little if any head-to-head comparative performance evaluation of these tests exists, a variety of different approaches are employed by companies that are perhaps driven more by the familiarity with the test rather than clear evidence-based choice of which system is most suitable for the detection of drug-induced mitochondrial liability [33] [34].

More mechanistic insight can be provided by the measurement of the oxygen consumption rate (OCR). The XF96 Extracellular Flux Analyser (Seahorse Bioscience) uses label-free fluorescence technology to measure OCR and extracellular acidification rate (ECAR). OCR is primarily a measure of mitochondrial respiration, while ECAR is a measure of lactic acid release indicating glycolysis (Table 3). In addition to basal respiration and glycolysis rates, specific mechanistic endpoints can be measured with stress tests, e.g. ATP production, uncoupling and spare respiratory capacity [26] [35]. The assay often performs well in routine use with cell lines such as HepG2. It is also possible to use OCR and ECAR with other cell types and cell models (e.g. disease models, 3D cell cultures or isolated mitochondria). Changes to mitochondrial DNA (mtDNA), in certain individuals with pre-existing liver pathologies, such as diabetes or non alcoholic fatty liver diseases (NAFLD) [36] [37] [25], may predispose these individuals to DILI. However, as a mechanism for study of drug-induced liver toxicities, it remains unidentified in short-term assays currently employed within the industry. This acknowledges a gap in the tools currently available in the industy for the assessment of drug safety. The use of cell lines or 2D/3D models amenable to long-term cultures has succeeded at identifying the deleterious effect of some drugs on mtDNA replication even if the toxicity is apparently difficult to detect. Finally, mitochondrial protein synthesis can be measured by a lateral flow immunoassay, typically using HepG2 cells, that monitors the ratio of complex IV:frataxin. This is known to decrease with drugs that inhibit either mtDNA replication or mtDNA-encoded protein synthesis [26], or indirectly through measurement of mtDNA and mtRNA levels. However, despite evidence of the impact of many drugs on mitochondrial function in *in vitro*, little information on *in vitro*-*in vivo* correlation of mitochondrial toxicants has been reported to date, possibly in part due to the technical challenges associated with existing assays and the high rates of false positives in these assays due to the high drug concentrations used [38] [39] [40] [41]. Recent developments might improve the *in vitro-in vivo* correlation through the use of cell models amenable to incubation with drugs of between 10-15 days at therapeutic relevant concentrations. Such changes may reflect greater relevance to the *in vivo* situation [42] and can address issues associated with the use of short-term assays and high drug concentrations.

*2.2.3. In vivo models for the detection of mitochondrial toxicity*

Evidence ofmitochondrial impairment in the liver of preclinical species is often lacking and consequently there remains a disconnect between *in vitro* studies of mitochondrial dysfunction and *in vivo* effects in preclinical animal models [23] [43]. In many instances, animal models fail to reveal such toxicity, as is the case for troglitazone, nefazodone and cerivastatin, all of which passed the requisite regulatory animal studies without any signs of liver injury. The lack of response is likely multifactorial and related to the fact that *in vivo* toxicity studies are conducted using limited numbers of young drug-naïve animals with high mitochondrial reserves and exhibiting low genetic diversity [44]. In addition, the traditional endpoints used in these in vivo toxicity studies do not offer the sensitivity to detect changes in mitochondrial function not correlated with functional organ changes.Therefore, animal models that are predictive of the clinical outcome need to be developed. Several transgenic and humanized animal models have been used to explore drug-induced toxicities,. [45] [46] [47]. For example, the heterozygous SOD 2+/- mouse model with reduced expression of the mitochondrial isoform of the antioxidant Mn-SOD has been shown to detect troglitazone induced liver toxicity in contrast to wild-type mice [46]. Similarly, nimesulide, an NSAID that potently uncouples respiration *in vitro*, also elicits liver mitochondrial damage, loss of ATP and ensuing apoptosis in the SOD 2+/- animals but not in wild-type animals [45]. However, contrasting findings have been reported on the use of SOD 2+/- model for the dection of liver toxicants [48] [49]. The use of several rodent strains to increase genetic diversity the toxicologic preclinical species has equally been proposed [50]. Yet much further work is required alongside the need to identify sensitive and specific biomarkers of mitochondrial toxicity. Routine measurement of serum lactate levels has been suggested, since the liver is primarily responsible for lactate clearance and lactate clearance may be impaired in the presence of significant liver dysfunction. Studies have shown the acutely injured liver may itself act as a source of lactate, but as a biomarker of mitochondrial toxicity, elevation of lactate does not appear to show a clear correlation with lactic acidosis [51].

**3. Cholestasis**

*3.1. Current understanding & importance*

Cholestasis is defined as an impairment of bile flow that can manifest clinically as jaundice or icterus (due to increase in total bilirubin). Elevated serum total bilirubin levels combined with increased serum transaminase levels are the components of Hy’s Law and are relevant commonly used clinical indicators of severe liver injury. Hyperbilirubinemia can be caused by multiple mechanisms that fall into the following three broad categories: (1) overproduction, such as in hemolytic anemia; (2) inadequate hepatic uptake, conjugation or secretion of bilirubin; and (3) intrahepatic or extrahepatic impairment of bile flow (i.e., cholestasis). Detection of cholestasis in toxicology species or during clinical testing frequently impacts the development of novel pharmaceutical compounds. In addition, injury of the biliary epithelium, with or without cholestasis, can be species specific and can occur only after months of dosing of a test compound [52]. The injury can be slight, only detectable by histopathological evaluation or can be severe with concomitant cholestasis and hepatocellular necrosis. When present in toxicology studies, its relevance to human is often difficult, if not impossible, to ascertain. Frequently, this type of lesion in toxicology species only becomes apparent after the initiation of clinical enabling toxicology studies and following substantial investment of resources. No reliable biomarkers or clinical pathology correlates exist for detection of biliary lesions, thus assessing the risk for translation to human based on observations in preclinical species is difficult (Table 4). As such, advancement of a candidate to clinical trials can be hindered with the finding of biliary lesion or cholestasis arising in sub-chronic and chronic toxicology studies.

