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Transcriptional, functional and mechanistic comparisons of stem cell-derived hepatocytes, HepaRG cells and 3D human hepatocyte spheroids as predictive *in vitro* systems for drug-induced liver injury

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Abbreviations

PHH, Primary human hepatocyte; DILI, Drug-induced liver injury; ADR, Adverse drug reaction; ADME, Absorption, distribution, metabolism and excretion; iPSC, Induced pluripotent stem cell; HLC, Hepatocyte-like cell; FBS, Fetal bovine serum; DMSO, Dimethyl sulfoxide; ATP, Adenosine triphosphate; FDR, False discovery rate; GST, Glutathione-S-transferase; UGT, UDP-glucuronosyltransferase; CYP, Cytochrome P450; BSEP, Bile salt export pump; NCTP, Na⁺-taurocholate cotransporting polypeptide; BCRP, Breast cancer resistance protein; C_{max}, Maximal serum concentration; APAP, Acetaminophen; NAPQI, N-acetyl-p-benzoquinone imine; CPT1, Carnitine palmitoyltransferase I; ROS, Reactive oxygen species

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Abstract

Reliable and versatile hepatic *in vitro* systems for the prediction of drug pharmacokinetics and toxicity are essential constituents of preclinical safety assessment pipelines for new medicines. Here, we compared three emerging cell systems, hepatocytes derived from induced pluripotent stem cells (hiPS-Hep), HepaRG cells and 3D primary human hepatocyte (PHH) spheroids at transcriptional and functional levels in a multi-center study to evaluate their potential as predictive models for drug-induced hepatotoxicity. Transcriptomic analyses revealed widespread gene expression differences between the three cell models, with 8,148 out of 17,462 analyzed genes (47%) being differentially expressed. Expression levels of genes involved in the metabolism of endogenous as well as xenobiotic compounds were significantly elevated in PHH spheroids, whereas genes involved in cell division and endocytosis were significantly upregulated in HepaRG and hiPS-Hep cells, respectively. Consequently, PHH spheroids were more sensitive to a panel of drugs with distinctly different toxicity mechanisms, an effect that was amplified by long-term exposure using repeated treatments. Importantly, toxicogenomic analyses revealed that transcriptomic changes in PHH spheroids were in compliance with cholestatic, carcinogenic or steatogenic *in vivo* toxicity mechanisms at clinically relevant drug concentrations. Combined, the data reveal important phenotypic differences between the three cell systems and suggest that PHH spheroids can be used for functional investigations of drug-induced liver injury *in vivo* in man.

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Introduction

Drug-induced liver injury (DILI) poses a serious threat to patients, accounting for 13% of acute liver failures and 15% of liver transplantations (Ostapowicz et al., 2002; Russo et al., 2004). Idiosyncratic DILI events, which are typically delayed in onset and restricted to predisposed individuals, account for 10% of these cases (Kaplowitz, 2005; Lauschke and Ingelman-Sundberg, 2016) and occur with an overall incidence of about 13-19 per 100,000 individuals (Sgro et al., 2002; Björnsson et al., 2013). Adverse drug reactions (ADRs) significantly increase the length and costs of hospitalization by 1.9 days and 2262-3244 US\$, respectively, and are associated with a 1.9-fold increased mortality risk (Bates et al., 1997; Classen et al., 1997). Hepatic liabilities are moreover important cost-drivers for the pharmaceutical industry that can result in late-stage attrition of drug candidates or post-marketing withdrawals, as exemplified by bromfenac, troglitazone, ximelagatran and pemoline (Park et al., 2011; Cook et al., 2014). In addition, decreased prescription due to black box warnings reduces sales, and 10 out of 45 compounds that were endowed with such boxed warnings between 1975 and 2000 received their label due to hepatotoxicity (Lasser et al., 2002).

Toxicity prediction of newly developed compounds in pre-clinical stages encompasses an array of *in silico*, *in vitro* and *in vivo* studies. Animal testing has long been the cornerstone for safety assessments of novel chemical entities. Yet, the liver is an organ with pronounced species differences with regards to expression and catalytic activities of factors involved in drug absorption, distribution, metabolism and excretion (ADME). Therefore, animal models do not accurately replicate aetiology and pathogenesis of human liver injury. Thus, due to growing recognition of the limited predictive validity of animal models and increasing legislative pressure to reduce, refine or replace (3 R-concept) the use of animal models, there is a clear

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need for predictive *in vitro* models, which faithfully reflect human liver physiology and function (Chapman et al., 2013).

Hepatic cell lines are frequently employed in pre-clinical screening assays due to their ease-of-use, ready availability and low costs. Importantly though, most hepatic cell lines lack relevant hepatic phenotypes, due to limited expression of drug-metabolizing enzymes, which makes extrapolation of the results to man questionable (Gerets et al., 2012). The HepaRG cell line presents a cell system that has been reported to be phenotypically stable, thus allowing long-term culture and repeated-exposure studies (Klein et al., 2013). Induced pluripotent stem cells (iPSC) have the advantage that they can be generated from any human cell type, which allows the retrospective acquisition of cellular material from individuals with a particular genotype or phenotype of interest, such as an idiosyncratic ADR, providing an interesting model for deciphering mechanisms of genetically determined DILI reactions (Kia et al., 2013).

Primary human hepatocytes (PHH) are considered the gold-standard for studying liver function (Gómez-Lechón et al., 2014). However, their rapid dedifferentiation in conventional 2D monolayer cultures, paralleled by a loss of hepatic functionality, renders them unsuitable for long-term studies and significantly impairs their predictive power for DILI risk (Gerets et al., 2012; Heslop et al., 2016; Lauschke et al., 2016c; Sison-Young et al., 2016). To prevent dedifferentiation, an array of 3D culture techniques has been developed in which hepatic phenotypes are maintained for extended periods of time (Lauschke et al., 2016a). One promising strategy is the culture of PHH as 3D spheroidal aggregates in which hepatocyte-specific functions

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can be retained for several weeks (Bell et al., 2016), thus enabling repeated-exposure experiments.

In this study, we characterized the transcriptomic signatures of HepaRG cells, PHH spheroid cultures and hepatocyte-like cells (HLCs) derived from iPS cells (hiPS-Hep). While expression patterns in PHH spheroids resembled freshly isolated hepatocytes, HepaRG and hiPS-Hep cells exhibited wide-spread differences in gene expression, particularly in genes involved in the metabolism of endogenous and xenobiotic compounds. These gene expression differences translated into functional differences as assessed by the sensitivity towards six different hepatotoxic compounds, with PHH spheroids constituting the most sensitive model that detected hepatotoxicity at clinically relevant concentrations. Importantly, toxicogenomic analyses revealed that transcriptional responses elicited by compounds causing inhibition of mitochondrial respiration, perturbation of β -oxidation, cholestatic injury or genotoxicity *in vivo* were faithfully reflected in this model. Combined, our data indicate that phenotypes and sensitivities to hepatotoxic agents differ considerably between pre-clinical cell models and that PHH spheroids are more physiologically relevant and mechanistically accurate in detecting and investigating hepatic liabilities of drugs as compared to HepaRG and hiPS-Hep cells.

