

SUPPLEMENTARY MATERIAL

Accompanying the article entitled:

Characterizing the Reproducibility in Using a Liver Microphysiological System for Assaying Drug Toxicity, Metabolism and Accumulation

Andres Rubiano¹, Amruta Indapurkar¹, Ryosuke Yokosawa¹, Alina Miedzik², Barry Rosenzweig¹, Ayesha Arefin¹, Chloe M. Moulin¹, Keri Dame¹, Neil Hartman^{1Ψ}, Donna A. Volpe¹, Murali K. Matta¹, David J. Hughes², David G. Strauss^{1,3}, Tomasz Kostrzewski², Alexandre J.S. Ribeiro^{1*}

¹ U.S. Food and Drug Administration, Center for Drug Evaluation and Research, Office of Translational Sciences, Office of Clinical Pharmacology, Division of Applied Regulatory Science, Silver Spring, MD, USA

² CN Bio Innovations Limited, Cambridge CB4 0WN, United Kingdom

³ U.S. Food and Drug Administration, Center for Drug Evaluation and Research, Office of Clinical Pharmacology, Silver Spring, MD, USA

Ψ Deceased

* Corresponding author: Alexandre J.S. Ribeiro, Alexandre.Ribeiro@FDA.HHS.GOV

10903 New Hampshire Avenue
WO 64, Room 2036
Silver Spring, MD 20993
Tel: (301) 796-0126

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Figure S1

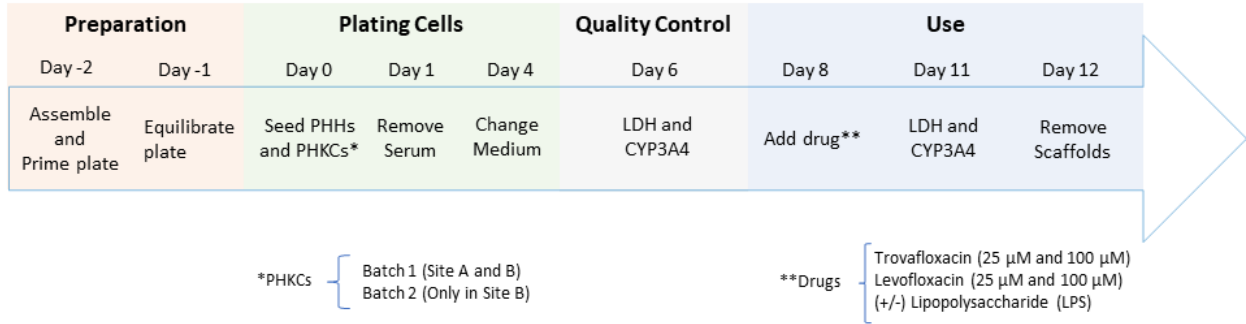


Figure S1. Workflow of the experiment for testing the toxicity of trovafloxacin at different sites and with different batches of PHKCs. Liver MPS plates were assembled two days before seeding cells, and assembled plates were equilibrated in plating medium a day before seeding cells. Co-cultures of PHHs and PHKCs were maintained in plating medium for one day after cell seeding. One day after seeding, cells were cultured in serum-free maintenance medium, which was changed two days later. LDH production and CYP3A4 activity were measured on day six. Solutions of trovafloxacin and levofloxacin were prepared and co-dosed on day eight with LPS to activate PHKCs. Wells not co-dosed with LPS were also evaluated. LDH production and CYP3A4 activity were analyzed on day 11. On day 12, scaffolds were removed from the liver MPS.

Figure S2

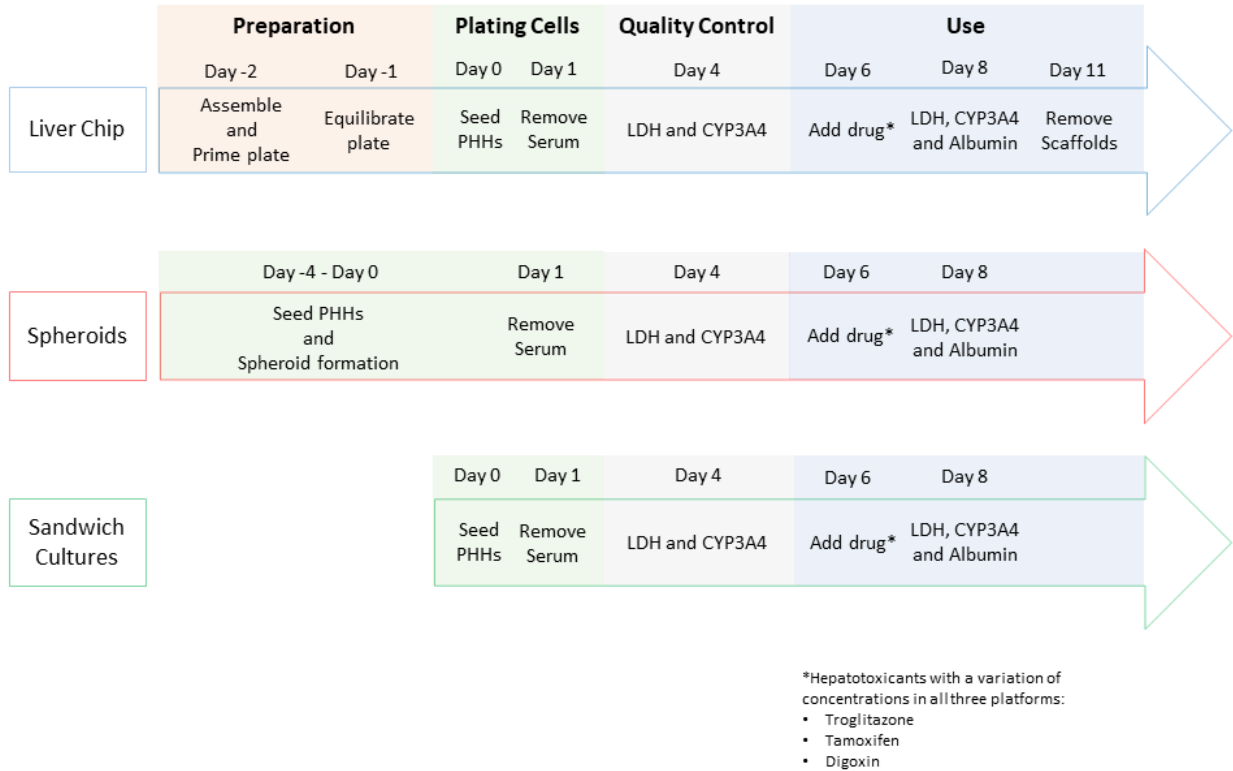


Figure S2. Workflow describing the experiment that tested differences in sensitivity to toxicants between liver MPS and other platforms for culturing PHHs. Liver MPS plates were assembled two days before seeding cells and plates were equilibrated in the plating medium one day before seeding cells. PHHs were seeded in the liver MPS and sandwich cultures one day before shifting from plating medium to serum-free maintenance medium. Spheroids were kept in plating medium for five days before changing to maintenance medium. On day six, compounds (troglitazone, tamoxifen and digoxin) were added to wells at varying concentrations between wells. LDH production, CYP3A4 activity, and albumin production were measured. MPS was disassembled after day 11.