When a drug candidate causes biliary hyperplasia or cholangiopathies in preclinical species, no general *in vitro* assays have been available to test back-up candidates to screen-out this chemical liability (Table 3). Consequently, multiple *in vivo* studies may be required to determine if an alternative compound can be found. Depending on the time it takes for the lesion to manifest, which can be months, substantial investment of resources will have been committed with no guarantee of success. Clearly, effective *in vitro* methods of translational relevance would provide a substantial advantage toward the hit-to-lead, lead optimization and candidate back-up strategies.

The development of a reliable, predictive screening method has proven difficult for two reasons: firstly the tissue architecture required to establish the hepatocellular - cholangiocyte networks and the bile flow and secondly the still largely unknown mechanistic nature of biliary injury and association with intrahepatic cholestatic mechanisms which are multifactorial in nature. The underlying pathology often manifests differently and these mechanisms are likely to depend on intra- and extra-hepatic functionality of bile flow and bile formation. A greater knowledge of the MIE in drug-induced cholestasis and hepatobiliary injury as well our current knowledge on the role of transporters form a central tenet to manage drug-induced biliary injuries.

Intrahepatic cholestasis evaluation routinely uses cultured hepatocytes and cell or vesicle systems with heterologously expressed membrane transporters. A number of *in vitro* assays are available to industry for the study of DILI and drug effect on hepatobiliary transporters on bile acid uptake and efflux. The widely recognized and physiologically most relevant systems are cultured human hepatocytes. These are deemed valuable for use in detailed late discovery work requiring close examination of the mechanistic understanding of drugs with suspected chemical safety liabilities to cause intrahepatic cholestasis. An alternative cell model is the HepaRG cell line with several of the physiological characteristics of human hepatocytes that are essential for the study of hepatobiliary transporter function and cholestasis. These include the polarity of transporters associated to the basolateral and canalicular membranes, regulation and expression of transporters, and well-formed bile canaliculi and production of bile acids. Despite the relevance of human hepatocytes and HepaRG cells for use in the study or detection of cholestatic drugs, high demand in the hit-to-lead and lead optimization stages of discovery have fueled the development of high-throughput screening platforms. Such platforms have included use of vesicles with heterologous expression of human transporters to rapidly identify bile acid transporter inhibitors. These have both advantages and limitations that are important to understand for proper decision making [53] [54].

3.2. *Routinely used endpoints*

Ex vivo analyses for the quantitation of conjugated and unconjugated bilirubin and serum bile acids represents physiologically relevant biomarkers. Overproduction and excessive accumulation of bile acids can be theoretically linked to biliary injury and techniques used for the detection of bile acids are sensitive and specific. However, in practice, bile acid concentrations and efflux into bile are associated with significant variability, exhibit diurnal variation and inter-animal variability of the composite bile acid pool, leading to high probability of missing relevant signals of hepatobiliary injury, particularly for more chronic injuries. Nevertheless, appreciation for the appropriate use of controls affords a utility in the use of bile acid measurements to detect chemical safety liabilities.

The use of vesicle or insect cell preparations to study transporter inhibition is a component of current *in vitro* test systems employed in discovery research. In part, this has been fueled by the technological ability to express human hepatocellular transporter proteins and adapt these to relatively high-throughput screening platforms. Nevertheless, this technology has both advantages and limitations to identify and select drug candidates with optimal safety profiles. The use of BSEP (ABCB11) inhibition has been proposed [55] and employed by some pharmaceutical R&D organizations to identify hazard of human DILI [54]. Nevertheless, the high number of false positives is now appreciated when solely screening for liabilities associated with BSEP inhibition [55]. More recent studies have strived to improve the predictive outcomes by including the MRPs transporter proteins in screens with varying degrees of success [53] [54]. Whilst these studies shed light on perhaps improving the predictive outcome, transporter inhibition remains only one portion of the numerous mechanisms associated with intrahepatic cholestasis and the direct and indirect effects that drugs may have on hepatobiliary function [56] [57] [58]. A noteworthy limitation in transfected transporter model systems (e.g. insect cell systems) is the inability to assess the effect of transporter redundancy [55]. Furthermore, understanding the relevant concentrations for evaluation is a major challenge at the early stages of drug discovery. Hence, the complexity of hepatobiliary function points to the need to assess selected compounds in definitive studies with well-characterized hepatocellular systems.

Tissue slices have also been used to study the relevance of transporter inhibition [59]. In tissue slices, incubation of a compound in the presence of a pan-inhibitor of transporters followed by analytical quantitation of drug uptake allows for an indirect analysis of all potential transporters (unpublished observations). Liver slices also represent an opportunity to interrogate effects across animal species and humans, since slice cultures can be readily prepared and used to classify toxicants [60]. Use of PHH sandwich cultures and the HepaRG cell line are suitable for hepatobiliary studies [61] [58] . The adoption of hepatocyte-based assays for routine employment in drug discovery will greatly benefit from the identification and use of novel transporter selective probe substrates. In addition, biomarkers for use *in situ* hepatocyte studies will facilitate greater use of cellular assays in drug discovery such as microRNAs (miRNAs). These miRNAs either released or upregulated, hold potential as biomarkers of theoretical promise (see section 4).