Materials and Methods

Cell culture

Cryopreserved PHH 3D spheroids were cultured in culture medium (Williams E medium supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μ g/ml

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streptomycin, 10 µg/ml insulin, 5.5 µg/ml transferrin, 6.7 ng/ml sodium selenite, 100 nM dexamethasone) as previously described (Bell et al., 2016). Four days after seeding, 50% of culture medium was substituted with fresh FBS-free medium and subsequently, medium was exchanged daily until the start of treatment at day 7. Hepatocytes in monolayer culture were seeded into plates coated with 5 µg/cm² Rat Tail Collagen Type I (Corning) in culture medium with 10% FBS. After two hours of attachment, the medium was replaced with serum-free culture medium. hiPS-Hep cells were obtained by differentiation from the human induced pluripotent cell line ChiPSC18 (DEF-hiPSC™ ChiPSC18, Cellartis, Takara Bio Europe AB) using the Cellartis® DE Differentiation Kit and Cellartis® HEP Differentiation kit (Takara Bio Europe AB) according to manufacturer's instructions. After initiation of differentiation at day 22, the HLCs were dissociated and reseeded in an appropriate cell culture format for transcriptional analyses and viability assessments. HepaRG cells (Biopredic International) were cultured and maintained in culture medium (William's E Basal Medium + GlutaMAX containing phenol red; Invitrogen) with Additive 710 (Biopredic International). For differentiation, cells were cultured in culture medium with Additive 720 (Biopredic International). Cells were maintained in growth medium for 2 weeks followed by 2 weeks of differentiation medium. Medium was changed to culture medium without phenol red and DMSO one day prior to the initiation of treatment.

Compound exposure and generation of toxicity curves

Compounds were dissolved in DMSO and diluted in FBS-free medium to a final DMSO concentration of 0.4%. Treatment was performed every 2-3 days in FBS-free medium. In the acute setting, viability was determined following a single-dose exposure for 2 days. Under long-term treatment, cells were repeatedly treated for 7 (three exposures) and 14 days (six exposures). Viability, as assessed by cellular

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ATP levels, was determined using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Sweden). Luminescence was measured and the samples were blank corrected and normalized to vehicle control. IC₅₀ values were calculated using a sigmoidal dose-response regression model constrained at viability 0 and 100 (Prism, GraphPad). IC₁₀ values were calculated as follows: $IC_x = (((100-x)/x)^{(1/Hill\ Slope)}) * IC_{50}$ with $x = 10$.

Transcriptomic analyses

After 2, 7 and 14 days in culture, cells were harvested in RNeasy Protect Cell Reagent (Qiagen, Sweden). RNA was extracted with the RNeasy Protect DNA/RNA Mini Kit according to the manufacturer's instructions (Qiagen, Sweden). Total RNA samples (45 ng) were labeled with cyanine 3, hybridised on Agilent Whole human Genome Oligo Microarray slides 8x60K, washed, and scanned on an Agilent MicroArray Scanner. Images were processed using Agilent Feature Extraction Software v10.7.3.1. Gene expression differences are expressed relative to the respective spheroid DMSO control samples at the same time point. Microarray data was uploaded to the Gene Expression Omnibus database (submission number GSE93840).

Data analysis

Expression data were analyzed in Qlucore Omics Explorer 3.1. Gene set enrichment analyses were performed using WebGestalt (Wang et al., 2013). To assess statistical significances, heteroscedastic, two-tailed, unpaired Student t-tests were performed and p-values below 0.05 were considered significant. To correct for multiple tests, the Benjamini-Hochberg algorithm was utilized with false discovery rates (FDRs) as indicated.

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Results

Transcriptional characterisation of hepatic cell models

When cultured in 2D monolayers, PHH rapidly dedifferentiate within hours at least in part due to wide-scale miRNA-mediated inhibition of drug metabolizing enzymes, transporters and other hepatic genes (Elaut et al., 2006; Lauschke et al., 2016b; Lauschke et al., 2016c). In contrast, expression levels of most important phase I (*CYP2C8*, *CYP2C9*, *CYP3A4* and *CYP2D6*) and phase II (*GSTT1* and *UGT1A1*) drug metabolizing enzymes, drug and bile transporters (*ABCB11*, *ABCC1* and *SLCO1B1*), ligand-activated nuclear receptors (*CAR*, *PXR* and *PPARA*) and other genes with importance for hepatic functions (*ALB* and *HNF4A*) were preserved in 3D PHH spheroid cultures, approximating levels found in the corresponding freshly isolated cells (Figure 1A). When hepatocytes from the same donors were cultured in 2D monolayers, expression of the same genes was downregulated up to 1800-fold, directly demonstrating the drastic effect of dedifferentiation on hepatic gene expression (Figure 1B).

We then benchmarked the mRNA expression patterns of PHH spheroids, HepaRG and hiPS-Hep cell systems using transcriptomic analyses (Figure 2). Importantly, we found pronounced gene expression differences between the three models with 8,148 out of 17,462 genes (47%) being differentially expressed over the course of three weeks in culture (Figure 2A, FDR<0.05, Benjamini-Hochberg correction). Genes involved in DNA replication ($p_{\text{adj}}=5*10^{-7}$), mismatch repair ($p_{\text{adj}}=6*10^{-6}$) and purine metabolism ($p_{\text{adj}}=0.0013$) were significantly upregulated in HepaRG cells, whereas genes implicated in endocytosis ($p_{\text{adj}}=8*10^{-10}$), focal adhesion signaling ($p_{\text{adj}}=0.0001$)

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and lysosomes ($p_{\text{adj}}=0.0001$) were overexpressed in hiPS-Hep cells. In addition, pathways with general importance for cellular functions, such as ribosome ($p_{\text{adj}}=0.0034$), cell cycle ($p_{\text{adj}}=0.0083$) and RNA transport ($p_{\text{adj}}=0.0083$) were upregulated in both HepaRG and hiPS-Hep cells. Importantly, genes involved in the metabolism of endogenous as well as xenobiotic compounds were expressed at significantly elevated levels in PHH spheroids compared to HepaRG and hiPS-Hep cells ($p_{\text{adj}}=3 \times 10^{-33}$). While principal component analyses revealed pronounced changes over culture time in HepaRG and hiPS-Hep cells, gene expression signatures in PHH spheroids were stable over the course of two weeks (Figure 2B).

When focussing on genes with importance for drug ADME, we found that variations between the cell systems differed by gene class (Figure 3). Levels of most phase I enzymes including major *CYPs*, such as *CYP1A2*, *CYP2B6*, *CYP2C8*, *CYP2C9* and *CYP2D6*, were much higher in PHH spheroids compared to HepaRG and hiPS-Hep cells (Figure 3A). *DPYD*, which encodes the rate-limiting enzyme in pyrimidine metabolism, was expressed at similar levels in PHH spheroids and HepaRG cells. In contrast, *CYP3A7* and *CYP3A5*, which constitute the major *CYP3As* expressed in fetal liver (Hakkola et al., 2001), were highly expressed in hiPS-Hep cells.

