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Advanced hepatic cell culture techniques

Drug-Induced Liver Injury (DILI) toxicology models throughout the discovery pipeline

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Drug-induced liver injury (DILI) remains a leading cause of attrition in drug development, underscoring the need for robust safety testing assays. Effective evaluation of both acute and chronic DILI relies on advanced toxicological models across all stages of drug discovery. Early-stage screening benefits from advanced cell culture techniques such as 3D spheroid models, which increase the physiological relevance of cell line-derived cultures. In later drug development stages, culturing primary human hepatocytes (PHHs) in spheroid formats and the addition of non-parenchymal cells (NPCs) can better approximate *in vivo* conditions. This study compares the outcomes of three different 3D spheroid hepatocyte culture models (spheroids derived from the HepaRG® Hepatic Cell Line, PHHs in monoculture, and PHHs in co-culture with NPCs) challenged with a panel of known hepatotoxins of varying severity and modes of action. Spheroids were challenged with an eight-point dose titration of acetaminophen, troglitazone, diclofenac, fialuridine, and cyclosporin A, and toxicity was measured using the Lonza ViaLight® Plus Bioassay Kit. All five hepatotoxins elicited DILI toxicity across the three cell culture models. The severity and timeline of hepatotoxicity were dependent on the cell culture model, as increasing physiological relevance led to more complex interactions between the effects of the compound being tested and the cells. This study shows that all three culture models are suitable for DILI-tox assays, covering a wide range of toxicity mechanisms and severity. NoSpin™ HepaRG® (HepaRG® hereafter) spheroid cultures eliminate variation due to donor characteristics and are suited for early-stage drug screens, showing expected cytotoxic responses to all compounds. Conversely, PHHs allow for donor variation to better approximate *in vivo* results, including idiosyncratic DILI responses. PHHs co-cultured with NPCs in spheroid format offer the highest diversity and provide more nuanced, physiologically relevant responses suited for late-stage screening.

Introduction

The recent FDA Modernization Act 2.0 calls for the development and validation of advanced cell culture models to replace or supplement animal models in drug discovery and safety testing and maximize physiological relevance. Achieving this goal would prevent the attrition of drug candidates that fail in less-relevant models and increase the accuracy of drug safety screens during both early- and late-drug discovery. To this end, 3D primary human hepatocyte (PHH) cell culture models have been shown to provide a more physiologically relevant outcome when compared to 2D models.[1-3] This same finding extends even to cell lines such as HepaRG® Cells, which are of great value in early drug screening, where maintaining minimal variance across experiments is vital.[4] For late-stage drug development and safety testing, co-culturing PHHs with non-parenchymal cells (NPCs) in a 3D format increases both the complexity as well as the physiological relevance of cultures, allowing for the inclusion of inflammatory feedback pathways between liver cells and liver-resident immune cells when testing drug safety and efficacy.[5,6]

This study aimed to develop robust protocols for culturing 3D spheroid HepaRG® cells, PHHs in monoculture, and PHHs in co-culture with NPCs (Kupffer Cells (KCs), Stellate Cells (SCs), and Liver Endothelial Cells (LECs)) and screening them against a panel of hepatotoxins of varying severity and modes of action. The resulting protocols can be used to establish 3D spheroid cultures optimized for early- to late-stage drug discovery and safety screening.

Methods

Materials used to create the optimized 3D spheroid cultures can be found in Table 1.

HepaRG® Cell Line 3D Spheroids

Two media were formulated prior to thawing cryopreserved HepaRG® cells. First, a Thawing and Plating Medium was formulated by combining 1 mL of 100X GlutaMax® Supplement and one tube (11.3 mL) of HepaRG® Thawing and Plating Additive (MHTAP) per 100 mL of Williams E. Medium. Second, an Induction Medium was formulated by combining 1 mL of 100X GlutaMax® Supplement and one vial (0.6 mL) of HepaRG® Serum-free Induction Medium supplement (MHIND) per 100 mL of Williams E. Medium. Both media were kept at 4°C until ready to use.