**4. Role of the immune system in human DILI**

4.1.. *Current understanding & importance*

DILI can be broadly classified into predicable dose-dependent ADRs and unpredictable ADRs (idiosyncratic). Although idiosyncratic ADRs often present with a complex concentration-response relationship, and occur with only a few drugs, a high concentration of a given drug has a higher probability of initiating an immune response. The immune system is highly implicated in most cases of idiosyncratic toxicities, yet the direct evidence and implication of the immune system in the initiation, progression and resolution of DILI is only recently making progress with experimental evidence and clinical reports (Table 5).

Compared to the intracellular events associated with mitochondrial toxicity, cytotoxicity and intrahepatic cholestasis, immune-mediated DILI (iDILI) is a complex association of both intra- and extra-hepatic signalling [62]. Thus, hepatocellular damage and dysregulation of the microenvironment of the liver are believed to play a critical role in the initiation of the immune response. The potentiation and resolution of immune response attributed to resident innate immune cells and infiltrating neutrophils and lymphocytes [63] [64]. Figure 4 illustrates the cellular aspects of immune-mediated DILI. The role of the adaptive immune response is supported by evidence related to delayed onset of symptoms and a rapid onset upon re-challenge [65], as well as by the clear association with certain human leukocyte antigens (HLA) [66] and the presence of antibodies to drug and/or modified drug-peptide antigens [67]. Some examples of established DILI associated with specific HLA alleles or HLA haplotypes include amoxicillin-clavulanate (HLA-DRB1\*15:01-HLA-DQB1\*06:02) [68] flucloxacillin (HLA-B\*57:01) [69] [70], lapatinib (HLA-DRB1\*07:01, HLA-DQA1\*02:01) [71], and ximelagatran (HLA-DRB1\*07:01) [72]. Although the expression of these alleles predisposes some patients to DILI, the exact molecular mechanism by which the presence of a particular HLA allele increases a patient’s susceptibility to DILI remains undefined and is an area of continuing research. Furthermore, information is lacking on the mechanism of tolerance in individuals that express the implicated alleles but do not suffer from DILI upon drug exposure. These obvious knowledge gaps remain major limiting factors in the use of HLA alleles as predictive biomarkers during the pre-clinical stages of drug development.

4.2.. *Model systems in use for detection of immune-mediated DILI*

Given the unpredictable nature of idiosyncratic DILI and associated severity of ADRs in patients, there is an urgent need for early *in vitro* screening and non-clinical tests to aid prediction and risk assessment of iDILI before clinical development. Unfortunately, *in vitro*-based models for application in drug discovery are distinctly lacking. The predictive capacity of *in vitro* tests to assess iDILI potential using covalent binding, glutathione trapping and cell viability assays is limited by low sensitivity and poor predictive value of liver injury. However, the elimination of potential chemical liabilities, such as covalent binding, could simplify drug discovery if an alternative lead compound with the requisite pharmacology is already available. Nevertheless there is an unmet need to develop predictive *in vitro* test systems for risk assessment when it is necessary to progress a compound with such a chemical liability. The development of co-cultures of parenchymal cells and non-parenchymal cells is offering the potential for the assessment of innate response to chemical insult, however these models are still at an early stage and much has yet to be accomplished to define the significance of the physiological, pharmacological and toxicological relevance to the *in vivo* situation in humans.

4.3*. In vivo non-clinical models for the detection of immune-mediated DILI*

Despite the difficulties in developing animal models of hepatotoxicity, progress has been made using experimental mouse models to understand the pathogenesis and the role of the immune system in DILI [73] [74]. The susceptibility of rodents to trovafloxacin in the presence of an inflammatory stress induced by LPS demonstrates a role for the innate immune system in the induction of DILI and is potentially a critical feature for adaptive immune activation [75] [76] [76]. In the mouse model of isoniazid-induced liver injury developed by Metushi and Uetrecht [77], the authors suggested that overcoming the dominant immunological tolerance in the liver was critical to the development of a valid animal model of idiosyncratic DILI. In subsequent experiments involving PD-1 (-/-) mice treated with amodiaquine and antiCTLA4, the authors reported features of liver injury similar to idiosyncratic DILI observed in patients [73]; Also, C57BL/6 CD4+-deficient mice devoid of the major histocompatibility complex (MHC) class II molecules have been used to study flucloxacillin sensitization [74]. Furthermore, it has been demonstrated that inhibition of immune tolerance by depleting myeloid derived suppressor cells in Balb/cj mice resulted in halothane-induced hepatitis [78]. These animal models of DILI demonstrate a role for the immune system in drug-induced hepatotoxicity. The immune-mediated toxicity of flucloxacillin is further supported by work showing the upregulation of immune and inflammatory genes in treated mice [79]. These murine models with a perturbed immunological function have been selected in the pursuit of an animal model that might mimic idiosyncratic DILI. Nevertheless, such models cannot be employed prospectively by the pharmaceutical industry until the models have been validated with appropriate sets of positive and negative test compounds.

The lymphocyte transformation test, which measures *in vitro*T cell proliferation in response to chemical insult, and the ELISpot cytokine secretion assay have been successfully used to confirm clinical diagnosis of drug hypersensitivity[70]. In addition, the generation of drug antigen specific T cells from peripheral blood mononuclear cells of either healthy volunteers or hypersensitive patients has been reported and offer an avenue for improved mechanistic understanding of the role of T cell activation in iDILI [68] [70]. These tests have greatly enhanced our understanding of the pathogenesis of DILI through the adaptive immune response, but the diversity of HLA phenotypes does not at this time allow the assay to be used *ab initio* in drug discovery to identify back-up candidates or to design-out immune activation of candidates identified from clinical research.