Distinctly different sets of phase II enzymes were expressed in the three cell models. Expression of most transcripts encoding GST enzymes was highest in hiPS-Hep cells and levels of *UGTs* and *TPMT* were elevated in 3D-cultured PHH (Figure 3B). Notably, phase II gene expression was generally low in HepaRG cells, suggesting a lower capacity of this cell model to accurately reflect and predict complex drug ADME patterns. While relevant transporter genes were expressed in all three cell models, their relative abundances differed drastically (Figure 3C). In PHH spheroids, high

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levels of physiologically important transporters, such as the bile acid transporters BSEP and NTCP encoded by *ABCB11* and *SLC10A1*, respectively, steroid and thyroid hormone transporters (*SLCO1B1* and *SLCO1B3*) and MDR2/3, the phosphatidylcholine transporter encoded by *ABCB4*, were observed. In contrast, transporters implicated in drug resistance of cancer cells were upregulated in hiPS-Heps, including *ABCB1* (MDR1) and *ABCG2* (BCRP) (Takara et al., 2006; Natarajan et al., 2012).

Toxicity in hepatic cell systems under repeated-exposure regimes

Next, we investigated functional consequences of the observed expression differences. Previous studies have indicated that although PHH provide a more predictive model than other hepatic cell lines, their predictive power in acute single-exposure studies in 2D cultures is significantly limited, at least in part due to the rapid loss of hepatic gene expression (Gerets et al., 2012; Sison-Young et al., 2016). Furthermore, with respect to the clinical profile of *in vivo* toxicity events, assessment of chronic drug induced hepatotoxicity is of particular importance. Thus, we here investigated the effect of repeated-exposure regimens and analyzed the sensitivity of the three cell models to six hepatotoxic compounds that cause toxicity by distinctly different mechanisms (Figures 4-5). We focused on 1) acetaminophen (APAP), which primarily causes hepatotoxicity due to reactive metabolite formation, 2) aflatoxin B1 as a genotoxic agent, 3) the anti-arrhythmic drug amiodarone, which inhibits acyl-CoA transport and mitochondrial respiration, 4) the cholestatic agent chlorpromazine, 5) troglitazone as an inhibitor of β -oxidation that also causes direct opening of the mitochondrial permeability transition pore and 6) the anticoagulant ximelagatran as a respiratory chain inhibitor.

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The three cell systems showed drastic differences in their sensitivity to APAP toxicity. hiPS-Heps were insensitive to APAP toxicity, even after 14 days treatment (14 d IC_{50} = 9,439 μ M). In contrast, the HepaRG cell line detected toxicity already in the acute setting at high concentrations (48 h IC_{50} = 5916 μ M) and the sensitivity increased further upon repeated exposures to approximate plasma levels in patients after acute APAP overdose (14 d IC_{50} = 1,311 μ M; APAP plasma concentration for which immediate treatment is stipulated: >0.7-1.3 mM depending on additional risk factors (Vale and Proudfoot, 1995)). In PHH spheroids, a drastic increase in sensitivity to APAP toxicity was apparent with chronic exposures, indicating toxicity slightly below typical overdose concentrations after 14 days of exposure (14 d IC_{50} = 644 μ M; therapeutic c_{max} = 136 μ M; (Sevilla-Tirado et al., 2003)).

Aflatoxin B1 toxicity showed substantial increases in toxicity over time in all cell systems. PHH spheroids were the most sensitive system in the acute as well as chronic setting, indicating toxicity at exposure levels detected in exposed individuals (28.5 nM, (Hassan et al., 2006)), followed by HepaRG cells.

hiPS-Heps cells were the only system to indicate amiodarone toxicity already after 48 hours albeit only at high concentrations. After chronic exposure all three cell models detected amiodarone-induced hepatotoxicity at similar concentrations with PHH spheroids being the most sensitive, approximating exposure levels reported as toxic in patients (14 d PHH IC_{50} = 11.9 μ M, 14 d HepaRG IC_{50} = 18.5 μ M, 14 d hiPS-Hep IC_{50} = 15 μ M, human toxic c_{max} = 3.8 μ M, (Regenthal et al., 1999)).

While chlorpromazine-induced hepatic injury was detected by all three models, PHH spheroids were the most sensitive at all time points investigated and at

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concentrations approaching clinical exposure levels (14 d PHH IC_{50} = 4.6 μ M, 14 d HepaRG IC_{50} = 34.1 μ M, 14 d hiPS-Hep IC_{50} = 24.6 μ M, human toxic c_{max} = 1.6 μ M, (Regenthal et al., 1999)).

Similarly, all three cell systems indicated troglitazone toxicity at clinically relevant concentrations with IC_{50} values in PHH spheroids reaching therapeutic levels after chronic exposures (14 d PHH IC_{50} = 1.5 μ M, 14 d HepaRG IC_{50} = 34.6 μ M, 14d hiPS-Hep IC_{50} = 18.7 μ M, therapeutic c_{max} = 2.82 μ M, (Loi et al., 1999)).

PHH spheroids were the only system to indicate toxicity of ximelagatran after prolonged treatment (7 and 14 days) but only at relatively high concentrations that significantly exceeded therapeutic levels (14 d IC_{50} = 165 μ M, therapeutic c_{max} = 0.3 μ M, (Schützer et al., 2004)). It has previously proven difficult to detect ximelagatran toxicity in various *in vitro* systems (Kenne et al., 2008) and the mechanisms underlying this toxicity are still unclear, although evidence that ximelagatran inhibits mitochondrial respiration has recently been presented (Neve et al., 2015).

In summary, while sensitivities differed between cell models for the hepatotoxic model compounds in the acute, single-dose setting, the PHH spheroid system was the most sensitive cell model after long-term exposure to all compounds tested (Figure 5).

Toxicogenomic analysis of gene expression changes preceding compound toxicity

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Next, we examined whether relevant compound-specific toxicity mechanisms were reflected using toxicogenomic profiling. To this end, we focused on the PHH spheroid model as the most sensitive system that detected toxicity of most tested compounds at clinically relevant exposure levels. In order to uncouple toxicity mechanisms and outcomes, i.e. study the changes in transcriptional signatures that precede the induction of cell death, we chose subtoxic concentrations (IC_{10}) of the six model compounds. After 14 days of treatment, no significant expression changes were observed in APAP, troglitazone and ximelagatran treated samples (data not shown), suggesting that these compounds trigger cell death directly without extensive transcriptional perturbations.

In contrast, pronounced changes of gene expression signatures were evident upon treatment with aflatoxin B1, amiodarone and chlorpromazine (Figure 6). Aflatoxin B1 induced nucleotide excision repair, apoptosis and DNA replication (Figure 6A), in agreement with its genotoxicity, in agreement with previous *in vivo* findings in aflatoxin-exposed rats and tree shrews (Ellinger-Ziegelbauer et al., 2004; Li et al., 2004; Jossé et al., 2012). We detected significant downregulation of *FHIT*, a tumor suppressor repressing canonical Wnt-signaling by inhibition of β -catenin, whose activity is commonly impaired in preneoplastic lesions (Weiske et al., 2007). Similarly, we detected a reduction in levels of the methyltransferase *SMYD3*, which is implicated in hepatocellular carcinoma (Hamamoto et al., 2004) (Figure 6B). Moreover, p53 signaling target genes, such as the p53 effector *TP53I3*, *RRM2B* and *DDB2*, which play roles in DNA damage repair, and *SESN1*, a protein mediating the tumor-suppressive effect of p53 by inhibiting mTOR, were increasingly upregulated with prolonged exposure.