Figure S3

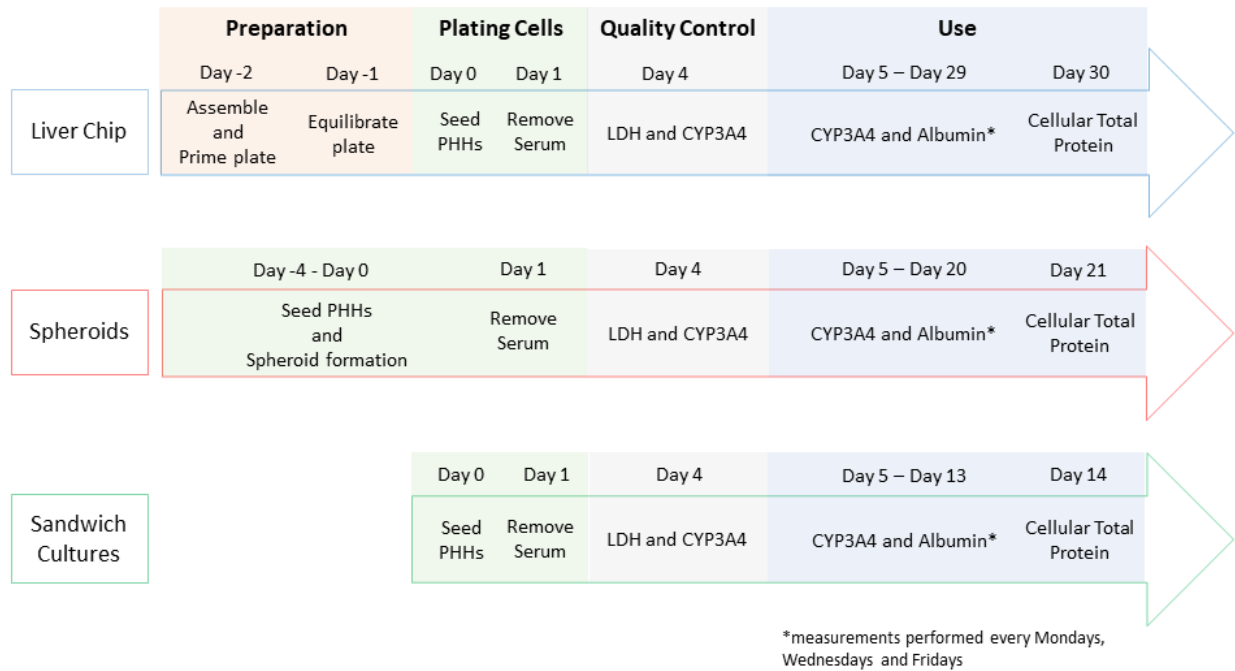


Figure S3. Workflow describing the evaluation of hepatic function at different days of culture in different platforms: liver MPS, spheroids and sandwich cultures. Liver MPS plates were assembled two days before seeding PHHs and plates were equilibrated in the plating medium one day before seeding cells. PHHs were seeded in the liver MPS and as sandwich cultures a day before shifting from plating medium to maintenance medium. With spheroids, this medium change was performed five days after seeding PHHs. CYP3A4 activity and albumin production were assayed every Mondays, Wednesdays and Fridays for all platforms. Total protein was measured at the end of the experiment.

Figure S4

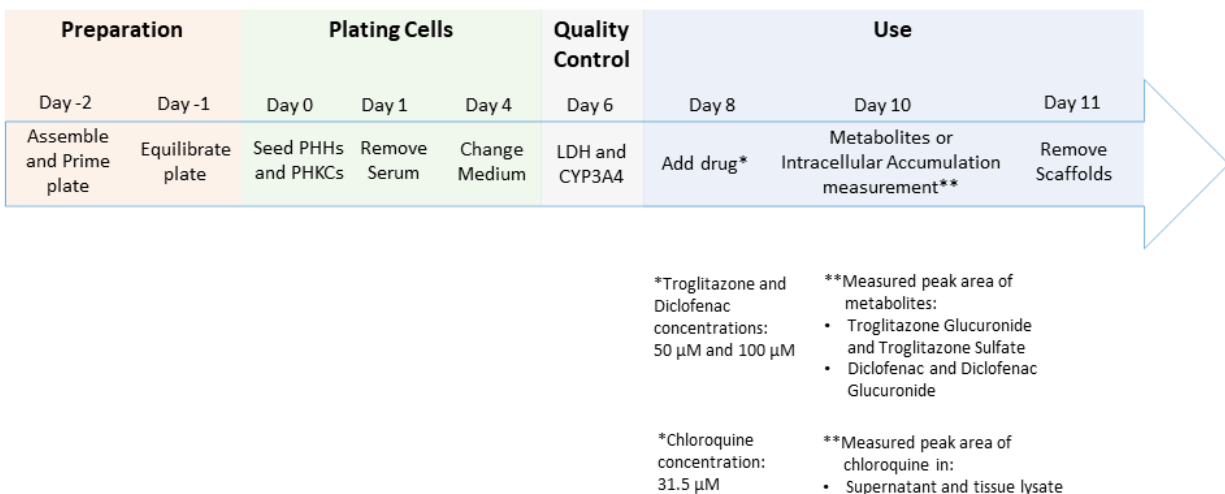


Figure S4. Workflow of the experiment to measure phase II metabolites and intracellular accumulation of compounds with the liver MPS. Liver MPS plates were assembled two days before seeding cells, and plates were equilibrated in the plating medium a day before seeding cells. PHHs and PHKCs were co-cultured and the cell culture medium changed the next day from plating medium to serum-free maintenance medium. After measuring LDH production and CYP3A4 activity on day six to evaluate cellular and hepatic function, compounds were added on day eight to wells for later measuring the formation of phase II metabolites from troglitazone or diclofenac, or the intracellular accumulation of chloroquine. Scaffolds were removed on day 11 and MPS was disassembled.