***5. In vitro* transcriptomic approaches to DILI**

Among different ‘omic approaches, the use of miRNAs and transcriptomic profiling have been investigated extensively as mechanistic markers of liver injury [80] [81] [82]. Release or overexpression of miRNAs, such as miRNA122, is diagnostic of liver injury. Tissue specific miRNAs can be compared in human and animal tissues to develop miRNA panels reflective of liver injury [83] [84]. The miRNAs are stable in plasma and thus allow opportunities for quantitation. However, miRNAs have limitations similar to other biomarkers, such as bile acids, which are also highly dependent on time of sampling. Despite these limitations, evaluation of miRNAs and newer biomarkers of liver injury, as potentially fit-for-purpose for use in drug discovery and investigative toxicology, is gaining traction to help bridge pre-clinical and clinical safety evaluations in risk assessment. What will greatly help the future uptake and consolidation of these novel biomarkers will be the availability of control baseline datasets validated for animal and human populations. Gene signatures are sets of transcripts identified using machine learning algorithms from databases of transcriptomic profiles of sets of training and testing compounds [82]. These signatures can help categorize the MoA of novel experimental compounds (chemogenomic approaches as chemical classifiers), but may also provide mechanistic and physiological insight into toxic changes*.* Unsupervised network methods organize transcriptomic data into networks using coalescent properties of the biological systems, e.g. co-expression [85]. In many cases, co-expression networks recapitulate known biological response pathways but may also define novel responses networks. Co-expression networks are highly perturbed in cultured hepatocytes in the absence of chemical treatment contributing to poor concordance in network responses to chemical comparing *in vitro* to *in vivo* results [86]. Network methods may provide a systems level approach to monitoring the integrity of biological response networks in next generation hepatocyte culture models. Thus, genomic approach applied to *in vitro* liver models hold promise not only for elucidating mechanistic markers of DILI and predicting DILI potential for new chemical entities, but as a method to evaluate the similarity of responses in improved next generation liver culture *in vitro* systems to intact liver compared to simple 2D culture models commonly employed in mechanistic studies [87].

1. **Chemically reactive metabolites**

Chemically reactive metabolites are recognized as a potential causative factor for DILI [88]. Several groups have published that quantification of reactive metabolite formation by covalent binding assessment in *in vitro* human liver models and calculation of body burden by use of daily dose and *in vitro* intrinsic clearance values can distinguish hepatotoxic from non-hepatotoxic compounds [89] [90] [91].

In a drug discovery setting, covalent binding of reactive metabolites to liver proteins is typically not assessed due to lack of availability of radiolabeled compound at this stage. Instead, pharmaceutical companies have implemented reactive metabolite trapping assays and mechanism based CYP inactivation screens to help identify reactive metabolite formation and elucidate the structure of reactive metabolites to guide medical chemistry to reduce and/or avoid the formation of reactive metabolites [88]. Sakatis *et al.* [92] have published the concordance between clinical hepatotoxicity liability, dose and bioactivation potential by use of glutathione trapping assays, mechanism based CYP inactivation screens or covalent binding assessment. Immune-mediated DILI are not yet fully understood. Among several hypotheses is the high proportion of drugs involved in immune reactions which will form reactive metabolites and covalently bind to proteins to form immunogenic haptens which in many cases forms a first step in the generation of an idiosyncratic response [93] [94].

**7. Cell-based models for DILI risk assessment**

Liver-derived *in vitro* models are widely adopted for use in ADMET studies in pharmaceutical R&D. Over recent years, many of these models have been very thoroughly and extensively reviewed [3]. Freshly isolated and cultured primary hepatocytes and cell lines are currently the most commonly accepted cell models for use in toxicological studies. Changes in phenotype cell function and gene expression are nevertheless a feature associated with 2D primary human hepatocytes (PHH) cultures. These phenotypic changes may influence the toxicological interpretation of results. However, evidence of marked differences in both sensitivity and specificity in detection of DILI *versus* non-DILI compounds in monocultures of 2D PHH *versus* hepatoma cell lines appears limited in short-term tests [95]. Such data point to potential improvements for the detection of hepatotoxicants by use of more advanced genetically engineered cells, 3D or microphysiological (MPS) systems. Examples of genetically modified cells, include the Upcyte™ technology to promote the proliferation of PHH without influencing the phenotype [96], and recombinant adenoviruses encoding DMEs to allow the rapid high-efficiency and controlled infection of HepG2 cells for use in metabolite-dependent DILI hazard identification [4]. However, such alternatives appear poorly adopted by industry at present. Induced pluripotent stem (iPS) cells have attracted much attention to complement existing hepatocellular models. Nevertheless, while providing opportunities to develop genetically characterized cells, their low level of DMEs and immaturity still represent critical limitations for use at present [97]. 3D cell models of PHH, cell lines and genetically modified hepatoma cells have been suggested to demonstrate greater physiological and pharmacological parity with human liver tissue [98]. These characteristics are expected to confer improvements in their use for detection of hepatoxicants. However, from an industry perspective much remains unknown about the true performance characteristics of advanced cell models for routine use by industry; either as 3D monocultures or co-cultures of hepatocytes with non-parenchymal cell (NPCs), and whether these are more suited for use in detection of toxicological endpoints. Clearly the advantage of these 3D models alongside microphysiological systems will be the opportunity to perform studies over extended periods (e.g., 7-14 days) to allow for the detection of low level and time-dependent drug effects. Unfortunately, there remains a lack of quantitative comparative benchmarking for many of these advanced models, especially where it relates to cellular differentiation state. Consequently, there are no industry standards or common acceptance of which cell models are more suitable for the detection of certain forms of human DILI.

Given that current and likely future models will not exactly replicate the complexity of the human liver, it appears critical to define what mechanistic endpoints relevant to human pathologies are best represented by each test system. In the context of hazard and risk assessment, this would allow to better define what purpose each cell model can be used for and establish reproducible bench-marking data and decision cut-off points rather than striving to develop a single test system that encompasses the spectrum of *in vivo* human DILI events. The future implementation and use of the more advanced cell models will inevitably incur further resourcing requirements and costs associated with lower throughput, compound requirements, study duration and technical competences. This will likely define a tiered approach towards early compound triage with 2D screens to rank-order promising subsets for further confirmatory tests in more advanced test systems.