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PPAR signaling was significantly upregulated following chronic amiodarone exposure, mimicking *in vivo* gene expression modulations in mice (McCarthy et al., 2004) (Figure 6A), resulting in increased expression of e.g. *CPT1A*, a PPAR α target gene whose gene product is inhibited by amiodarone (Kennedy et al., 1996). Furthermore, we detected a progressive upregulation of key genes involved in lipid and cholesterol metabolism, such as *HADHA*, *ACSL4* and *HMGCR* (Figure 6C). Moreover, expression levels of *G6PD*, the central regulator of the pentose phosphate pathway that controls generation of NADPH was significantly increased.

Prolonged chlorpromazine treatment caused the most pronounced perturbations of expression signatures, with 6,755 genes identified as being differentially expressed (compared to 1,520 for aflatoxin B1 and 863 for amiodarone). Among the deregulated pathways were bile acid metabolism ($p=1*10^{-5}$), reflecting the cholestatic mechanism of chlorpromazine toxicity (Horikawa et al., 2003). Higher expression of *CYP1A2*, whose gene product is involved in chlorpromazine metabolism (Yoshii et al., 2000), increased with chlorpromazine treatment, whereas transcript levels of *CYP7A1*, the key enzyme in bile acid synthesis, as well as of the bile transporters *SLC22A1* (OCT1) and *SLC10A1* (NTCP) decreased. Moreover, expression levels of SLC and ABC transporters were broadly repressed following 14 days of treatment (Figure 6E), suggesting major alterations of underlying transcriptional networks.

Discussion

In this study we compared the phenotypes of three emerging cell culture models for pre-clinical safety assessments of drugs and drug candidates: PHH spheroids, HepaRG and hiPS-Hep cells. We found that mRNA expression levels of genes with

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importance for hepatic functionality in PHH spheroids pivoted around levels found in freshly isolated hepatocytes. These data corroborate the results of previous studies showing that 3D spheroid culture conditions improve the gene expression signatures and phenotypes of PHH, resulting in an approximation of their physiological counterparts *in vivo* in man (Tostões et al., 2012; Bell et al., 2016). Importantly, transcriptional signatures of HepaRG and hiPS-Hep cells drastically differed with 8,148 out of 17,462 genes (47% of the assessed transcriptome) being differentially expressed between the three cell models (FDR<0.05). Importantly, expression of genes encoding enzymes involved in xenobiotic metabolism was strongly reduced in HepaRG and hiPS-Hep cells compared to PHH spheroids ($p_{\text{adj}}=3*10^{-9}$). Furthermore, HepaRG and hiPS-Hep cells exhibited impaired expression of genes involved in the metabolism of endogenous compounds, such as fatty acids ($p_{\text{adj}}=3*10^{-10}$) and retinol ($p_{\text{adj}}=2*10^{-9}$). Combined, these differences suggest impaired capacities of these two cell models to metabolize drugs and to faithfully mimic the mechanisms underlying compound toxicity.

When focusing on ADME genes, we detected highly elevated transcript levels of genes characteristic of the mature human liver, such as *CYP1A2*, *CYP2C8*, *CYP3A4*, *ABCB11* and *SLC10A1*, in PHH spheroids. In contrast, hiPS-Hep cells showed increased levels of the fetal CYPs *CYP3A5* and *CYP3A7*, as well as high expression of the most important fetal GST (*GSTP1*) and transporters whose expression correlated with dedifferentiation during carcinogenesis, such as *ABCB1* and *ABCG2* (Hakkola et al., 2001; Raijmakers et al., 2001; Takara et al., 2006; Natarajan et al., 2012). The data revealed that gene expression signatures in PHH spheroids closely resembled those detected in isolated hepatocytes. In contrast, reduced expression of many important hepatic genes was evident in HepaRG and hiPS-Hep cells, indicative of deficits in maturation.

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To relate changes in transcription patterns to functional consequences, we examined the differential sensitivities of the three cell models to hepatotoxins. APAP toxicity is primarily due to reactive metabolite formation catalyzed by CYP2E1 and CYP3A4 causing subsequent glutathione depletion but also immune-mediated mechanisms have been linked to APAP-induced liver injury (reviewed in (Krenkel et al., 2014)). In agreement with high *CYP2E1* and *CYP3A4* expression levels and physiological but comparatively low expression of glutathione-S-transferases (GSTs) involved in NAPQI detoxification, PHH spheroids detected APAP toxicity after 14 days at concentrations below typical overdose levels. The finding that APAP toxicity was already detected at concentrations that are clinically considered safe (Bradley et al., 1991; Geba et al., 2002) is consistent with previous clinical reports showing liver damage, as indicated by serum alanine aminotransferase elevations above three times the upper limit, in 31-44% of healthy volunteers receiving 4 g acetaminophen daily for 14 days (peak acetaminophen serum level average = 99.2 μ M) (Watkins et al., 2006).

Similarly, hepatotoxicity of the mycotoxin aflatoxin B1 requires metabolic activation by CYP1A2 and CYP3A4 to a highly reactive 8,9-epoxide, which can lead to the development of hepatocellular carcinoma or, in rare cases, acute hepatotoxicity (Johnson and Guengerich, 1997; Macé et al., 1997; Williams et al., 2004). Sensitivity to aflatoxin B1 toxicity was strongly pronounced in PHH spheroids, which show physiological expression levels of the respective metabolizing enzymes (Figure 3A). Combined these data suggest that physiological and temporally stable expression levels of ADME genes are required to detect hepatotoxicity of compounds that require metabolic activation.

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The lipophilic benzofuran derivative amiodarone causes mitochondrial uncoupling due to influx of protonated amiodarone into the mitochondrial matrix (Fromenty et al., 1990). Furthermore, it impairs the respiratory chain complexes I, II and III and inhibits CPT1, thus limiting the import of fatty acids into the mitochondria and reducing the flux through mitochondrial β -oxidation (Fromenty et al., 1990; Kennedy et al., 1996; Spaniol et al., 2001). Sensitivity to amiodarone hepatotoxicity did not drastically increase over time and was similar between the three cell models. While amiodarone is extensively metabolized by CYP3A4 and CYP2C8, its therapeutic as well as toxicological effects seem to be caused by both the parent compound as well as its dealkylated metabolite (Trivier et al., 1993; Soyama et al., 2002). Consequently, amiodarone toxicity does not depend on bioactivation, which could provide an explanation for the similar sensitivity levels between the cell systems. These findings are in agreement with previous reports showing lipid accumulation in hiPS-Hep and HepaRG cells already after short-term amiodarone exposures (Anthérieu et al., 2011; Pradip et al., 2016).