HepaRG® Vials were thawed at 37°C and transferred to a tube containing 7.5 mL Thawing and Plating Medium. Using the guaranteed yield for the given lot of HepaRG® Cells found on the certificate of analysis, the volume of the cell suspension was adjusted with Thawing and Plating Medium so that the final cell density equaled 1×10^6 cells/mL. A 2 mL aliquot of this cell suspension was then diluted in 98 mL of Thawing and Plating Medium to a density of 20,000 cells/mL. Cells were then seeded from this aliquot at a density of 2,000 cells/well in an ultra-low attachment 96-well plate

Vendor	Catalog no.	Description	Size
HepaRG® 3D Spheroid Culture			
Lonza	NSHPRG	Cryopreserved NoSpin™ HepaRG® Cells	≥ 8 million cells
	MHTAP	NoSpin™ HepaRG® Thawing and Plating additive	11.3 mL
	MHIND	NoSpin™ HepaRG® Serum-free Induction additive	0.6 mL
Gibco	35-050-061	100X GlutaMax® Supplement	100 mL
Any		Williams E Medium (without phenol red)	
PHH and PHH+NPC 3D Spheroid Culture			
Lonza	HUCPI – spheroid qualified	Cryopreserved Primary Human Hepatocytes (plateable, DDI qualified)	≥ 5 million cells
	HUCPG – spheroid qualified	Cryopreserved Primary Human Hepatocytes (plateable)	≥ 5 million cells
	HUCLS-200K	Cryopreserved Human Stellate Cells (SC)	≥ 0.2 million cells
	HLKC-500K	Cryopreserved Human Kupffer Cells (KC)	≥ 0.5 million cells
	HLECP1	Cryopreserved Human Liver-derived Endothelial Cells (LEC)	≥ 1 million cells
	MCHT50	Human Hepatocyte Thawing Medium	50 mL
	CC-3199	HBM Basal Medium	500 mL
	CC-4182	HCM SingleQuots® Supplements	1 kit
	CC-3198	HCM Hepatocyte Culture Medium BulletKit®	1 kit; contains both CC-3199 and CC-4182
	BEBP17-737E	1M HEPES Buffer	100 mL
Any		Fetal Bovine Serum (FBS)	
For All Models			
Any		PBS (no Ca^{2+} or Mg^{2+})	
		0.4% trypan blue	
		96-well ultra-low attachment cell culture plate	
Lonza	LT07-321 LT07-121 LT07-221	Lonza ViaLight® Plus BioAssay Kit	10,000 tests 1,000 tests 500 tests

Table 1.
Materials required for 3D spheroid cell culture and biochemical analysis of DILI toxicity. Note that primary human hepatocytes must be pre-qualified to form spheroids.

Donor no.	Catalog no.	Lot no.
1	HUCPI	HUM200101
2	HUCPI	HUM222051
3	HUCPI	HUM182531
4	HUCPG	HUM222621
KC* SC* LEC*	HLKC-200K HUCLS-200K HLECP1	HKC231781 HSC231781 HLE231781

Table 2.
Primary human hepatocyte part and lot numbers (donors) used in this study. All donors were pre-qualified for spheroid formation. *Non-parenchymal cells used in this study were all from the same donor, mismatched to the four hepatocyte donors. KC = Kupffer cells, SC = Stellate Cells, LEC = Liver Endothelial Cells.

(100 µL per well) and incubated for five days without disturbance. A 50% medium change was made using Induction Medium on days five through seven and then every two–three days thereafter until the last culture timepoint (day 21). A more detailed protocol can be found on the Lonza website.[7]

Primary Human Hepatocyte 3D Spheroids (Monoculture and Co-culture with Non-Parenchymal Cells (NPCs))