DILI in human is almost always a result of chronic chemical aggravation of the hepatocyte, which linked in some way to individual patient factors. This further supports the development of *in vitro* models suitable for longer use (i.e., > 1-2 weeks) than currently in practice. Such typically more sophisticated test systems require more resources and have a cycle time not consistent with the pace of medicinal chemistry. Thus, this would need to be integrated as part of a tiered system (from simple and rapid to complex and lengthier models) during the lead optimization process. Ultimately, sophisticated and evidence-based quantitative systems toxicologymodels, appropriately validated with consensus reference compounds, will have to be developed. This will enable rationale translational predictions based on multiple chemical properties of experimental compounds determined as quantitative or categorical measures in a well-designed preclinical screening battery or generated through computerized approaches. Coupled with greater evidence-based knowledge of human variation in DILI susceptibility should maximize the probability of minimizing DILI in the clinic.

**8. Conclusions**

Achieving a productive drug discovery pipeline and tackling unacceptable levels of safety-related, late-stage attrition merits continued efforts and resources to develop predictive *in vitro* test systems for use in drug discovery. From an industry perspective, *in vitro* test systems for the hazard identification and risk assessment of small molecules with potential to cause liver toxicity will remain an essential part of this process. This will need to take into account the new classes and biological types of therapeutic agents that the pharmaceutical industry is developing for the next decade. What is becoming increasingly apparent is the need for: (1) test systems with levels of sufficient sensitivity and selectivity to detect compounds with potential to induce moderate and low-level hepatotoxicities over longer incubation periods; (2) modelling tools to better estimate pharmacologically relevant clinical blood and liver concentrations; and (3) a clear bench-marking of existing systems robustly evaluated for performance. In early screening paradigms, stable cell phenotype(s) permits consistent intra- and inter-assay readouts to probe the subtle effects of novel chemical entities on biological response, rather than non-drug related variables introduced as artefacts in these test systems. This ensures that in early lead optimization, test systems are reproducibly applied within and across chemical series prior to preclinical candidate selection. Improvements in robustness and characterisation of cell models is certainly needed, coupled with a much improved mechanistic understanding and identification of translationally relevant biomarkers to define chemical risks and to use these for accurate extrapolation to humans [99]. The development of test systems equally requires the selection of compounds which can be used as reference chemicals to calibrate readouts for the risk assessment of novel molecules. Above all, it is important to avoid elaborate systems that do not offer a clear step change over the simplest functional test systems for risk assessment. Likewise, when using advanced systems such as spheroids and MPS, steps to ensure that the properties of experimental compounds are amenable for use (e.g. solubility and stability, effects of buffer conditions and non-specific binding) will be needed. Underlining this are the requirements to generate insights into signalling pathways and quantifiable endpoints to enable pharmacokinetic-toxicokinetic modelling of exposure and biological responses, upstream of hepatocellular response(s) to chemical insult.

9. **Expert opinion (summing-up)**

Endpoints commonly employed in 2D cell culture systems for the assessment of drug-induced cytotoxicity are broad, yet their value to predict human DILI remains questionable. This is in part attributable to (1) a lack of harmonization of protocols [100], (2) a lack of consensus on the most appropriate test systems and the most clinically relevant mechanisms of liver toxicity, and (3) the reagents to calibrate these test systems. It is widely accepted that these simple test systems enable an acceptable assessment of ‘cell health’ and serve well to prioritize and rank compounds (Table 1). The next generation of cytotoxicity test systems ideally should include evidence of a DILI selective measure of risk, with flexibility to perform not only short-term incubations, but equally incubations over several days or even weeks at concentration multiples reflecting the intended pharmacological exposure range(s) in liver. Spheroid models offer promise in this direction by enabling longer-term exposure at pharmacologically relevant doses. In addition, they offer a greater chance to mimic the disease state and understand the potential safety risks in specific patient populations. In this respect, more physiologically relevant 3D models allow for the possibility to study in greater depth the mechanisms of drug toxicity.

In contrast to evidence necessitating short- and long-term cell cultures for cytotoxicity, recent investigations [32] show that acute metabolic modification, via glucose-deprivation over a 4-hour period immediately prior to compound addition is sufficient to allow the identification of drugs which induce mitochondrial dysfunction. This “glu-gal” approach gives no detailed information on the mechanism of mitotoxicity and the correlation with *in vivo* effects remains to be firmly established. Measurement of OCR and ECR combined with stress tests can give mechanistic information about mitochondrial toxicity with relatively high throughput [26] [35]. In a typical assay set-up, effects are measured directly after addition of the compound to isolated mitochondria or HepG2 cells. Both models nevertheless have inherent merits and limitations. Use of isolated mitochondria may give mechanistic insight for the study of direct drug effect on the mitochondrion, but do not account for drug hepatocellular uptake, distribution and elimination that can contribute to the amount of drug reaching the mitochondrion. HepG2 cells are largely devoid of drug-metabolising enzymes to assess the effect of both parent drug and metabolites, but have thus far remained the model of choice for mitochondrial toxicity testing to achieve reproducible readouts (*section 2*). Given the above, there is a need to: (1) develop robust models for the assessment of mitochondrial toxicity of both parent drug and metabolites, (2) define an integrated work flow of short-term (hours) testing for screening and long-term tests (days) for parent and metabolite, (3) identify mechanisms requiring longer treatment periods (e.g. mtDNA damage), and (4) have access to assays for mechanistic insight to screenout chemical safety liabilities associated with specific mitochondrial targets within project series.