Chlorpromazine causes primarily cholestatic liver injury and multiple toxicity mechanisms have been suggested, including perturbation of oxidative phosphorylation (Nadanaciva et al., 2007), inhibition of bile export (Horikawa et al., 2003), glutathione depletion (Xu et al., 2008), phospholipidosis due to inhibition of phospholipases (Anderson and Borlak, 2006) and hypersensitivity (Ayd, 1956). Clinicopathologically, chlorpromazine toxicity presents as self-limited jaundice in approximately 1 in 100 patients prescribed chlorpromazine (García Rodríguez et al., 1997) that typically manifests within one to five weeks after starting of treatment, in three-quarters of individuals in combination with eosinophilia (Selim and Kaplowitz, 1999). Most patients recover within weeks after discontinuation of treatment but few

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experience progression of cholestatic injury to hepatic ductopenia. Toxicity of chlorpromazine has been reported to be caused by its 7-hydroxylated metabolite, whereas the sulfoxidized metabolite appeared less toxic (Ros et al., 1979; Watson et al., 1988). PHH spheroids exhibited the highest sensitivity towards chlorpromazine and detected toxicity already at therapeutic concentrations, which was paralleled by increased expression of *CYP1A1* and *CYP1A2*, as previously reported (Parmentier et al., 2013). Furthermore, expression of genes with importance for bile acid synthesis, such as *CYP7A1*, which catalyzes the rate-limiting step in the classical bile acid synthesis pathway, and bile transport, such as the canalicular transporter BSEP (encoded by *ABCB11*) and the sinusoidal transporters NTCP (*SLC10A1*) and OCT1 (*SLC22A1*) were strongly downregulated, mirroring expression alterations seen in cholestatic patients *in vivo* (Zollner et al., 2001; Zollner et al., 2007; Chen et al., 2008; Nies et al., 2009). Interestingly, transcriptional changes indicative of chlorpromazine-induced cholestasis preceded apoptosis by two weeks, suggesting the potential of the spheroid system to aid biomarker discovery.

The thiazolidinedione troglitazone is a PPAR γ agonist used as an insulin sensitizer for treatment of diabetes that also exhibits weak affinity to PPAR α (Lehmann et al., 1995). After regulatory approval in 1997, troglitazone was withdrawn from the US market in 2000 due to idiosyncratic hepatotoxicity. Troglitazone causes parent compound-mediated steatosis by inhibition of long-chain acyl-CoA synthetase (ACS) and opening of the mitochondrial permeability transition pore (Fulgencio et al., 1996; Tirmenstein et al., 2002; Lim et al., 2008). In addition to parent compound toxicity, troglitazone metabolites, primarily troglitazone sulfate, can cause cholestatic liver injury by inhibition of the bile salt export pump (BSEP) with an IC₅₀ of 0.4 μ M (Funk et al., 2001). Furthermore, reactive metabolites and oxidative stress have been implicated in troglitazone toxicity, although their role remains controversial

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(comprehensively discussed in (Masubuchi, 2006)). The high sensitivity across models is consistent with troglitazone toxicity being largely caused by the parent compound itself. Nevertheless, toxicity in PHH spheroids is enhanced as compared to the other two models, potentially due to additive effects of toxic metabolites, such as troglitazone sulfate.

Notably, previous studies demonstrated improved phenotypes, functionality and sensitivity to various hepatotoxins in spheroid culture systems of hepatic cell lines (Fey and Wrzesinski, 2012; Gunness et al., 2013; Ramaiahgari et al., 2014), stem cell-derived HLCs (Takayama et al., 2013; Tasnim et al., 2016) and primary hepatocytes from rat (Sakai et al., 2010; Schutte et al., 2011; Purcell et al., 2014) and human (Tostões et al., 2012; Bell et al., 2016). Yet, toxicity in most of these studies was only tested under short-term exposure and only detected at elevated concentrations (Table 2). Furthermore, it was not evaluated whether the mechanisms of compound toxicity were recapitulated *in vitro*. Our study reinforces the positive effects of 3D culture on expression levels of hepatic genes and provides evidence that spheroids from PHH can recapitulate human *in vivo* toxicity mechanisms in an *in vitro* setting.

Combined, the data presented here suggest that cytotoxicity studies in which long-term treatment regimens are employed improve the sensitivity of diverse hepatic *in vitro* models. PHH spheroids in particular were found to be the model that most accurately reflected *in vivo* expression signatures in human liver. Consequently, 3D cultured PHH were the most sensitive system to detect drug hepatotoxicity at clinically relevant concentrations. Furthermore, our results show that the 3D spheroid system faithfully reproduced transcriptional toxicity responses observed in human

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livers *in vivo*, particularly for drugs that require metabolic activation, act via ROS or inhibit bile flow. Thus, development and characterization of the 3D PHH spheroid model constitutes a promising step toward a much-needed physiologically replicative system that is mechanistically predictive of human drug response.

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Author contributions

Participated in research design: Andersson, Ingelman-Sundberg

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Conflict of interest

V.M.L and M.I.-S. are co-founders and owners of HepaPredict AB.

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Footnotes

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C.C.B and V.M.L contributed equally.

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Figure legends:

Figure 1: PHH cultured in 3D spheroids resemble freshly isolated cells regarding expression patterns of drug metabolizing enzymes, drug transporters and hepatic markers. **A**, Expression of phase I (*CYP2C8*, *CYP2C9*, *CYP2D6* and *CYP3A4*) and phase II (*GSTT1* and *UGTA1*) metabolic enzymes, drug transporters (*SLCO1B1* and *ABCB11*), ligand-activated nuclear receptors (*CAR*, *PXR* and *PPARA*) as well as the critical hepatic transcription factor *HNF4A* and the main hepatocyte secretory product, albumin (*ALB*), were quantified in PHH spheroids by qPCR and normalized to expression in freshly isolated cells of the same donors (n=3-4 donors; donor demographics are shown in Table 1). Importantly, with the exception of *CYP2C8* (33% of expression of freshly isolated cells, p=0.001) and *CYP2C9* (40%, p=0.004), no significant differences in expression levels between freshly isolated cells and PHH spheroids were detected. Error bars indicate SEM. n.s. not significant, p>0.05, * p<0.05, ** p<0.01, *** p<0.001 heteroscedastic two-tailed t-test. **B**, Expression levels of genes analyzed in **A** were elevated up to 1834-fold in the 3D spheroids compared to 2D cultured PHH from the same donors after 7 days in culture.

Figure 2: Transcriptomic profiling of hepatic *in vitro* models reveals widescale differences in global gene expression. **A**, Heat map depicting differentially expressed genes in PHH spheroids (donor 1; blue), HepaRG (red) and hiPS-Hep cells (green) at 2, 7 and 14 days. Overall, 8,148 out of 17,462 genes analyzed were found to be differentially expressed after multiple testing correction (Benjamini-Hochberg FDR<0.05). PHH spheroids showed elevated expression of genes involved in endogenous and xenobiotic metabolism ($p_{\text{adj}}=3*10^{-33}$), whereas HepaRG and hiPS-Hep cells exhibited, among others, elevated transcript levels of genes involved in proliferation ($p_{\text{adj}}=0.0083$) and ribosomes (p=0.0034). Average values of

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three technical triplicates are presented as mean-centred and sigma-normalized. **B**, Principal component analysis revealed clear separation of the three cell models, which even increased over time (time progression is indicated as shades of purple). Notably, temporal changes of the transcriptomic signatures were more evident for HepaRG and hiPS-Hep cells during the culture period (indicated by arrows), whereas the transcriptomes of PHH spheroids remained temporally stable.

Figure 3: Expression levels of important ADME genes differ substantially between the three hepatic *in vitro* models. PHH spheroids, HepaRG and hiPS-Hep cells showed pronounced expression differences in phase I (**A**) and phase II enzymes (**B**) and drug transporters (**C**). Median expression values of three technical replicate microarray measurements are shown for each cell system and time point. Data is presented as mean-centred and sigma-normalized.

