Toxicogenomics of drug induced liver injury – from mechanistic understanding to early prediction.

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ABSTRACT
Despite rigorous preclinical testing, clinical attrition rates in drug development remain high with drug-induced liver injury (DILI) remaining one of the most frequent causes of project failures. To understand DILI mechanisms, major efforts are put into the development of physiologically relevant cell models and culture paradigms with the aim to enhance preclinical to clinical result translation. While the majority of toxicogenomic studies have been based on cell lines, there are emerging trends toward the predominant use of stem cell-derived organoids and primary human hepatocytes in complex 3D cell models. Such studies have been successful in disentangling diverse toxicity mechanisms, including genotoxicity, mitochondrial injury, steatogenesis and cholestasis and can aid in distinguishing hepatotoxic from nontoxic structural analogs. Furthermore, by leveraging inter-individual differences of cells from different donors, these approaches can emulate the complexity of polygenic risk scores, which facilitates personalized drug-specific DILI risk analyses. In summary, toxicogenomic studies into drug-induced hepatotoxicity have majorly contributed to our mechanistic understanding of DILI and the incorporation of organotypic human 3D liver models into the preclinical testing arsenal promises to enhance biological insights during drug discovery, increase confidence in preclinical safety and minimize the translational gap.

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Introduction
Drug-induced liver injury (DILI) constitutes an important phenomenon in drug development and clinical practice. DILI is idiosyncratic and rare with DILI risk estimates for individual medications ranging from 1 in 10,000 to 1 in 100,000 individuals upon exposure (Larrey 2002; de Abajo et al. 2004; Navarro and Senior 2006; Bell and Chalasani 2009; Björnsson 2010). Similar results were obtained for liver injury due to herbal or dietary supplements (Navarro et al. 2017). Overall, DILI accounts for 52% of acute liver failure cases of which around 75% are due to intentional and unintentional acetaminophen overdoses (Ostapowicz et al. 2002). Excluding acetaminophen, overall DILI incidence at the population level is considerable with crude annual incidence rates in the general population of 1 in 5,000 to 1 in 10,000 (Sgro et al. 2002; Björnsson et al. 2013). In addition to its clinical relevance, hepatotoxicity constitutes a major hurdle in drug development and remains a leading cause of drug failure during both preclinical and clinical stages (Watkins 2011; Cook et al. 2014). Furthermore, hepatotoxicity constitutes the most common reason of drug withdrawals in post-marketing stages accounting for 81 medicinal products between 1953 and 2014 out of a total of 462 withdrawals (18%) (Onakpoya et al. 2016).

Importantly, prediction of DILI propensity for a new compound is difficult using animal models due to extensive species-specific differences in expression patterns, isoform composition, and activities of drug metabolizing enzymes and drug transporters (Martignoni et al. 2006; Chu et al. 2013). Consequently, in vitro models using human liver cells are integral, well-established tools for a multitude of toxicological and pharmacological applications in drug development, including the identification of hepatotoxic liabilities and the delineation of the underlying toxicity mechanisms (Figure 1 and Lauschke et al. 2016; Lin and Khetani 2016; Kuna et al. 2018; Lauschke et al. 2019; Zhou et al. 2019). In the past decade, there has been a growing body of literature utilizing comprehensive expression profiling of human liver cell systems to investigate hepatotoxicity mechanisms. While most studies are based...
on the hepatoma cell lines HepG2 and HepaRG, there is an increasing number of studies evaluating toxicity in stem cell-derived hepatocyte-like cells (HLC) and primary human hepatocytes (PHH) in conventional 2D monolayers and, more recently, in 3D cultures (Table 1).

HepG2 cells

HepG2 cells have been the predominant cell model for toxicogenomic analyses. While phenotypes and sensitivity to toxic insults differ drastically between HepG2 and primary human liver cells (Wilkening et al. 2003; Hart et al. 2010; Gerets et al. 2012; Sison-Young et al. 2017), these cell models have nevertheless proved useful for transcriptomics-based hepatotoxicity testing. By comparing gene expression patterns in HepG2 cells exposed to 13 Ames test-positive genotoxic compounds with 21 controls, transcriptomic signatures were identified that were highly predictive for genotoxicity with 89% accuracy and 91% specificity using an additional independent test set of 28 chemicals (Magkoufopoulou et al. 2012). Gene expression profiling moreover allowed to distinguish between carcinogens that activated the oxidative stress response and those that acted via different mechanisms (Deferme et al. 2015). Transcriptomics were similarly successful to identify a gene expression signature of 36 genes that was sufficient to accurately (91% accuracy) flag molecules with hepatotoxic liability on a small validation panel of 11 compounds (8 hepatotoxins and 3 nontoxic controls; Figure 2(a)) and to sub-classify compounds into cholestatic and non-cholestatic mechanisms (Figure 2(b)) (Van den Hof et al. 2014). Interestingly, hepatotoxins that synergized with pro-inflammatory cytokine signaling, such as diclofenac and carbamazepine, caused dysregulation of endoplasmic reticulum (ER) stress and Nrf2-mediated antioxidant signaling in HepG2 cells, which aligned well with data from 80 drugs in PHH (Fredriksson et al. 2014). In contrast, compounds whose toxicity was inflammation-independent, including ketoconazole, nefazodone, and methotrexate, did not perturb these pathways.