Cryopreserved Lonza PHHs qualified for spheroid formation (four donors; see Table 2 for part number and donor number) were thawed and counted according to manufacturer instructions. PHHs were then seeded in a Spheroid Formation medium (consisting of Hepatocyte Culture Medium (HCM™ Medium) with 20% FBS and 25 mM HEPES added to encourage spheroid formation) at a density of 1,500 cells/well in an ultra-low attachment 96-well plate. For co-cultures, Lonza NPCs were thawed and counted according to manufacturer instructions, then combined with PHHs in Spheroid Formation medium in the following ratio: 1 PHH : 0.15 Kupffer Cells (KCs) : 0.125 Stellate Cells (SCs) : 0.225 Liver Endothelial Cells (LECs). Cells were then plated at a density of 1,500 PHHs, 225 KCs, 188 SCs, and 337 LECs per well in an ultra-low attachment 96-well plate. Plates were incubated for five days without disturbance. A 50% media exchange with serum-free HCM™ Medium was performed on days five through seven and then every two to three days thereafter until the last culture timepoint (day 21). The Lonza website contains more detailed protocols for monoculture[8] and co-culture[9] of PHH 3D spheroids.

Drug panel and dosing

This study aimed to validate and compare the behavior of all three cell culture model types (HepaRG® Cell Line vs. PHH monoculture vs. PHH-NPC co-culture) when challenged with a panel of diverse hepatotoxic compounds, allowing for a harmonized workflow from early drug screens (using HepaRG®) to later-stage drug screens (PHHs in monoculture or co-culture). For all culture types and compounds tested, three to six spheroids were dosed with an eight-point titration of each drug starting on day seven of culture during the media exchange step. Each drug was re-administered during subsequent media changes to maintain its concentration in the medium. Table 3 shows each drug tested in the initial tox panel, their mode of action, severity of toxicity, and the doses used in this study. Albuterol was included as a negative control, as well as a drug-free DMSO vehicle control.

Biochemical analysis

During method development, samples were taken from each cell culture model on days 10, 14, and 21 of culture to examine spheroid morphology and measure CYP3A4 induction to show that each model could maintain long-term functionality. CYP3A4 induction was measured after 72-hour exposure to DMSO (basal) or 10 µM Rifampicin (induced) using a luminescence-based assay (Promega).

EC50 values for each drug were determined by measuring total cellular ATP as a proxy for cell viability. Measurements were made in three to six individual spheroids per condition at days 10, 14, and 21 (representing 3, 7, and 14 days of exposure) using Lonza's ViaLight® Plus Bioassay Kit per the kit instructions. EC50 values were normalized to respective vehicle controls on each day, and dose-response curves were generated using log (drug concentration) vs normalized response with a 3PL fit. EC50 values were averaged across donors. Two-way ANOVA with Tukey's multiple comparison test was used to determine statistical differences in EC50 values between culture models and between days.

Results and discussion

Spheroid morphology and functionality

In all three cell culture models (HepaRG®, PHH monoculture, and PHH + NPC co-culture), spheroids formed within seven days of culture initiation and maintained proper morphology throughout the 21-day culture (Figure 1). CYP3A4 fold change between basal and induced (by 10 µM Rifampicin) states was much lower in HepaRG® Spheroids than in PHH-based spheroids. However, this was due to the much higher underlying basal CYP3A4 activity found in HepaRG® Cells (Figure 2A vs. B – C). High CYP3A4 induction is typical of HepaRG® Cells, making them an excellent candidate model for studying responses to drugs metabolized through the CYP3A4 pathway.[4] Furthermore, this model uses media supplemented with the HepaRG® Serum-free Induction Medium supplement, which further increases basal CYP3A4 activity (Figure 2A). Interestingly, the fold change between baseline and induced CYP3A4 activity was much higher in PHH-NPC spheroids compared to monoculture PHH spheroids (Figure 2B vs 2C) due to baseline CYP34A expression in co-culture formats being nearly absent. Downregulation

Compound	Toxicity mechanism	Severity	Cmax
Acetaminophen[10]	Oxidative stress	Low	136 µM
Troglitazone[1]	Mitochondrial toxicity / Bile acid-mediated toxicity	Severe; Idiosyncratic	6.39 µM
Diclofenac[1]	Multiple; Immune mediated	Moderate; Idiosyncratic	9.43 µM
Fialuridine[11]	Mitochondrial toxicity	Severe	0.64 µM
Cyclosporin A[1]	Bile acid accumulation; Cholestatic injury	Moderate	0.77 µM

Table 3.
DILI toxicity panel used to challenge 3D spheroid cell culture models in this study. Severity: "Idiosyncratic" indicates compounds where toxicity is idiosyncratically donor dependent. Cmax = the highest possible serum concentration for a given compound.

of CYP450 enzymes (including CYP3A4) by NPCs, which can potentially alter the toxicity of compounds metabolized by CYP450 enzymes, has been observed in other studies as well.[12] All three models maintained healthy morphology and functionality during 21 days in culture, making them excellent candidate models for testing compounds on both acute and chronic timescales.