The use of 2D cell culture models and heterologous transporter expression as vesicles or cell-based assays permit the study of drug effect on transporter function by use of PHH, or cell lines with appropriate expression of the hepatobiliary transport proteins on the basolateral and apical membranes. Only where slow onset of hepatobiliary effects may be suspected to occur would the effect of drugs or metabolites on bile acid transport require longer incubation than those currently employed. Microphysiologial systems with well-formed bile canaliculi and biliary cell ductule structures, which would facilitate elimination of bile acids and avoid bile acid accumulation within the microtissues, would offer a test system to complement those currently available to study intrahepatic cholestasis and biliary toxicities associated with extrahepatic cholestasis. Co-cultures with non-parenchymal cells could allow exploration of innate responses for the initiation or potentiation of cholestasis. With detailed time-resolved studies of cholestasis, the subsequent generation of cirrhosis and fibrotic signals reported with some drugs and only in some individuals may be possible. Measurement of total bile acid and bile acid composition requires accurate, reproducible and sensitive techniques. The incorporation of dynamic flow systems to allow for the addition of bile flow variables and composition of the total bile acid pool with time of incubation for a better representation of liver physiology would be of value. Nevertheless, it is important to pinpoint those drugs affecting only certain classes of bile acids of quantitative importance and not others. Therefore, assessment of only one prototypical bile acid, such as taurocholic acid, as representative of the general bile acid formation and flow is perhaps misleading.

The rare and idiosyncratic nature of DILI requires predictive test systems to identify chemical risk and reduce clinical incidence. Progress has been made in developing *in vitro* and *in vivo* assays aimed at understanding the molecular mechanism of iDILI [67] [74] [101] [102]. However, developing test systems to predict the potential immune-mediated DILI liability of compounds in early drug discovery continues to pose challenges. This difficulty results from the multiple risk factors linked to idiosyncratic DILI and apparent complexities in heterogeneity of cell types and signalling involved in immune function. Although advancements have been made to comprehend the role of the innate immune system, the involvement of the adaptive immune system in iDILI has not been fully characterised and this will remain challenging. One important aspect to incorporate into predictive assays will be the impact of an individual’s genotype on the pathogenesis of drug-induced hepatotoxicity. Such an *in vitro* approach has been undertaken to design out HLA-associated toxicity with an established drug abacavir [103], but the step change to predict toxicity associated with 2000 HLA alleles *ab initio* represents a monumental technological challenge [104].

In some instances, pharmacogenetics has provided vital insights into the mechanisms of idiosyncratic drug toxicities and has been applied clinically to prevent or reduce the incidence of these reactions. For example, for abacavir hypersensitivity reaction, the HLA-B\*57:01 has a negative predictive value of 100% and a positive predictive value of 47.9%. However, it is still unclear why 52.1% of individuals expressing the HLA-B\*57:01 risk allele do not experience abacavir hypersensitivity. Therefore, it is important to continue in the pursuit to identify contributing non HLA factors (genetic and non-genetic) that predispose individuals to ADRs. In summary, the prediction of idiosyncratic DILI will require a more precise understanding of human individual risk factors, which can then be factored into preclinical test systems.

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**Conflict of interest**

There is no conflict of interest

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 Papers of special note have been highlighted as either of interest (\*) or of considerable interest (\*\*) to readers.

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| **ARTICLE HIGHLIGHTS** |
| * No standard approach exists in the pharmaceutical industry with respect to the systematic and sequential use of *in vitro* test systems for detection of human DILI
* Of the numerous tests for DILI, a first screen for the detection of cytotoxicity is broadly adopted across the industry
* No test systems have been robustly evaluated for performance and bench-marked across the industry
* A consensus among EFPIA members identifies several major gaps for improvement for the detection of DILI
* Greater Industry consensus is likely only achievable with systematic evidence-based pharmacological and physiological characterization of routinely used hepatocellular-based cell models and endpoints fit for decision-making in Drug Discovery
* Better understanding of DILI will lead to better, predictive in vitro tools in the future
* 3D microphysiological cell models are exciting, but still require characterization and validation for acceptance and application within EFPIA companies
 |
| This Box summarizes key points contained in this article |

**Table 1. Cytotoxicity and cell health endpoint assays**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **Functional**  | **Gene**  | **Mitochondrial**  | **Entire cell toxicity** |
| **ATP, MTT, Rezasurin** | **X** |  | **X** |  |
| **LDH release** | **X** |  |  | **X** |
| **CFDA-AM** | **X** |  |  | **X** |
| **Neutral Red** | **X** |  |  | **X** |
| **Impedance** |  |  |  | **X** |
| **DNA release** |  | **X** |  | **X** |
| **Albumin** | **X** |  |  | **X** |
| **Urea** | **X** |  |  | **X** |
| **miR122** |  | **X** |  | **X** |
| **Nrf2** |  | **X** |  | **X** |
| **High content imaging for cell health parameters** | **X** |  | **X** | **X** |

**Table 1. Cell health and Cytotoxicity endpoint assays**. Endpoints used for the assessment of cytotoxicity include: lactate dehydrogenase (LDH) release, membrane permeability; MTT (mitochondrial metabolism & respiratory toxicity); general physiological cell-state; Neutral red (lysosomal activity); Sulforhodamine B (cell proliferation); ATP content. In the presence of high glucose-containing medium, some endpoints will reflect general cytotoxicity in contrast to a galactose-containing media (see mitochondrial test systems). Impedance-based real time cell analyzer (RTCA) is a label-free method for screening cytotoxicity with potential application for mechanistic understanding of cellular targets. High content imaging can employ various cell stains to perform multiparametric assessment of cell health.