Besides overall patterns of toxicity, HepG2 cells have proven useful for the elucidation of the toxic mechanisms for candidate drugs of interest. Analyses of the gene expression changes caused by the genotoxic agent cisplatin revealed regulatory networks controlled by HNF4A, SP1, MYC and p53 that were conserved across species and cell models (human HepG2 cells, mouse primary hepatocytes and embryonic stem cells) (Rieswijk et al. 2014). Similarly, HepG2 cells revealed upregulation of SOS1 and oncogenic RAS as an important mechanism underlying toxicity of the non-genotoxic carcinogen 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Jennen et al. 2011).

In addition to carcinogens, toxicogenomic studies have contributed to the mechanistic understanding of acetaminophen (APAP) toxicity. By exploiting the fact that HepG2 cells do not express CYP2E1, the enzyme responsible for formation of the reactive APAP metabolite N-acetyl-p-benzoquinone imine (NAPQI), these cells provided a paradigm that allowed the investigation of NAPQI-independent mechanisms of APAP toxicity. Specifically, dysregulated expression of genes encoding the subunits of electron transport chain complexes and
downregulation of the mitochondrial ROS scavenger SOD2 were identified as mechanisms underlying APAP-induced oxidative stress (Jiang et al. 2015).

In recent years, comprehensive mechanistic evaluations of toxicity in HepG2 cells have extended beyond the use of transcriptomics. Using Maldi-TOF proteomics, van Summeren and colleagues identified ER stress and perturbed ER-Golgi transport as a potential mechanism underlying the hepatotoxicity of cyclosporine A (Van Summeren et al. 2011). Similarly, epigenomic and metabolomic analyses revealed persistent patterns of altered DNA methylation upon aflatoxin B1 exposure (Rieswijk et al. 2016) and dose-dependent metabolomic alterations as indicators of hepatotoxicity using a panel of 35 test substances (Ramirez et al. 2018).

**HepaRG cells**

HepaRG cells are bipotent hepatic progenitor cells that can be differentiated into cells with hepatocyte-like and biliary-like epithelial morphology and phenotypes (Parent et al. 2004). Upon differentiation, they are generally considered to better reflect human hepatocyte phenotypes and function compared to HepG2 cells (Hart et al. 2010; Jennen et al. 2010; Gerets et al. 2012; Sison-Young et al. 2017). Specifically, HepaRG cell differentiation results in proliferative quiescence and the expression of key drug metabolizing enzymes and transporters at much higher levels than in HepG2 cells (Jennen et al. 2010). Nevertheless, much fewer studies have used HepaRG cells for toxicogenomic evaluations. Using a cost-effective transcriptomic assay with reduced complexity (profiling of 2839 genes) in HepaRG cells exposed to six different drugs, it was possible to discriminate dose-dependent and compound-specific hepatotoxicity signatures (Limonciel et al. 2018). Specifically, these experiments confirmed mitochondrial injury and ER perturbations upon cyclosporine A exposure mediated by ATF4 gene activation. Similarly, valproic acid activated the NRF2 pathway in alignment with its mechanism of liver injury that involves elevated ROS levels due to mitochondrial dysfunction. Furthermore, by combining gene expression profiling of 47 genes with proteomics, candidate markers for injury of 30 different pesticides were identified already at subtoxic concentrations (Braeuning et al. 2020). However, whether those can systematically optimize liver safety assessments remains to be determined.

HepaRG cells have also proven useful for the mechanistic evaluation of compound-specific liver toxicity. Using integrative transcriptomic, proteomic, and metabolomic profiling, bosentan was shown to inhibit the bile salt export pump (BSEP), activate FXR signaling, and induce oxidative stress and inflammation (Rodrigues et al. 2018). Similar integrative approaches revealed that inhibition of fatty acid metabolism and activation of AhR signaling underlie the toxicity pathway of the chemical pollutant PCB126 at toxicologically relevant picomolar concentrations (Mesnage et al. 2018).