Figure 4: The sensitivity to model DILI compounds differs drastically between hiPS-Hep, HepaRG and PHH cell models. **A**, PHH spheroids, HepaRG and hiPS-Hep cells were treated with APAP, aflatoxin B1, amiodarone, chlorpromazine, troglitazone and ximelagatran in single dose (48h, black) or repeated exposure experiments (7d, brown and 14d, orange). Data is presented as percentage relative to the viability of vehicle-treated controls at the same time point. For PHH: two replicate experiments (both from donor 1) with six replicate measurements per concentration and time point. For HepaRG: three replicate experiments with 3 replicate measurements per concentration and time point. For hiPS-Heps: two replicate experiments with three replicate measurements per concentration and time point. Error bars indicate SEM. **B**, Semi-log plot showing the temporal evolution of sensitivity in PHH (blue), HepaRG (red) and hiPS-Hep cells (green). Dashed line

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indicates therapeutic exposure levels. Note that long-term exposure resulted in increased sensitivity towards the hepatotoxins used in all cell systems. PHH spheroids detected toxicity at clinically relevant exposure levels for all compounds with the exception of ximelagatran.

Figure 5: PHH spheroids constitute the most sensitive *in vitro* cell culture system tested. Heatmap summarizing the sensitivities of the three cell systems to cytotoxicity as shown in Figure 4. Data is presented as mean-centred and sigma-normalized and related to therapeutic (ximelagatran and troglitazone) or toxic (APAP, aflatoxin B1, amiodarone, chlorpromazine) exposure values. * sensitivity <30x C_{max} , ** sensitivity <10x C_{max} , *** sensitivity < 1x C_{max} . C_{max} or exposure values were obtained from the following references: APAP: 700 μ M (Vale and Proudfoot, 1995); aflatoxin B1: 0.03 μ M (Hassan et al., 2006); amiodarone: 3.9 μ M (Regenthal et al., 1999); chlorpromazine: 1.6 μ M, (Regenthal et al., 1999); troglitazone: 2.82 μ M (Loi et al., 1999); ximelagatran: 0.3 μ M, (Schützer et al., 2004).

Figure 6: PHH spheroids faithfully mimicked compound-specific transcriptional toxicity effects observed *in vivo*. Transcriptomic analyses of PHH spheroids treated chronically (14d) with subtoxic concentrations (IC_{10}) of aflatoxin B1, amiodarone and chlorpromazine. **A**, Venn diagram showing significantly dysregulated genes compared to DMSO controls (Benjamini-Hochberg multiple testing correction, $FDR < 0.05$). Gene set enrichment analysis revealed that compound-specific toxicity responses, such as DNA damage related pathways, perturbations of bile acid metabolism and PPAR signaling, were detected in aflatoxin B1, chlorpromazine and amiodarone treated spheroids. Targeted analysis of genes implicated in aflatoxin B1 (**B**), amiodarone (**C**) and chlorpromazine (**D**) toxicity *in vivo*. Genes whose

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expression was up- or downregulated *in vivo* are shown in shades of red and blue, respectively. **E**, Expression of cellular ABC and SLC transporters was broadly inhibited upon chlorpromazine treatment. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ heteroscedastic two-tailed t-test compared to DMSO control at the same time point.

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Table 1: Demographic Information of PHH Donors Used in this Study

Donor	Sex	Age	Race	Viability of isolated cells
1	Male	22	Caucasian	83%
2	Female	37	Asian	85%
3	Male	58	Caucasian	79%
4	Female	48	Polynesian	84%

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Table 2: Comparison of the sensitivity to compounds tested in this study with published spheroid models

Cell type	PHH	PHH	HepaRG [1,2]	HepaRG [2]	HepG2 [2-4]	HepG2 [3]	HLC [4,5]	C3A [6]	rat hep [7]
Setting	acute	repeated-dose (14d)	acute	repeated-dose (72h)	acute	repeated-dose (6d)	acute	acute	acute
APAP	>10mM	0.6mM	3mM	ND	24mM	9.4mM	30mM	40mM	40mM
AFB	0.4µM	0.03µM	1.6µM	ND	>200µM	ND	7µM	ND	ND
AMD	>100µM	11.9µM	178µM	ND	50 - >200µM	ND	25µM	260µM	ND
CPZ	16.6µM	4.6µM	98µM	43µM	43µM	ND	ND	ND	ND
TRO	37.4µM	1.5µM	400µM	ND	80 - 150µM	100µM	50µM	ND	ND
XIM	>400µM	165µM	ND	ND	ND	ND	ND	ND	ND

References: 1 - (Gunness et al., 2013), 2 - (Mueller et al., 2014), 3 - (Ramaiahgari et al., 2014), 4 - (Takayama et al., 2013), 5 - (Tasnim et al., 2016), 6 - (Fey and Wrzesinski, 2012), 7 - (Schutte et al., 2011). AFB = Aflatoxin B1, AMD = amiodarone, CPZ = chlorpromazine, TRO = troglitazone, XIM = ximelagatran. ND = not determined.