Figure S5

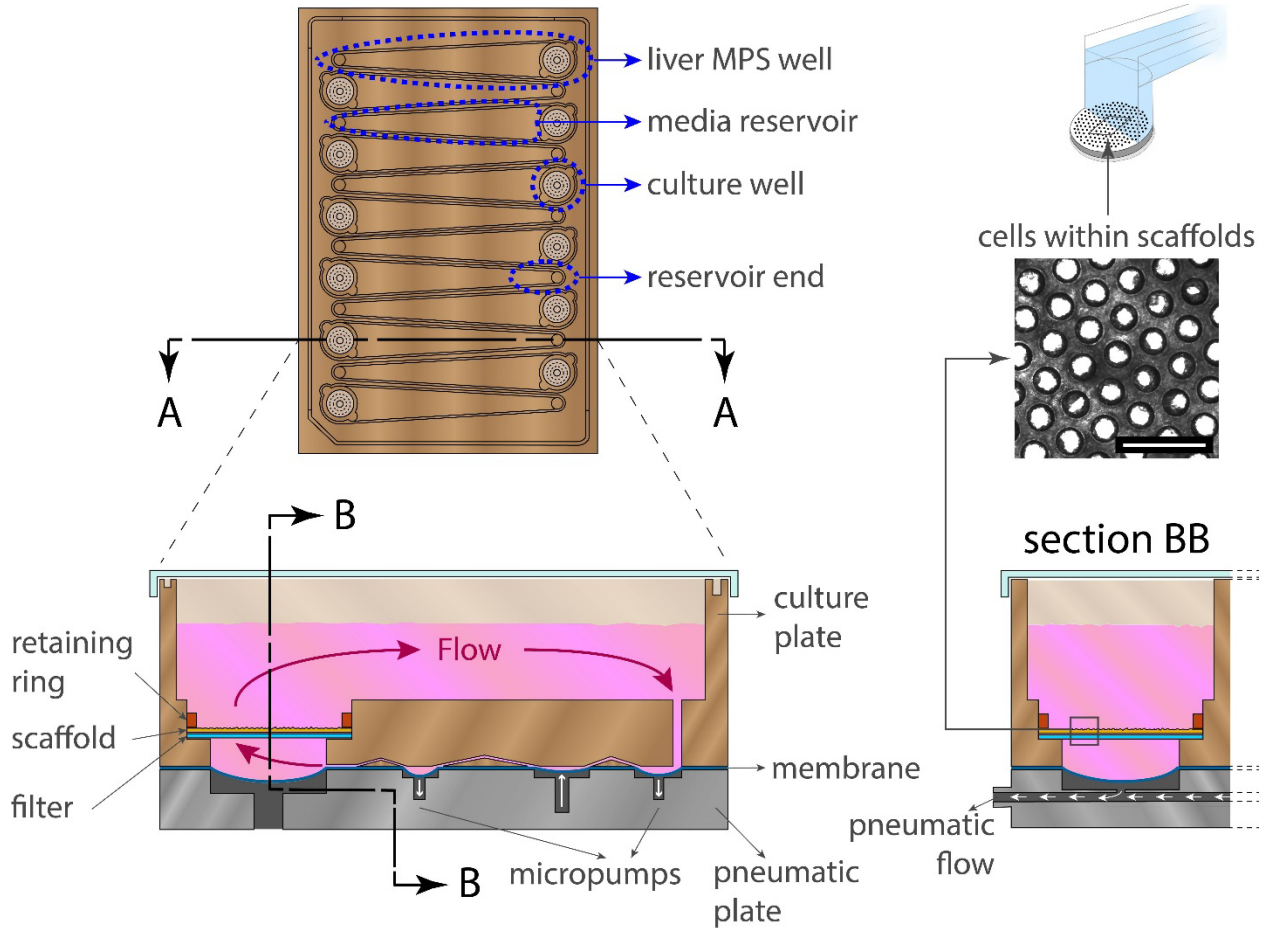


Figure S5. General design and operation of the liver MPS plate. The liver MPS plate was designed to contain 12 wells. Each well contains a media reservoir unit and a culture well unit. Media recirculates along the well and through the microfluidic channels between the culture plate and a deformable membrane. Media flow is produced by micropumps that displace pockets of fluid between the membrane and the culture plate, which is enabled by the air that flows inside the pneumatic plate. Cells are cultured within the scaffold and are exposed to flow. The liver MPS pump controller is connected to compressed air and vacuum lines, and regulates the air flow, the pressure and air mass flow within the microchannels of the pneumatic plate. For the LC12 MPS plates, compressed air and vacuum pumps are contained within the PhysioMimix™ controller, which regulates the operation of the plate. Scale bar in image of scaffold represents 1 mm.

Figure S6

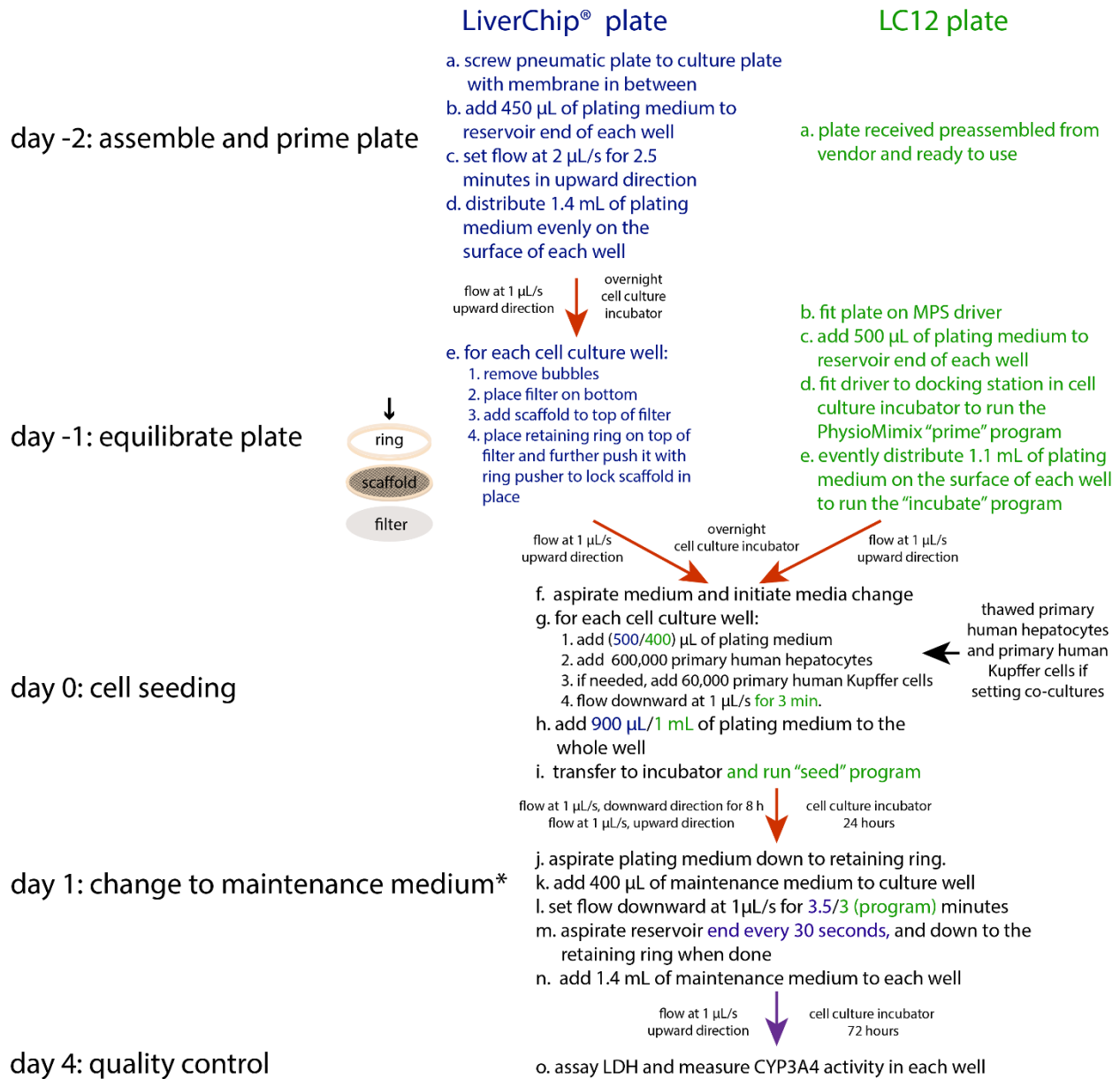


Figure S6. Preparation flow chart for the two types of the liver MPS plate shows the fundamental steps from initial assembly to the quality control stage, at which point the cultures are ready for experiments. Steps and quantities specific to LiverChip® MPS plates are highlighted in blue, while steps and quantities specific to LC12 MPS plates are highlighted in green. Red arrows indicate incubation at 37°C for 24 hours.