**Table2. *Endpoint measurements in cell health (cytotoxicity) tests***

|  |
| --- |
| * **Cell counting and indirect measurements of cell toxicity:** Cytotoxicity can be measured by direct cell counts using bright field imaging, by use of fluorescent DNA stains, correlating enzymatic reactions such as ATP, tetrazolium/formazan, and resazurin measurements [105] [13]
* **Loss of cell membrane integrity**: Reduced cell membrane integrity as a measurement for late apoptosis or necrosis. Can be performed by measurement of leakage of intracellular proteins such as lactate dehydrogenase (LDH), or by retention or penetration of fluorescent probes [106].
* **Lysosomal function:** Lysosomal function can be assessed using lysosomotropic fluorescent probes, such as LysoTracker, or indirectly by measurements of accumulation of neutral or phospholipids [107].
* **Cell morphology, proliferation**: Cell morphology and proliferation can be assessed using impedance assays, or by use of imaging with fluorescent probes [108].
* **Phospholipidosis**. Drug-induced phospholipidosis can be assessed by use of the LipidTox assay [109]. Lysosomal function may be severely perturbed at the onset of hepatocyte death [110]. Can be associated with phospholipidosis as a consequence of disturbed phospholipid breakdown in lysosomes. Occurrence of phospholipidosis often related to the physicochemical characteristics of compounds.
* **Glutathione:** Measures of glutathione levels as can be performed with fluorescent probes and used to measure oxidative status of cells [12].
* **Hepatocellular markers**: Selective endpoint assessment of cell function can be made by measurements of urea and albumin formation, or specific hepatocellular markers such as miRNA122
* **High content imaging**: Multiparametric imaging can be used to combine several of the above mentioned end points [13].
* ***Limitations & gaps in knowledge of endpoints and/or suitability***
* Most endpoints are broad assessments of cell health or a specific organelle function without insight into true molecular events.
* Structure activity relationships are typically hard to interpret as decreases in cell health can occur through disturbances of several pathways.
* Depending on the type of cell health measurements, there can be several interpretations of the result. As an example, loss of cell counts may be due to decreased cell health or a simple loss of cell attachment; tetrazolium/formazan reactions are not only dependent on cell health but also endocytosis/exocytosis. ATP measurements may be due loss of viability or decreased mitochondrial activity. Therefore, it is often prudent to assess cell health with multiple endpoints.
* Different endpoints may have different sensitivity and specificity towards DILI based on the technical method and endpoints in use to detect different mechanisms.
* Most cell health endpoints are similar across cell types and do not reflect specific organ toxicities. The value of the endpoints is highly dependent on the cell system.
* There is currently no consensus on time points for assessments, or estimated separations from therapeutic exposures to compromised cell health needed to avoid DILI. Most Companies rely on internal validations for their specific assays.
* Physicochemical properties of molecules may interfere with cell health measurements such as poor solubility, lipophilicity, auto-fluorescence *etc*.
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**Table3. *Endpoint measurements in mitochondrial toxicity tests***

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| * **Glucose vs galactose assay.** Cells cultured in galactose- or glucose-supplemented media facilitate the identification of mitochondrial-targeted chemical insult as opposed to general cytotoxicity, respectively [31] [30]. Different endpoints can be deployed (Figure 2).
* **Measurement of mitochondrial membrane potential** **(MMP)**. Fluorescent probes for MMP (e.g. TMRE, Mitotracker, JC-10, Rhodamine 123) in different cell types or isolated mitochondria can be measured using plate-readers or image analysis and multiplexed with other parameters of mitochondrial and cell function e.g. oxidative stress, apoptosis and Steatosis [111] [12].
* **Measurement of mitochondrial respiration.** Mitochondrial respiration can be measured by use of isolated mitochondria or cells, with fluorescence-based tests of mitochondrial oxygen consumption (e.g. Luxcell or XF analyzer). Specific mechanistic end-points by use of stress tests, e.g. ATP production, uncoupling and spare respiratory capacity can be employed [112] [113].
* **Polarography and** **Mitochondrial swelling**. Assessment of the morphological impact of drugs on mitochondria can be evaluated by use of isolated mitochondria. [33] [34].
* **Immunocapture Targeting**. Drug induced mitochondrial toxicity by use of immunocapture based OXPHOS allows target identification to identify drugs that directly inhibit four of the OXPHOS complexes: I, II, IV, and V activity. Assay can be applied for use in drug screening [114]
* **Beta-oxidation assays**. Measurement of oxygen consumption in parallel with polarography from beta-oxidation using isolated mitochondria, or by measurement of ketone bodies [115] with use of radiolabeled fatty acids. Inhibition of beta-oxidation can equally be determined by use of fluorescent neutral red with the detection and accumulation of neutral lipids in the cytoplasm.

***Limitations & gaps in ‘knowledge’ of in vitro test systems*** * **Glucose vs galactose assay.** Requires two parallel cell cultures. Cells cultured in galactose have tendencies of becoming fragile in long term galactose containing medium and no detailed mechanistic information is obtained.
* **MMP assays.** Probe drug interactions possible [116]No detailed mechanistic information. Multiparametric HCA requires careful design, extensive data analysis and good reference compound dataset.