Figure 1

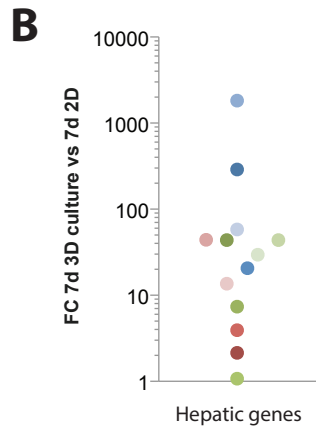
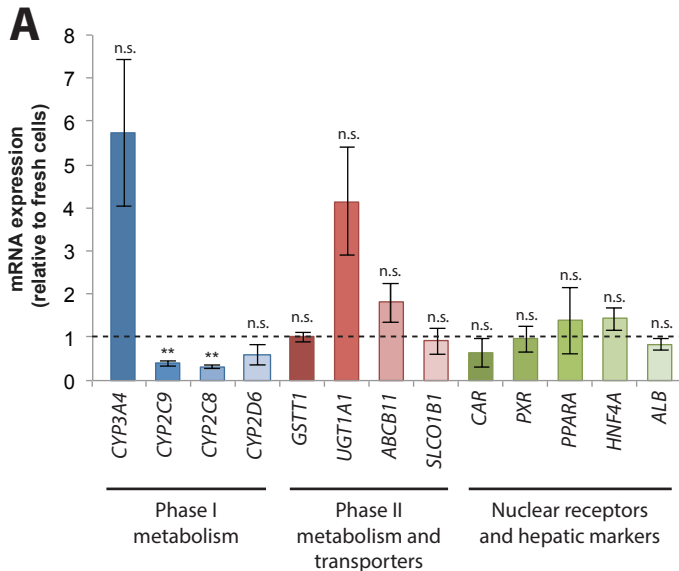
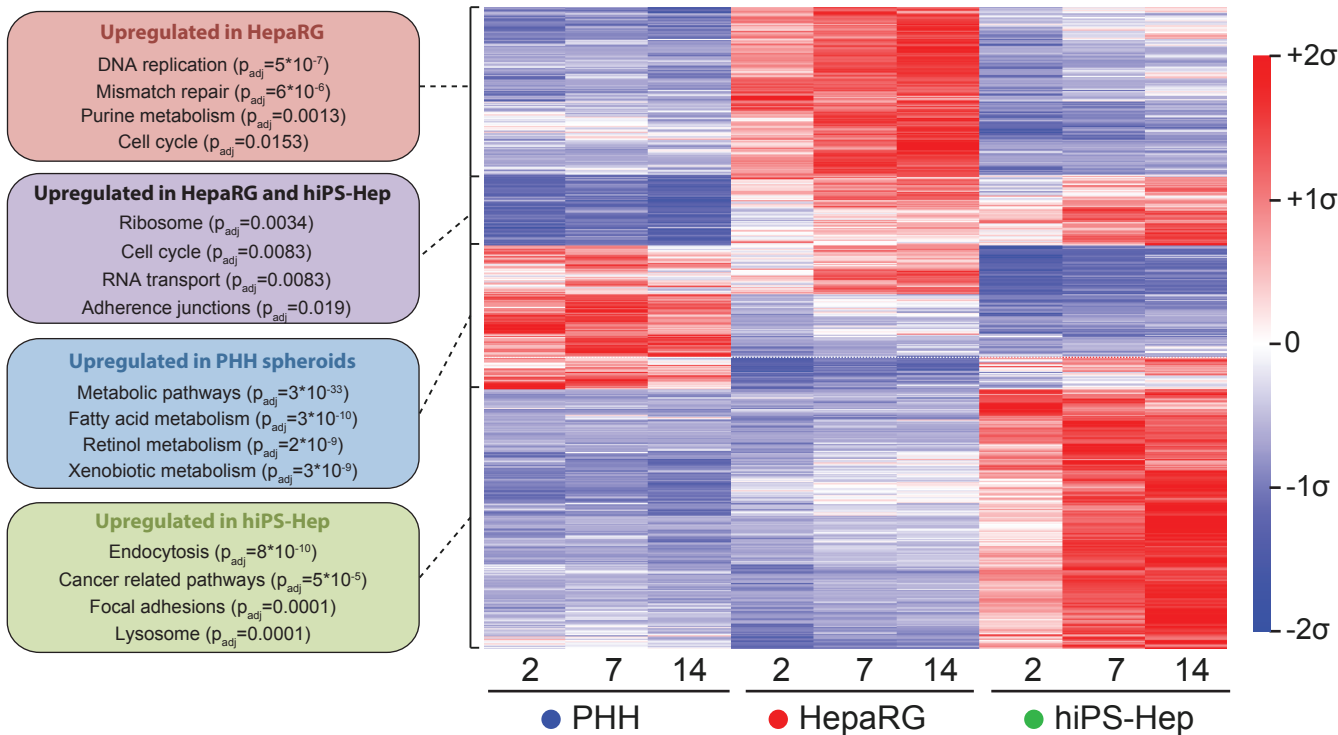


Figure 2

A



B

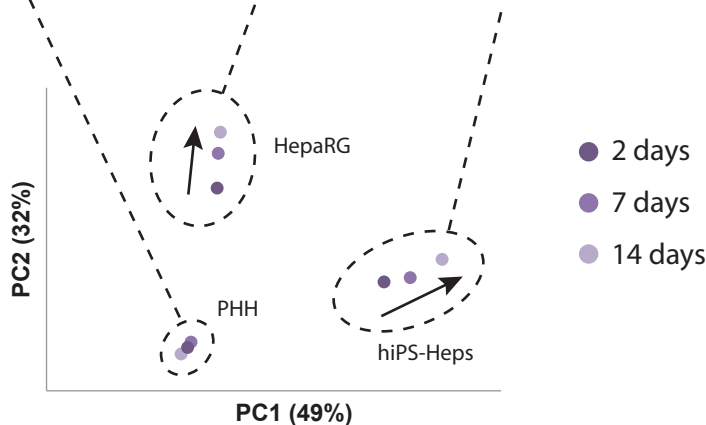


Figure 3

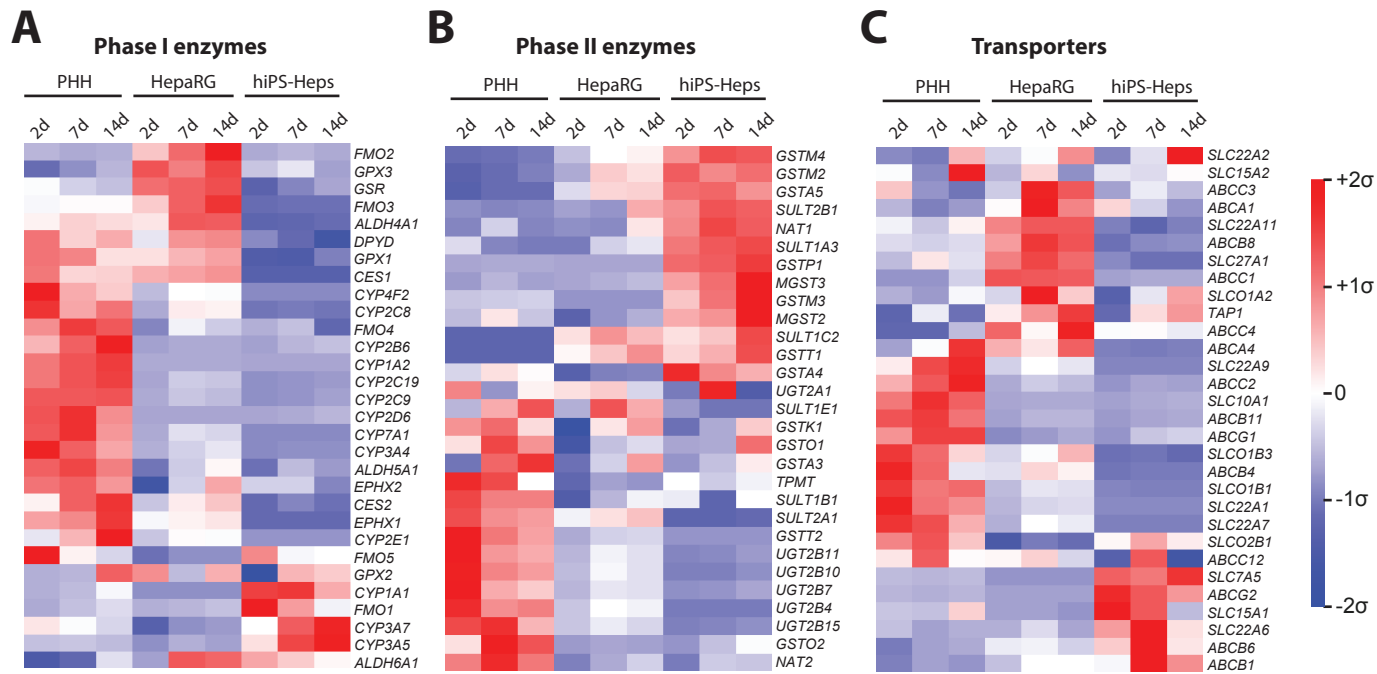
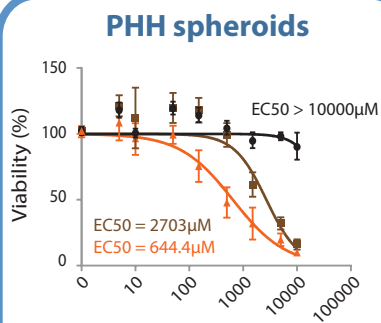