Figure S7

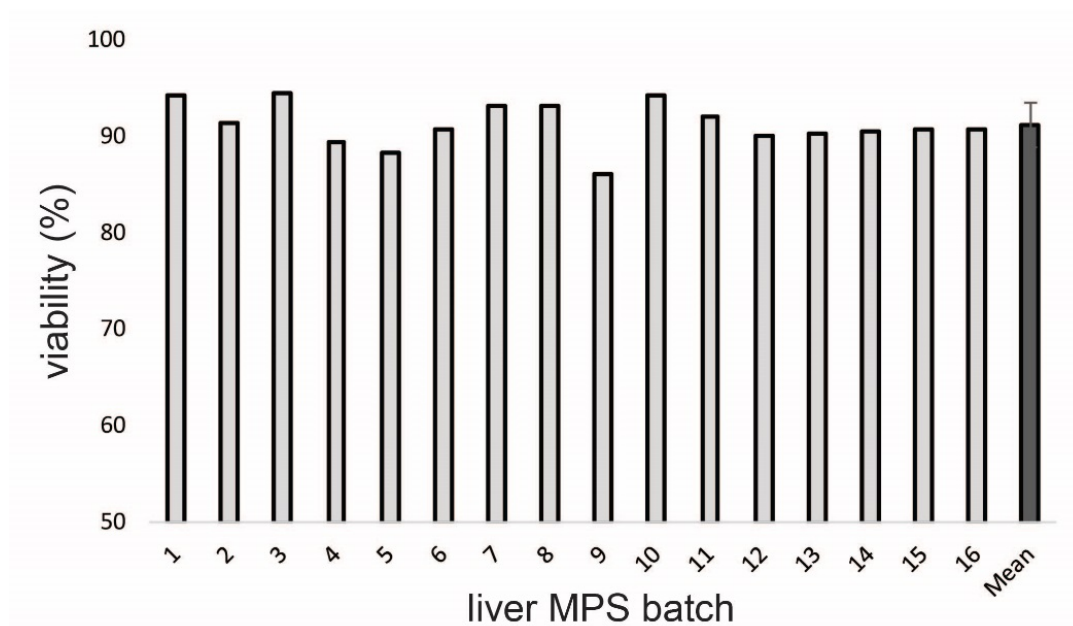


Figure S7. Viability of PHHs (human plateable hepatocytes, transporter qualified, Gibco, lot number HU8264) after being thawed to culture different liver MPS batches. Cell viability was assayed with the trypan blue exclusion method. The mean of all measurements and associated standard deviation is also presented.

Figure S8

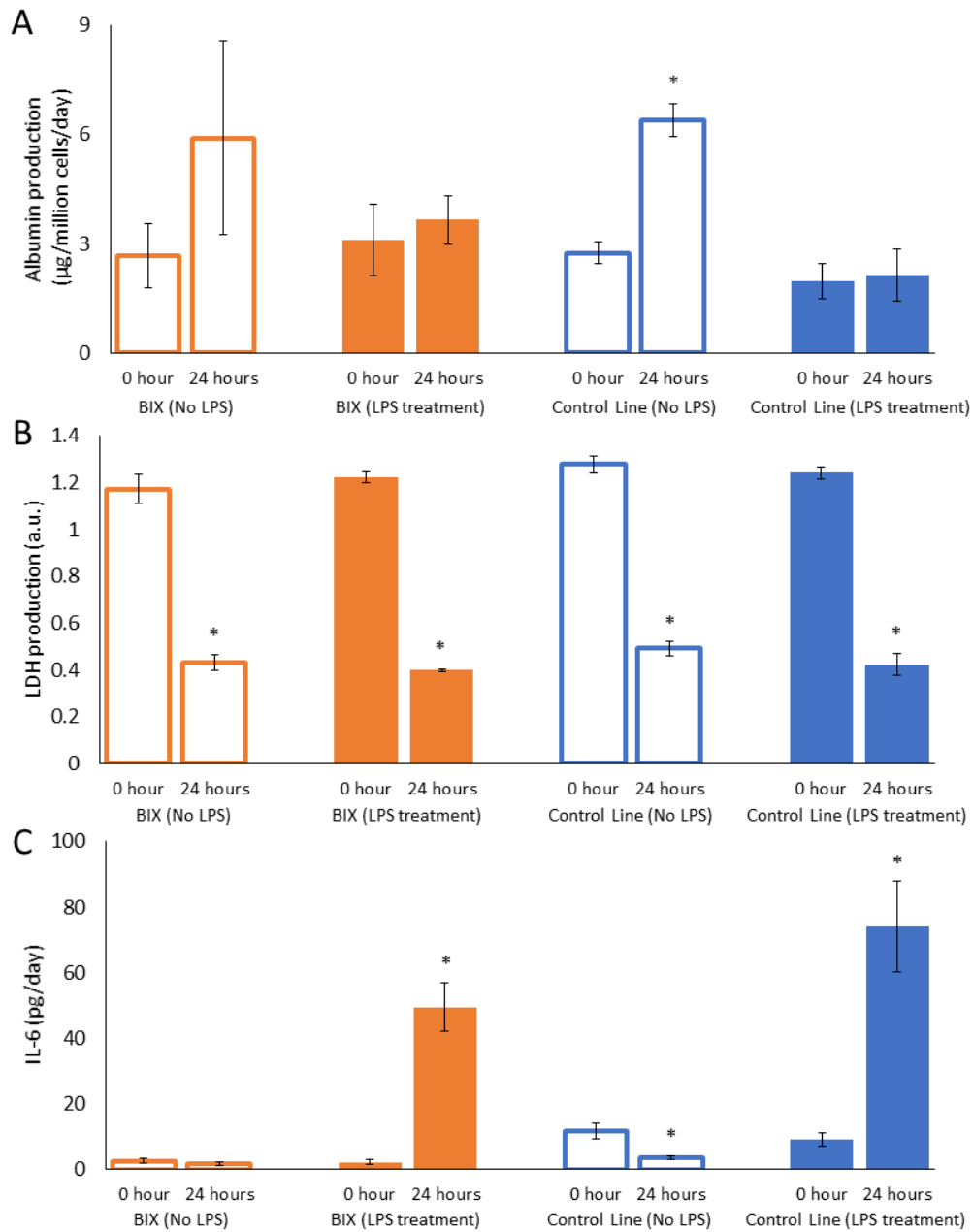


Figure S8. Functional properties of PHKCs (lot number BIX) relative to a previously qualified control lot of cells. In co-cultures of PHKCs and PHHs in the liver MPS, LPS was added to wells for 24 hours and production of (A) albumin, (B) LDH and (C) interleukin 6 (IL-6) were assayed for groups (n=3) of MPS wells that were treated with LPS relative to wells where no LPS was added. Adding LPS inhibited the production of albumin, had no effects in LDH secretion and activated IL-6 expression. *P-value < 0.05 calculated with student's t-test. Error bars represent the standard deviation of the mean.

Figure S9

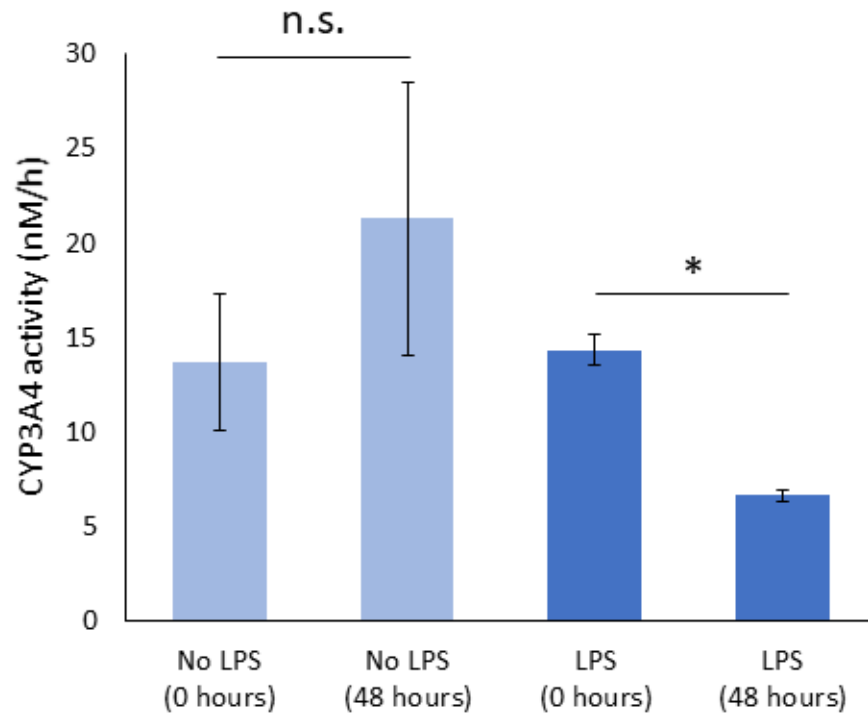


Figure S9. Effects of LPS in CYP3A4 activity of the liver MPS maintaining co-cultures of PHKCs (lot number HK8323) with the used batch of PHHs. LPS was added for 48 hours to wells (n=3) of a liver MPS where PHKCs were co-cultured with PHHs and CYP3A4 activity was assayed. LPS induced a decrease in CYP3A4 activity when compared with the wells (n=3) that were not exposed to LPS. *p-value < 0.01 calculated with student's t-test. Error bars represent the standard error of the mean.