**Stem cell-derived hepatocyte-like cells**

Human induced pluripotent stem cells (iPSCs) constitute a promising cell source that upon differentiation could

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**Table 1. Advantages and limitations of commonly used human hepatic cell models for toxicological studies.**

| Origin | HepG2 | HepaRG | iPSC-HLC | PHH |
|--------|-------|--------|----------|-----|
| Phenotype | HCC of Caucasian male | HCC with HCV etiology | Stem cell-derived | Primary |
| Functionality | * | ** | *** | --- |
| Knowledgebase | *** | *** | New donors require characterization | --- |
| Scalability | *** | *** | *** | High |
| Cost | Low | Low | Medium | High |
| Handling | Easy | Easy | More difficult | More difficult |
| Effects of 3D compared to 2D culture | Minor | Minor | Minor | Major |
| Advantages | Can be expanded; easy to maintain | Can be expanded; better phenotype than HepG2 | Can be established from donors of interest; can be expanded | Most relevant phenotype; different donors allow to study inter-individual variability |
| Limitations | Highly dedifferentiated; poor hepatic functions | Substantial molecular differences compared to mature hepatocytes | Establishment and differentiation of iPSCs can be difficult; phenotypes remain fetal | Finite material per donor, expensive |
| Recommendation for toxicogenomics | Not recommended as mechanistic analyses require adequate molecular phenotypes | Only recommended for mechanistic studies in specific donors without available liver samples | Recommended for maximal translatability of results | --- |

HCC: hepatocellular carcinoma; HCV: hepatitis C virus; iPSC-HLC: induced pluripotent stem cell-derived hepatocyte-like cell; PHH: primary human hepatocyte.

Classification: *low,* **medium,* ***high.*
Figure 2. Toxicogenomic studies in human hepatic cells can identify hepatotoxic compounds and delineate underlying toxicity mechanisms. (a) Heatmap showing a gene expression signature of 36 genes in HepG2 cells that can distinguish hepatotoxic from non-hepatotoxic compounds. The signature was identified in the training set and replicated reasonably well in a separate set of validation compounds. (b) Furthermore, using a separate signature of 12 genes, HepG2 cells could distinguish between cholestatic and non-hepatotoxic compounds. Panels a and b were adapted with permission from Van den Hof et al. (2014). Copyright 2014 American Chemical Society. (c) Heatmap visualization of a gene expression signature in patient-derived HLCs from three patients sensitive (Sens1-3) and two patients tolerant (Tol1-2) to pazopanib. Transcripts highlighted in red TFRC and SPINK1 are related to iron metabolism. (d) Gene set enrichment analysis (GSEA) for genes related to oxidative stress (top panel) or iron metabolism (bottom panel) in cells for which pazopanib was toxic (HT PZ) compared to nontoxic (NHT PZ). ES = enrichment score. Panels c and d were modified with permission from Choudhury et al. (2017). (e) Venn diagram showing the number of differentially expressed genes (FDR < 0.05) in primary human hepatocyte spheroids exposed to hepatotoxins with different toxicity mechanisms compared to vehicle controls. GSEA indicates compound-specific and overlapping toxicity responses. Panel e modified with permission from Bell et al. (2017).
provide an unlimited supply of liver cells. Particularly promising is the possibility to generate HLCs from individuals with specific genetic predispositions, such as genetic diseases or major histocompatibility complex (MHC) variants. Importantly however, while differentiation protocols have been substantially improved in recent years, current stem cell-derived liver systems still do not allow to closely recapitulate mature hepatic phenotypes and functions (Schwartz et al. 2014; Messina et al. 2020). Consequently, despite increasing use as cell models for toxicity testing (Wills and Rajagopalan 2020), only few toxicogenomic studies have been published that use HLCs for mechanistic evaluations.

A comparative study using HLCs, HepaRG, HepG2 cells, as well as PHH investigated expression signature alterations upon exposure to acetaminophen across cell models (Rodrigues et al. 2016). The authors use an acute toxicity setting with sub-toxic concentrations, i.e. acetaminophen concentrations that cause 10% cell death after 24 h of exposure (IC$_{10}$). Notably, IC$_{10}$ values differed drastically between models with HLCs being least sensitive (IC$_{10}$, HLC of 18 mM, which is approximately 18-fold higher than concentrations resulting in fulminant liver failure in vivo); however, all 2D models aligned overall poorly with transcriptomic signatures of in vivo acute liver failure samples.