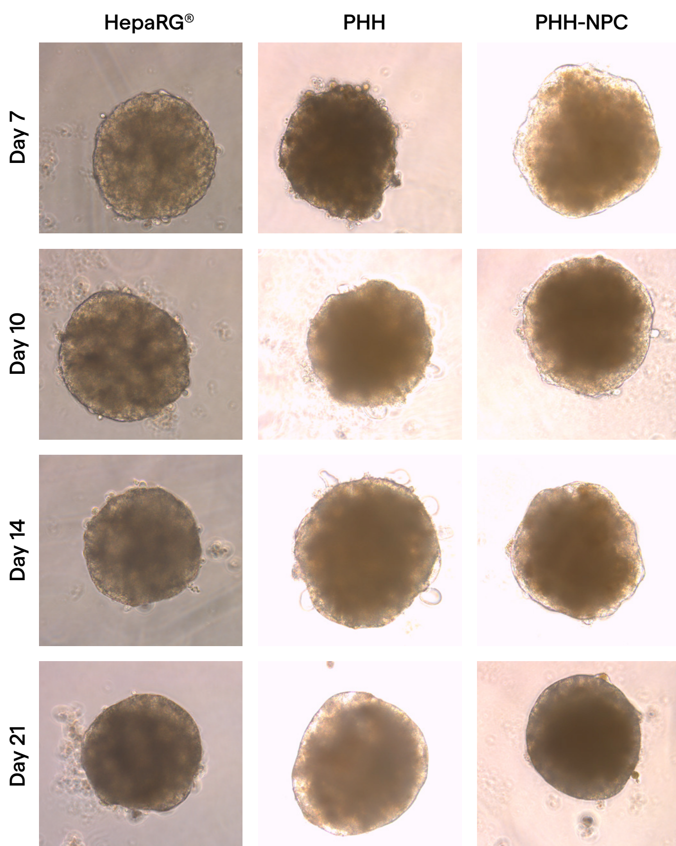

Figure 1.
Representative images of spheroid formation by primary human hepatocytes (Donor 1) in monoculture (PHH), co-culture with non-parenchymal cells (PHH + NPC) and by HepaRG® Hepatocarcinoma Cells. Spheroids formed by day seven for all culture conditions and were maintained throughout the 21-day culture.