Figure S10

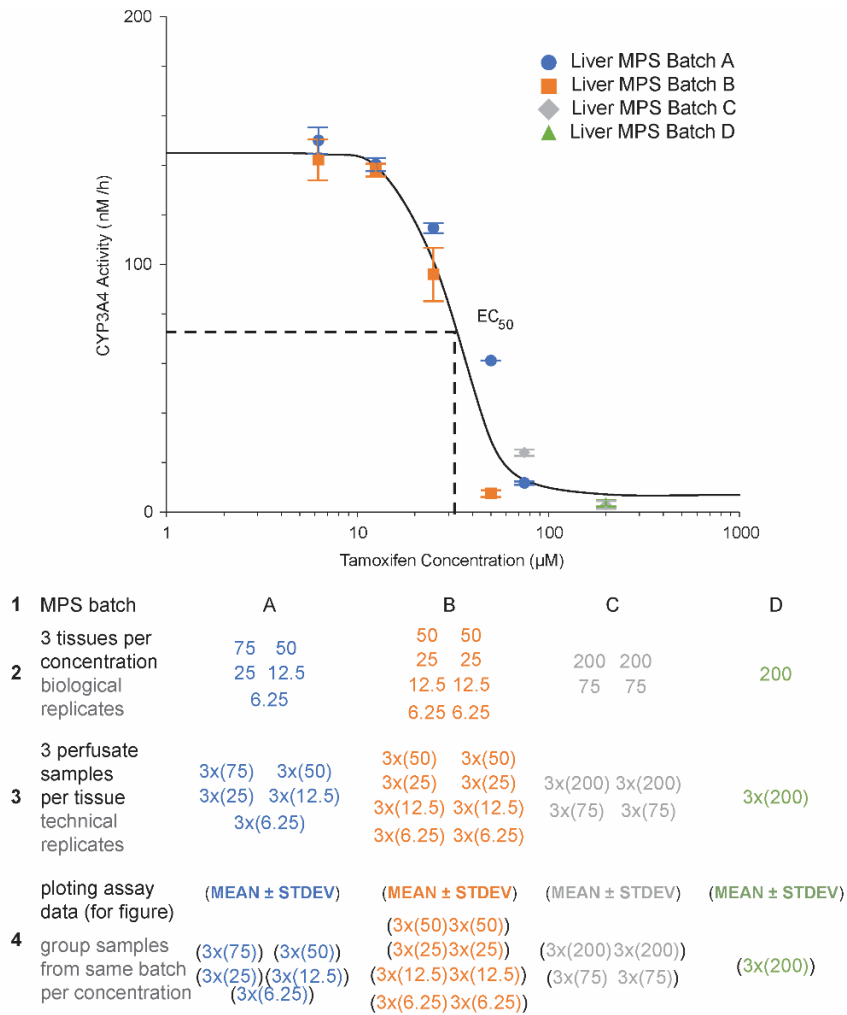


Figure S10. Example of data acquisition process to test the sensitivity of the liver MPS to an hepatotoxicant. Different MPS batches were used to test system sensitivity to hepatotoxicants. In the presented example, PHHs were cultured in 4 different liver MPS batches: A, B, C and D. The hepatotoxicant tamoxifen was added to wells in maintenance medium for 48 hours at various concentrations: 200 μM , 75 μM , 50 μM , 25 μM , 12.5 μM and 6.25 μM . CYP3A4 activity was measured for each well and the resulting data was fit to a dose-response sigmoid curve. EC_{50} for the presented data was derived from the curve and indicated with dashed lines ($\text{EC}_{50} = 31.12 \mu\text{M}$). Each data point shows the average of groups of three technical replicates for each drug concentration from one or two tissues from each liver MPS batch (A, B, C and D). Overall, three wells were used as biological replicates per experimental condition. Error bars show the standard error of the mean of groups of experimental triplicates for each batch used per condition. The bottom diagram illustrates the general process of data acquisition. 200 μM technical replicate samples were collected from one tissue from batch D and two tissues from batch C. 75 μM technical replicate samples were collected from one tissue from batch A and two tissues from batch C. 50 μM sample technical replicate samples were collected from one tissue from batch A and two tissues from batch B. 25 μM technical replicate samples were collected from one tissue from batch A and two tissues from batch B. 12.5 μM technical replicate samples were collected from one tissue from batch A and two tissues from batch B. 6.25 μM technical replicate samples were collected from one tissue from batch A and two tissues from batch B. Unless indicated otherwise, experiments were concluded after acquiring three replicates for each concentration.