**OCR/ECAR** **assays.** Acute measurements of toxicity and thus effects of compensatory mechanisms are limited and no long-term effects on mitochondria can be measured.. Preliminary assessment of cytotoxicity and effect of medium change is required before OCR/ECAR assays are amenable for use, in long-term testing.* **Polarography.** Extremely low throughput requiring high quantity of cells to isolate mitochondria. Devoid of the cellular environment..
* **General.** No thorough assessment of the most promising cell model coupled with appropriate endpoint measurement(s) exists. No proper in vivo-in vitro correlation, evaluation of predictivity and comparison between the methods exist.
* **Combining Endpoint Measurements**. Results from different mitochondrial endpoints (e.g. glu-gal, mitochondrial respiration, glycolysis and reserve capacity) or with other endpoints such as mitochondrial respiration and BSEP inhibition is likely to lead to the classification of drugs as hepatotoxins. Notably, however, these effects are typically only observed at *in vitro* concentrations markedly above the therapeutic Cmax of many drugs (31).
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**Table4. *Assessments by industry for the detection of cholestasis***

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| **Issues with preclinical hepatobiliary toxicants** * Detection of hepatobiliary injury frequently occurs only during the drug development, typically after initiation of Phase I, once substantial investment in non-clinical and clinical research has occurred [52].
* **Clinical characteristics**
	+ Poor correlation of clinical pathology readouts with no reliable or sensitive biomarkers available.
	+ Pathologies frequently manifest differently and maybe related to chemical space of the drug - include intrahepatic cholestasis**,** bile duct hyperplasia; implications of neoplasia/fibrosis [52]**,** hyperbilirubinemia**,** inflammation/ inflammatory infiltrate in portal tracts**,** bile duct necrosis**,** bile duct obstruction.
* **Mechanisms**
	+ Absence of detection of intrahepatic cholestasis in rodents is attributed to differences in the selectivity, specificity, relative abundance of transporters, and composition of the bile acid pool.
	+ Bile formation and flow measurements in animal species are often highly variable and low animal number limits effective use in assessment of risk**.**
	+ Mechanisms of hepatobiliary toxicants are likely multifactorial in nature, the complexity of which do not permit a single non-clinical approach to assess risk (intra- and extrahepatic).
	+ The role of transporters is highly implicated in cholestasis. Isolated membrane or insect cell preparations and hepatocyte cultures are typically *in vitro* test systems employed for use in drug discovery.

***Limitations & gaps in ‘knowledge’ of in vitro test systems*** * No test system(s) currently exists for the assessment of biliary toxicities
* Isolated membrane and cell-based transporter assays permit the study of drug inhibition on transporter function, but can differ in readout of drug liabilities; Vesicles preparations do not account for metabolites as inhibitors of transporter function, impact on gene regulation or up/down regulation and integration of transporters to the apical and basolateral cell membranes.
* Inhibition of BSEP activity may serve to triage large compound series with chemical risk, but false positive and negative results are expected. [58]

, * Florigenic analogues of bile acids, such as cholyl-l lysyl (CLF) and cholyglyacrylamide (CGamF), whilst useful for application to multiplex with other HCA formats have yet to be shown as specific markers of individual membrane transporters[117]
* Novel selective probes substrates for individual transporters are required for use in cell-based assays. Such probes should serve as florigenic analogues of naturally occurring bile acids with similar kinetics to the endogenous monovalent and divalent bile acid conjugates.
* Transcriptomic signatures by use of a database of compounds with concurrent and bile duct injury in the rat have been used, but issues of relevance to human are not fully described and validity of IVIVE transcriptomic profiles can be limited
* Role of adaptive immunity in cholestasis cannot be assessed in assays currently available and therefore cannot help identify association of cholestasis and immune-mediated idiosyncratic DILI.
* Selectivity, specificity and enzyme kinetics of bile acids across the membrane transporters for key classes of bile acids are incomplete; limits the detailed DDI assessment and knowledge of mechanisms (competitive and non-competitive inhibition) and impact on accumulation of 1° and 2° bile acid pool.
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**Table5. *Evidence for the role of immune function in human DILI***

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| **Evidence of immunity in progression and resolution of iDILI*** **Clinical characteristics**
	+ Liver is a highly immunologically tolerogenic organ and sometimes referred to as an immune privileged site [64].
	+ Dysregulation of the tolerogenic immune microenvironment of the liver is believed to play a critical role in the initiation, exacerbation, progression and resolution of iDILI [118]
	+ The cytokine microenvironment surrounding immune cells can determine immune cell polarisation but also the severity of DILI [119] [120].
	+ Infiltration of granzyme-B secreting lymphocytes for at least one drug, sulfasalazine, upon histological examination of inflamed human liver [121]
* **Mechanisms**
	+ Bio-activation of parent drug that generates protein-reactive metabolites can activate the immune system.
	+ The pro-inflammatory cytokines such as IFN-γ, TNF-α and IL-1β are strongly associated with pathogenesis of DILI. Increased levels of IL-6, IL-8 and monocyte chemoattractant protein (MCP-1) have been observed in patients with elevated ALT (>1000 IU/L) following acetaminophen overdose [122] In other studies, increased serum levels of IL-17, IL-21, IL-1β, IL-10, IL-12, IL-13 and TNF-α were associated with the onset and progression of DILI [123] [124]
	+ While IL-6 and TNF-α is linked to DILI progression, the expression of IL-10 is often associated with liver protection and resolution of symptoms. Nevertheless, the upregulation of IL-10 during the early phase of acute liver failure is correlated with poor disease prognosis in patients [125]
	+ Associations between susceptibility to iDILI and the expression of HLA have been established.
	+ Of three immune-mediated mechanisms of iDILI, the hapten hypothesis has been demonstrated in ADRs targeting the liver [70]

***Gaps in the knowledge and available in vitro tests for safety assessment**** It is unknown whether antigen presentation and activation of T cells occurs in the liver or whether dendritic cells migrate to the lymph nodes where they prime and activate T cells.
* Whilst the role of cytokines in in vitro studies by use of PHH remains unequivocal, use of the HepaRG cell-line with reproducible biochemical readouts permits measure of the role of IL6 signalling relative to corresponding controls [126]
* Capacity of in vitro covalent binding tests and cell viability is limited by poor sensitivity and prediction [102]
* Recently, *in vitro* assay capable of integrating inflammatory and immune gene signatures to predict the potential of a drug to initiate DILI have been developed [101] [127]
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