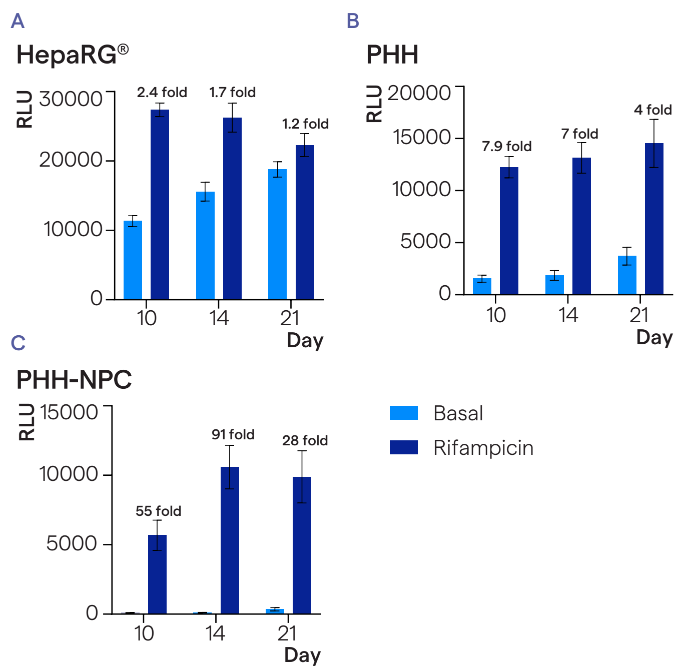

Figure 2.
Fold-change in CYP3A4 induction levels between average basal (DMSO control) and induced (10 µM rifampicin) states for HepaRG® Spheroids (A), primary human hepatocyte (PHH) spheroids in monoculture (B), and in co-culture with non-parenchymal cells (PHH-NPC) (C). HepaRG® results are an average of three experiments. PHH monoculture and co-culture results are an average across four donors. Note that each PHH donor in co-culture was paired with the same donor-mismatched NPCs. RLU = relative light units.

Challenging HepaRG®, PHH, and PHH+NPC 3D spheroid culture models with a DILI drug testing panel

All five hepatotoxic compounds elicited DILI toxicity across all three spheroid models, with EC50 values becoming significantly lower over time (Figure 3). However, the toxicity patterns, timing, and severity varied by culture model (Figure 3). Toxicity patterns were the most consistent across all drugs in HepaRG® Cells. For acetaminophen, troglitazone, and cyclosporin A , toxicity in HepaRG® Spheroids was both acute and severe (Figure 3A, B, E). EC50 values for troglitazone and cyclosporin A toxicity were significantly lower in HepaRG® Spheroids than both PHH-based culture formats at three days after initial dosing. Diclofenac and fialuridine toxicity was also consistent for HepaRG® Spheroids, with EC50 values declining significantly, but over longer timescales (Fig 3C, D). Consistent and severe toxicity across the entire drug panel was likely driven by high baseline CYP3A4 enzyme activity in HepaRG® Spheroids as well as the culture media that was designed to induce CYP3A4 enzymes (Figure 2).[4] Overall, these results suggest that HepaRG® Spheroid cultures are an excellent tool for screening drug candidates in early-stage drug development, as their high CYP3A4 enzyme activity predicts many hepatotoxic responses that may be sub-clinical or idiosyncratic in other culture models.

PHH monoculture spheroids responded similarly to HepaRG® Spheroids across all five hepatotoxins, showing significant declines in EC50 values. However, the declines were more gradual and over longer time scales for acetaminophen, troglitazone, and cyclosporin A compared to HepaRG® Cells, which may be due to differences in CYP3A4 activity (Figure 3). Furthermore, both troglitazone and diclofenac, which are both known to have idiosyncratic toxicity, showed strong donor-to-donor variability in EC50 values (Figure 4). This idiosyncratic response was also observed in PHH-NPCs, but for troglitazone only (Figure 4C). In comparison to the other two culture formats, the inclusion of NPCs in PHH spheroids greatly altered toxicity for immune-mediated compounds. EC50 values for diclofenac and cyclosporin A in PHH-NPC spheroids were significantly higher than either PHH monoculture or HepaRG® Spheroids at late timepoints, indicating that the presence of liver-resident immune cells greatly

moderated toxicity and liver damage by both drugs (Figure 3C, E).

All three spheroid models showed strong hepatotoxic responses to fialuridine (Figure 3D). 3D spheroid models are far better at detecting fialuridine toxicity than other culture models due to their long culture time and better approximation of *in vivo* conditions, demonstrating severe toxicity only after a period of about seven days.[6,11] This study demonstrates that all three spheroid models, including HepaRG®, are capable of detecting hepatotoxicity in compounds that require hepatic models with high degrees of cellular differentiation.[6,11]

Idiosyncratic and immune-modulated drug-induced liver injury

Myriad factors determine whether a drug or compound is hepatotoxic. As such, countless studies have explored the toxicity mechanisms of drugs such as troglitazone, diclofenac, and cyclosporin A to create better models that can predict drug candidate toxicity and safety. Until very recently, the causes of idiosyncratic troglitazone toxicity were not understood. A combination of genetic and environmental factors such as obesity, diabetes, or other conditions that might result in oxidative or mitochondrial stressors were thought to be involved.[13] A more recent study found that, in some individuals, troglitazone triggers immune cells to repress IL-12a production in PHH-immune cell co-cultures, leading to liver injury.[14]