Figure S11

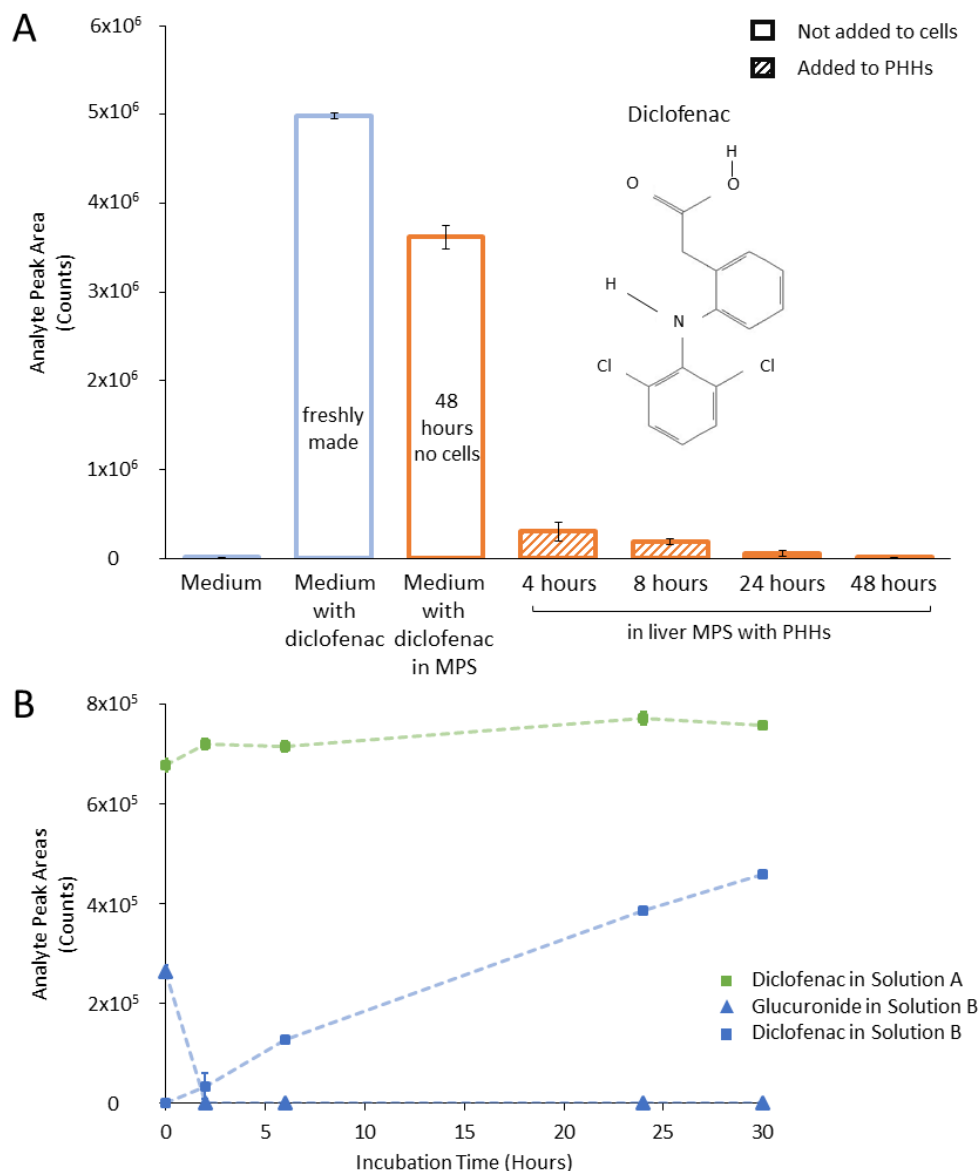


Figure S11. Quantification of diclofenac in maintenance medium. (A) Peak area of drug was detected from a blank solution composed of maintenance medium and from a 50 μM solution of drug in freshly made maintenance medium, incubated in the MPS for 48 hours and added to the liver MPS with PHHs for 4, 8, 24 and 48 hours. Each data point shows the average of 6 measurements, and error bars show the standard deviation. (B) Assessment of the stability of glucuronidated diclofenac. Peak areas of 50 μM of diclofenac and diclofenac glucuronide at different times after being dissolved in maintenance media and kept at 37 $^{\circ}\text{C}$. Two solutions were prepared: diclofenac diluted in maintenance medium (Solution A) and diclofenac glucuronide diluted in maintenance medium (Solution B). Solution A and B were incubated at 37 $^{\circ}\text{C}$ for 30 hours, and samples were collected at 0, 2, 6, 24 and 30 hours. Each data point shows the average of 6 measurements, and error bars show the standard deviation. Peak areas of diclofenac and diclofenac glucuronide were detected with liquid chromatography-mass spectrometry at each sampling time point.

Figure S12

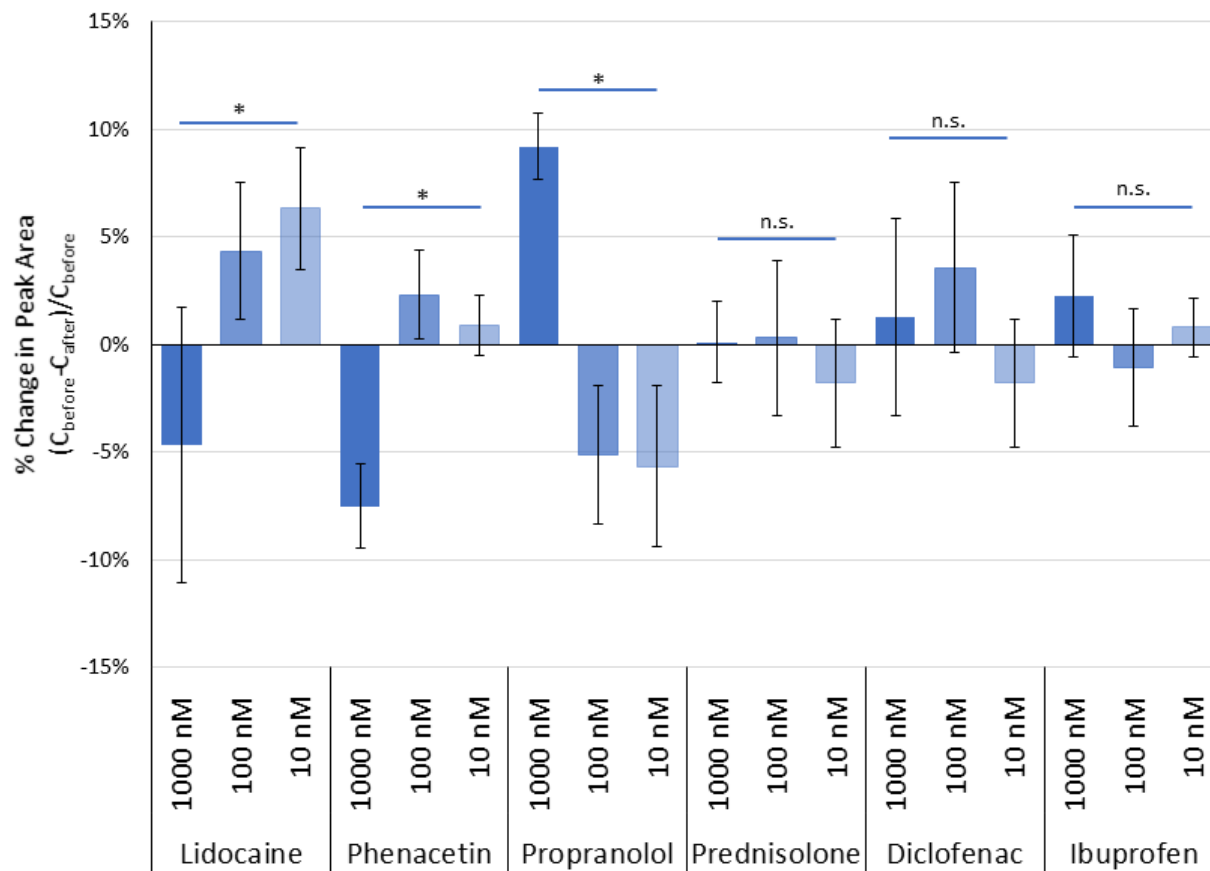


Figure S12. Quantified adsorption of compounds to the liver MPS materials. Lidocaine, phenacetin, propranolol, prednisolone, diclofenac and ibuprofen were incubated in the MPS after being diluted into three concentrations in maintenance medium: 1000 nM, 100 nM and 10 nM. Compound solutions were incubated in the MPS for 48 hours and samples were collected at 0 hours and 48 hours. Peak area of samples was detected with liquid chromatography-mass spectrometry and percent change of peak area at sampling time between 0 hours and 48 hours was calculated. * $p < 0.05$ one-way ANOVA and n.s. stands for no significance. Each data point shows the average of 6 measurements, and error bars show the standard deviation.