In an interesting study into the hepatotoxicity mechanisms of the tyrosine kinase inhibitor pazopanib, Choudhury and colleagues established iPSC lines from two and three patients treated with identical doses that tolerated pazopanib or experienced pazopanib-induced hepatotoxicity, respectively (Choudhury et al. 2017). Upon differentiation, HLCs from pazopanib-sensitive individuals showed higher pazopanib toxicity, whereas no differences were observed for acetaminophen as reference compound. Transcriptomic and functional analyses revealed that pazopanib depleted glutathione and increased levels of reactive oxygen species in all samples while further causing specific alterations of genes related to iron homeostasis and iron metabolism in susceptible-HLCs (Figure 2(c,d)), thus providing a nice demonstration of the power of stem cell derived liver models in linking clinical phenotypes to patient-specific molecular alterations.

**Primary human hepatocytes**

Primary hepatocytes are considered the gold standard for in vitro hepatotoxicity testing and their use is becoming more and more common. A multitude of studies have evaluated toxicogenomic responses in conventional 2D PHH cultures. One prominent example of the utility of this system is the identification of expression changes of genes involved in mitochondrial damage, RNA processing, transcription, and inflammation that clearly distinguish the idiosyncratic hepatotoxin trovafloxacin from structurally related nontoxic fluoroquinolones (Liguori et al. 2005; 2008). Similarly, PHH monolayer cultures have provided insights into the toxicity mechanisms of cyclosporine A (Wolters et al. 2016), diclofenac (Sarkar et al. 2017), valproic acid (Wolters et al. 2018) and triazole antifungals (Goetz and Dix 2009). Furthermore, by leveraging transcriptomic data from the Open TG-GATES repository, a toxicogenomic database containing data from PHH exposed to 158 compounds, NRF2 activation was identified as a good indicator of the intrinsic biochemical reactivity of a compound which remains an important component for the comprehensive evaluation of compound systems toxicology (Copple et al. 2019).

Notably however, in conventional 2D monolayer cultures, PHH very rapidly lose their mature characteristics with first gene expression changes being detectable as early as 30 min after the start of culture (Lauschke et al. 2016), which likely impacts toxicogenomic responses. Consequently, organotypic 3D cell culture methods that prevent or ameliorate this dedifferentiation have become increasingly prevalent. Comprehensive toxicity screens in spheroids and micropatterned co-cultures (MPCCs) using hundreds of compounds have demonstrated that PHH in 3D culture have high predictive accuracy (Khetani et al. 2013; Proctor et al. 2017; Vorrink et al. 2018). Furthermore, due to the extended viability and phenotypic stability that allows for chronic exposure studies for multiple weeks, the sensitivity of such 3D systems exceeds those of cells from the same donors cultured in 2D monolayers or 2D sandwich cultures (Bell et al. 2018).

In a toxicogenomic analysis of PHH in MPCC with stabilizing fibroblasts, differential gene expression analysis could distinguish hepatotoxic drugs troglitazone, nefazodone, ibufenac and tolcapone from their nontoxic structural analogues rosiglitazone, buspirone, ibuprofen and entacapone (Ware et al. 2017). Similarly, transcriptomic analyses of 3D spheroid cultures accurately identified the diverse toxicity mechanisms of aflatoxin B1 (genotoxicity), amiodarone (inhibition of mitochondrial respiration and steatogenesis) and chlorpromazine (cholestasis) based on gene expression and pathway activity differences (Figure 2(e)) (Bell et al. 2017). Furthermore, the same study showed that the expression of biomarkers correlated well with the available in vivo data.

**Conclusions and future perspectives**

Driven in part by changes in legislation and perception of animal research, the multi-faceted advances in
human cell culture methods and platforms have opened up new possibilities for the improved prediction and mechanistic analysis of DILI. However, important considerations remain. For instance, current culture models rarely embrace the inter-individual variability of human livers, as cell lines are derived from a single individual and studies using primary cells rarely use cells from more than five donors per study. While most clinically apparent cases of DILI are dose dependent and are primarily attributed to accidental or voluntary acetaminophen overdoses, the hepatotoxicity of most drugs is idiosyncratic and very rare, occurring only in 1 per 10,000 or fewer individuals. Consequently, only those toxic liabilities can be detected by toxicogenomic analyses where altered gene expression modules manifest in all or most individuals, though few would have experienced clinically manifest liver injury.

Along these lines, an interesting recent study developed a polygenic risk score for the susceptibility to DILI based on previous large-scale genome-wide association studies (Koido et al. 2020). Using primary hepatocytes and stem cell-derived organoids from multiple donors treated with over ten different drugs, this approach could flag individuals at risk of DILI, which can inform preclinical studies as well as patient selection in clinical trials. In conclusion, organotypic culture methods coupled with comprehensive multi-dimensional molecular profiling and an increasing understanding and appreciation of the polygenic architecture of DILI predisposition promises to improve early hepatotoxic risk prediction and reduce safety events in drug development.

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