Previous studies have indicated that idiosyncratic diclofenac toxicity could be due partly to variation in responses of liver-resident immune cells across individuals.[15] Polymorphisms in the expression of IL-10 and IL-4 can result in increased diclofenac-related neoantigen presentation by monocytes and activation of T cells in some individuals, leading to inflammatory liver injury.[15] At the same time, alternate phenotypes leading to higher IL-10 production in the liver could prevent toxicity.[15] We used the same mismatched donor for the non-parenchymal cells in each co-culture (Table 2), so it is possible that NPCs from donors with certain phenotypes may display the opposite pattern (a severe toxicity response to diclofenac) of what is observed here.

Reduced cyclosporin A toxicity observed in PHH-NPC co-culture models is likely due to several complex factors. Kupffer cells (KCs) in particular have been implicated in increased hepatotoxicity via promotion of inflammatory conditions in the liver in response to drug-related liver injury and subsequent immune-related damage to tissues.[16] KCs are also responsible for clearing apoptotic and necrotic cells from the liver and play a key role in both the progression and regression of diseases such as liver fibrosis.[5,17,18] Furthermore, we observed that the presence of NPCs downregulates CYP3A4 activity in PHHs (Figure 2), CYP3A4 being the primary enzyme responsible for metabolizing cyclosporin A.[12,19,20] Though the precise mechanisms of cyclosporin A hepatotoxicity are not fully understood, cyclosporin A toxicity in PHH-NPC spheroids was likely moderated by a complex interplay between KCs, SCs, LECs, and the hepatocytes themselves within the spheroid microenvironment that ultimately promoted anti-inflammatory and wound-healing processes and altered the levels of downstream metabolites generated via CYP3A4.

This study demonstrated multiple mechanisms of DILI across the three culture models, including both idiosyncratic donor-dependent toxicity and immune-mediated processes that prevented DILI. Taken together, the data presented in this study and the above examples illustrate the necessity of using multiple culture models in drug safety screening to holistically assess multiple mechanisms of toxicity as well as provide physiologically-relevant models for determining safe limits of drug dosing across many donors.