Figure S13

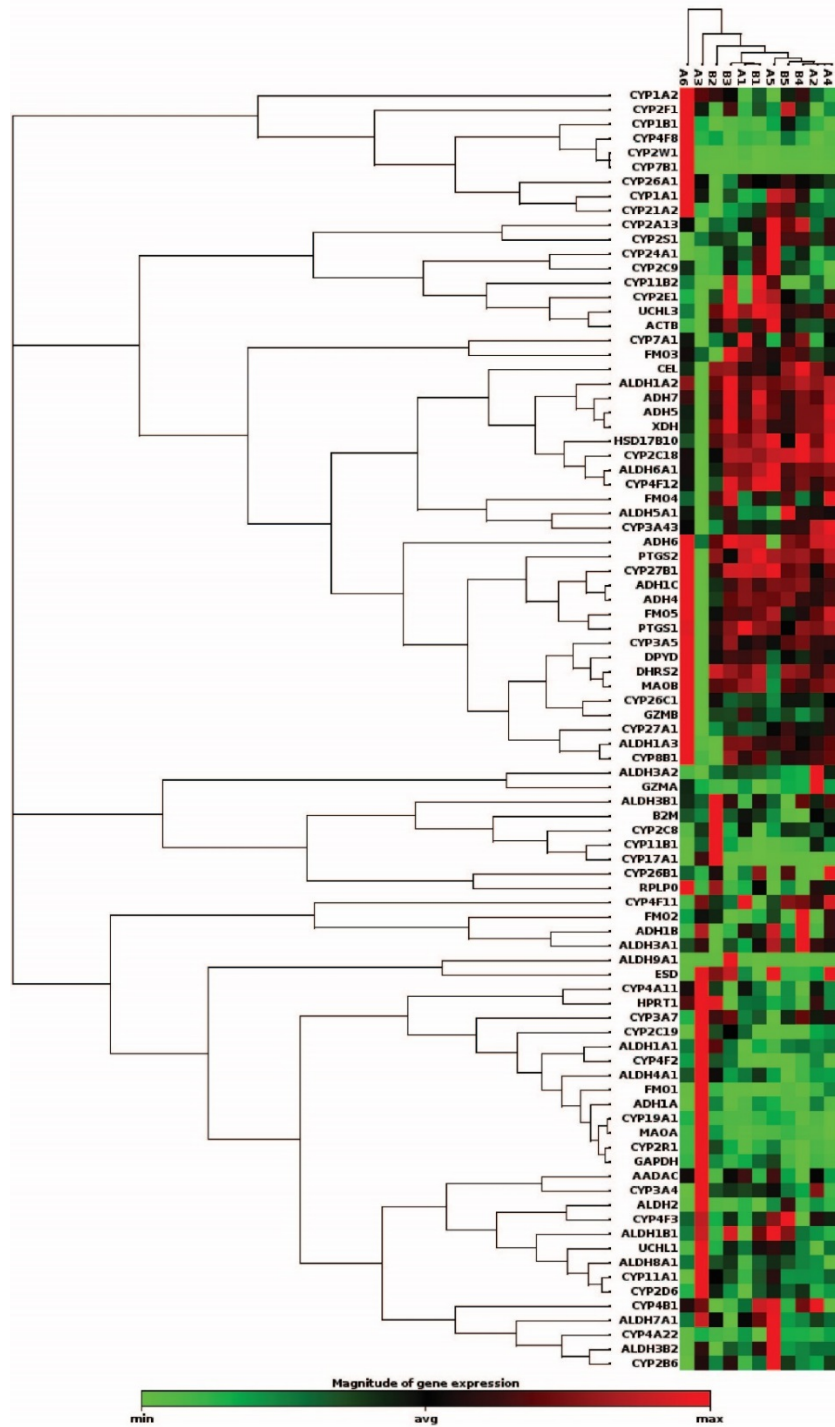


Figure S13. Unbiased clustering with geneglobe-generated scatter clustergram. Plot shows an unbiased clustering of an outlier test group composed of samples A3, A6 and B2 relative to the remaining samples represented in Figure 5. Gene expression magnitudes of samples A1, A2, A4, A5, B1, B3, B4 and B5 clustered together and represented a control group.

Figure S14

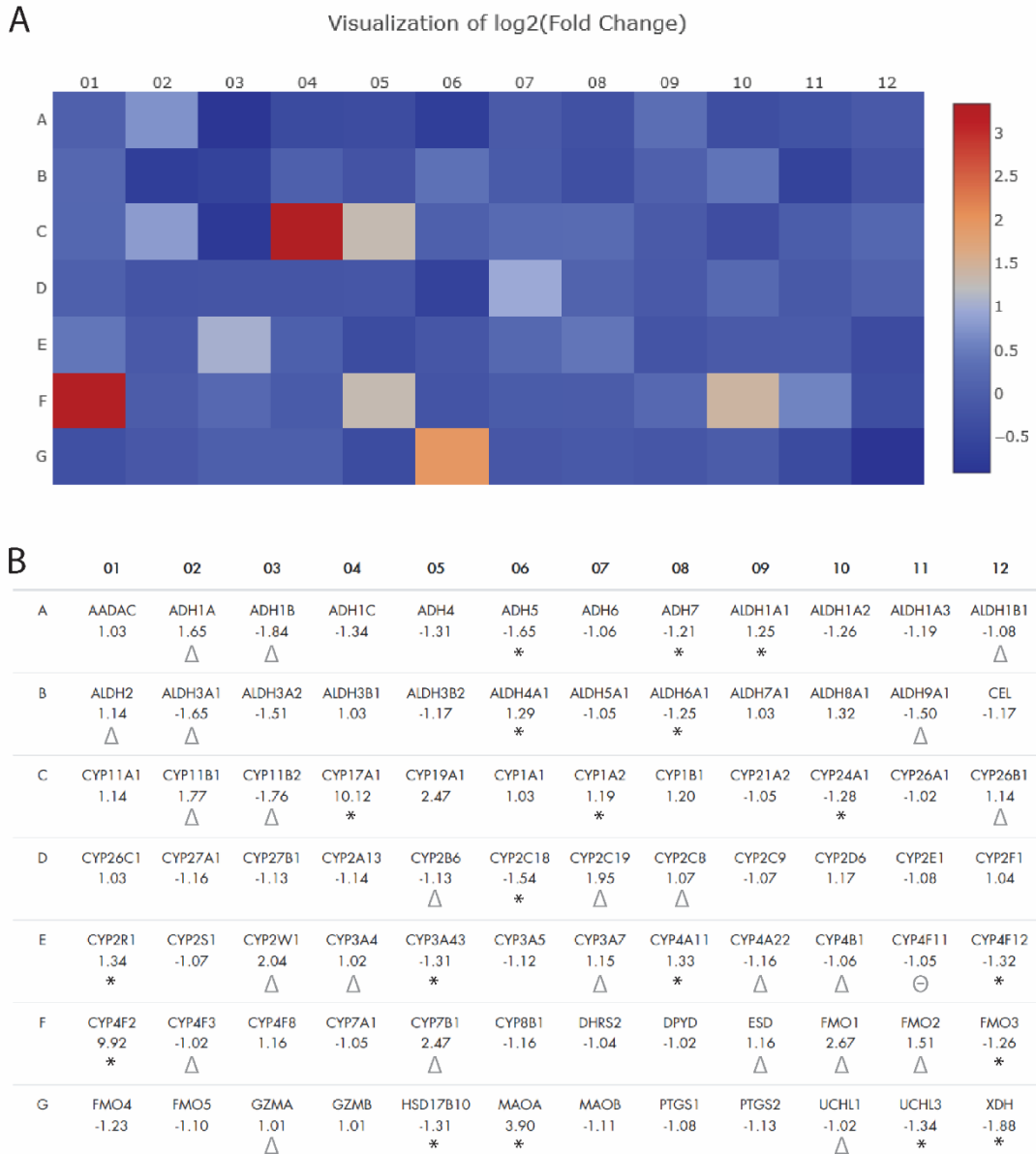


Figure S14. Fold change relative to a control group of samples in the expression of assayed genes for a test group of outlier samples that was unbiasedly determined as presented in Figure S13. (A) Geneglobe scatter matrix heat map of log₂(Fold Change) for the tested genes, which are specified in (B) along with the calculated magnitude of fold change for each gene. * p-value < 0.05 calculated from Student's t-test analysis. △: The average threshold cycle for this gene was relatively high (>30), implying that its relative expression level was low, in both control and test samples, and the p-value was unavailable or high (>0.05). ⊖: In both samples, the average threshold cycle for this gene was either not determined or greater than the default cut-off value of 35, meaning that its expression was undetected and making the fold change value erroneous.