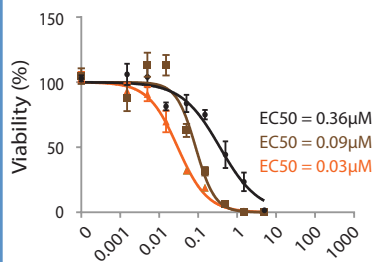
Figure 4

A

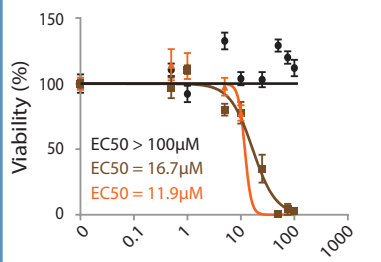
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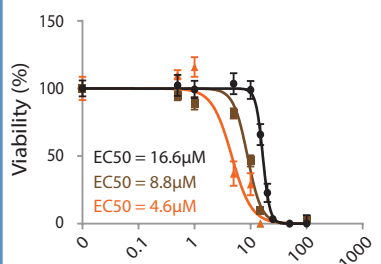
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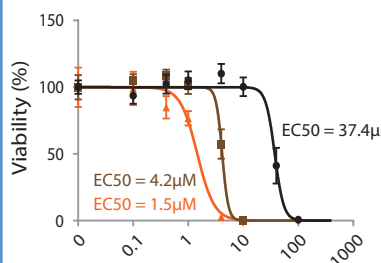
Amiodarone



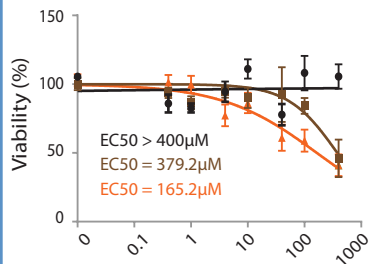
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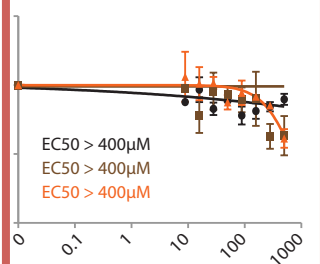
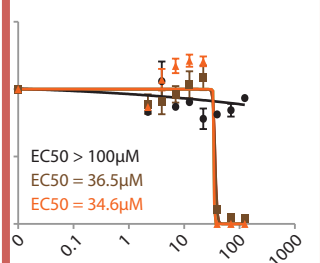
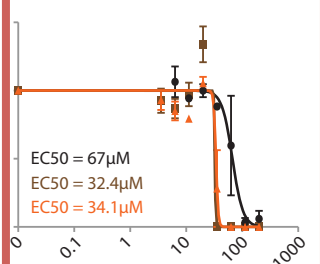
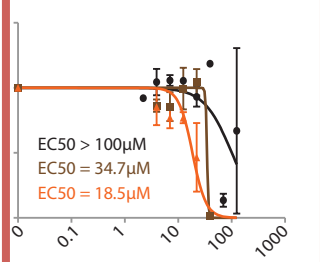
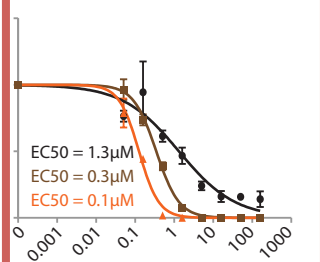
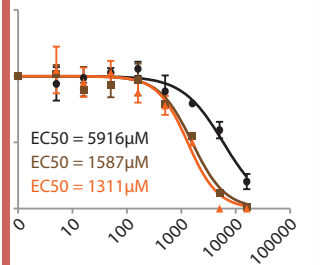
Troglitazone



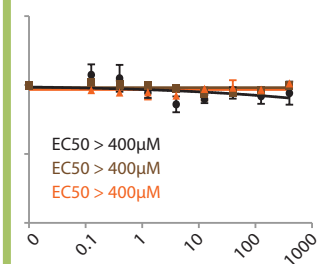
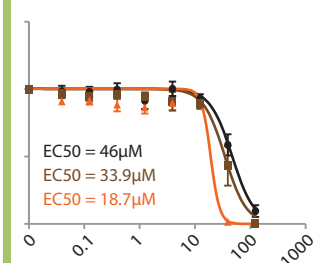
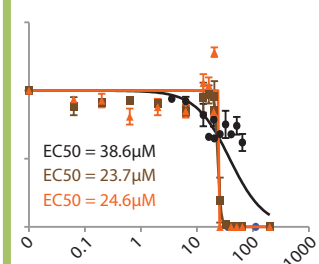
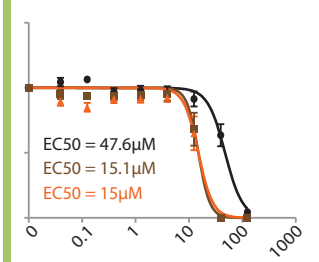
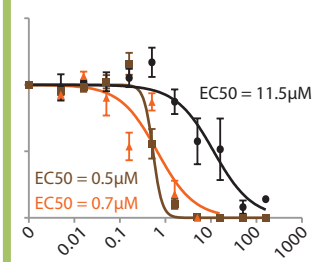
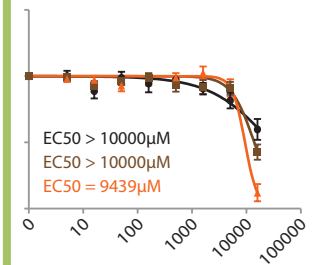
Ximelagatran



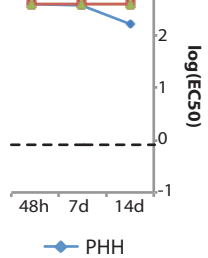
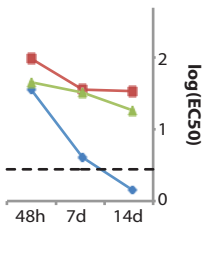
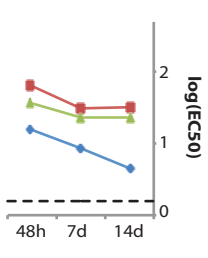
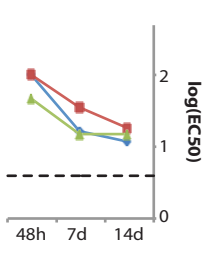
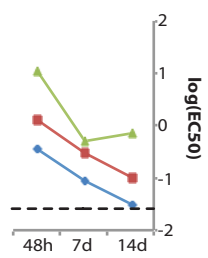
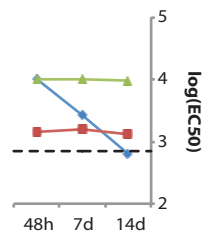
HepaRG



hiPS-Hep



B



● 48h ■ 7d ▲ 14d

◆ PHH
■ HepaRG
▲ hiPS-Hep

Figure 5