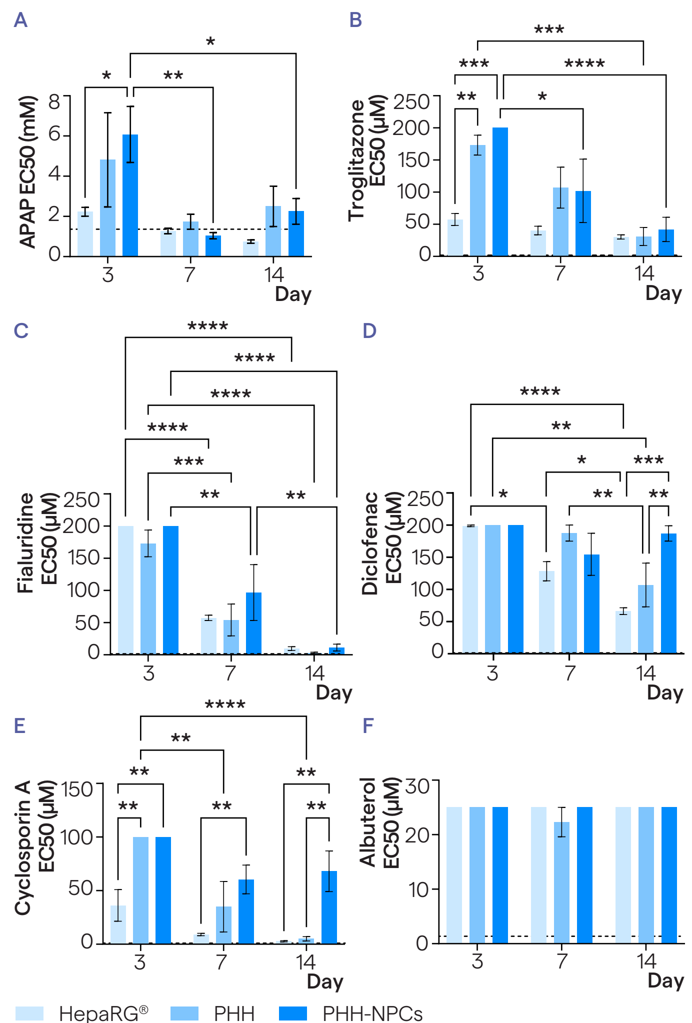

Figure 3.
EC50 values for five hepatotoxic compounds (A – E) and a non-hepatotoxic negative control (albuterol; F) administered to HepaRG®, primary human hepatocyte (PHH), and primary human hepatocyte and non-parenchymal cell co-culture (PHH-NPC) spheroids across three time points (3, 7, and 14 days of exposure). Dotted lines indicate 10x Cmax (10 times the maximum possible serum concentration for a given drug). Significant differences between time points and between culture conditions are indicated by the following: *$p < 0.03$, **$p < 0.002$, ***$p < 0.0002$, ****$p < 0.0001$.

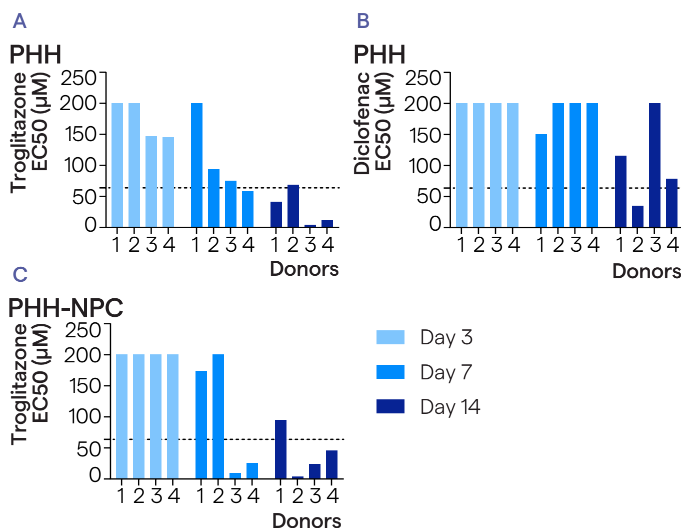

Figure 4.
Idiosyncratic responses by primary human hepatocyte-based spheroids to troglitazone and diclofenac.

Conclusions

Overall, this study showed that:

1. All three spheroid models maintained healthy morphology and metabolic functionality (CYP3A4 activity) throughout 21 days of culture. Dosing spheroids on day seven allowed measurements of toxicity across acute (3 days after initial dosing) and chronic (14 days after initial dosing) time periods.
2. HepaRG® Spheroids are a reliable and consistent model of DILI toxicity across all five drugs tested, which spanned a wide range of severity and modes of action. HepaRG® Cells responded particularly strongly to CYP3A4-mediated hepatotoxins due to their naturally high expression of CYP3A4. CYP enzyme induction can be further increased by culturing HepaRG® cells in a specialized induction medium that primes them for further induction. HepaRG® Spheroids provide a more physiologically relevant model suitable for early-stage drug screening where repeatability and consistency are crucial.
3. PHH spheroids provide greater physiological relevance, as they better reflect *in vivo* conditions, and allow for the donor-to-donor variation critical to identifying idiosyncratic DILI toxicity. PHH spheroids are ideal for late-stage drug screening and safety testing on smaller numbers of drug candidates as well as for establishing safe dosing limits.
4. PHH-NPC spheroids provide the greatest physiological relevance of all three spheroid models. While this study and others have shown that NPCs can reduce toxicity of immune-modulated compounds,[1] other studies have shown that inducing inflammatory pathways in NPCs can worsen hepatotoxic effects.[5,16] Co-culturing with NPCs allows for the inclusion of immune-mediated processes that are key to understanding DILI as a result of inflammatory vs. anti-inflammatory pathways in the liver. In addition, NPC co-culture can help identify or elucidate the causes of idiosyncratic toxicity, especially in compounds that modulate or interact with resident immune cells in the liver. Validation and use of co-culture models with high physiological relevance such as PHH-NPC spheroids will help drive compliance with the FDA Modernization Act 2.0 and eventually replace animal models with reliable human tissue analogs.

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