Figure S15

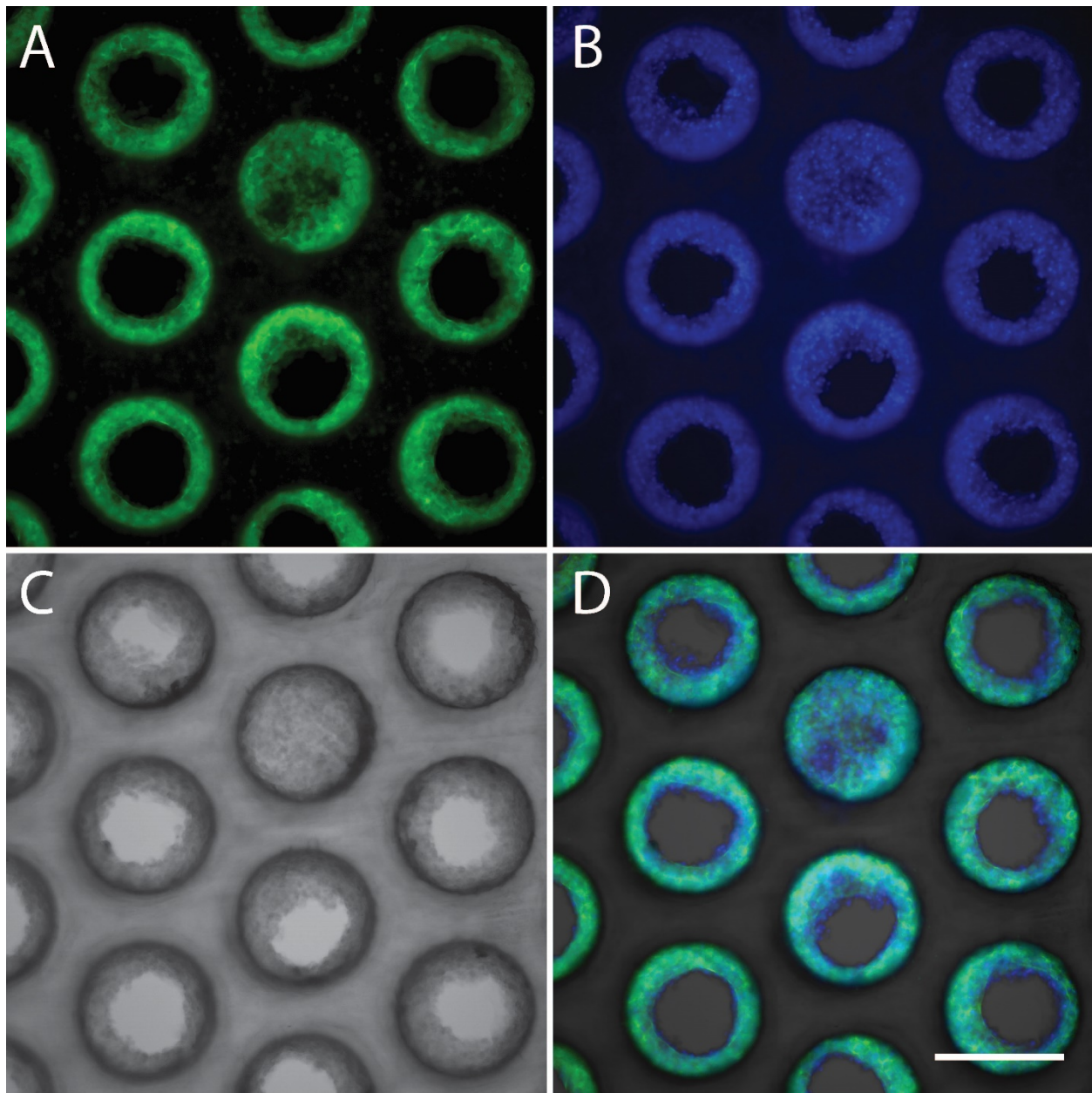


Figure S15. Microscope images of fluorescently labeled fixed PHHs within a scaffold of a liver MPS after 2 weeks in maintenance medium. For the same field of view, (a) actin labeled with Oregon Green 488 Phalloidin, (b) nuclei labeled with Hoechst, (c) bright field, and (d) merge of all the channels. Scale bar: 300 μm . Cells were cultured in 3D within the pores of the MPS scaffolds through which cell culture media flowed at a control rate.

Figure S16

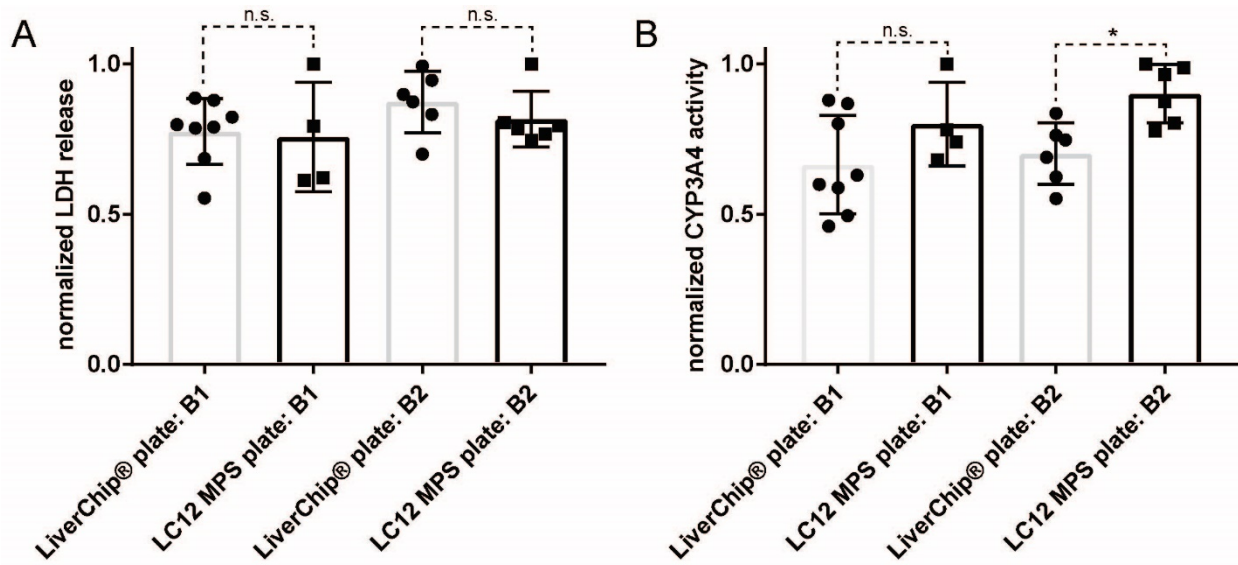


Figure S16. Cellular function of wells from two different modalities of liver MPS plates (LiverChip® and LC12) that were seeded at different times with PHHs from distinct cryopreserved vials (B1 and B2). MPSs were assayed after four days of seeding cells and values of (a) LDH production and (b) CYP3A4 activity were normalized to the maximum value obtained from the group of wells of both plate types with cells from the same vial (B1 or B2). Each dot corresponds to one MPS well and *P<0.05, n.s.P>0.05 by the unpaired t-test with Welch's correction.

Table S1. Types of cell culture medium used in this study. Different types of culture media were used depending on the stage of cell culture: i) thawing, ii) plating or iii) maintenance of cells in culture. In addition, we used different media compositions to co-culture primary human hepatocytes with PHKCs. For each type of medium, composition is described per 100 mL of volume. The thawing medium requires no preparation since it is acquired from a commercial vendor as a 50 mL tube of Cryopreserved Hepatocyte Recovery Medium (CHRM).

Culture Type	Medium Type	Base Medium	Platform	Supplements				
				Dexamethasone 10 mM	Cocktail A	Cocktail B	FBS	Hydrocortisone
PHHs Monoculture	Plating Medium	William's E Medium	liver MPS Sandwich Spheroids	10 μ L	3.6 mL	-	5 mL	-
	Maintenance Medium	William's E Medium	liver MPS Sandwich Spheroids	1 μ L	-	4 mL	-	-
PHHs & PHKCs Co-culture	Plating Medium	Advanced DMEM	liver MPS	-	3.6 mL	-	5 mL	-
	Maintenance Medium Day1-3	Advanced DMEM	liver MPS	-	-	4 mL	-	-
	Maintenance Medium > Day 3	William's E Medium	liver MPS	-	-	4 mL	-	50 μ L**
Both	Thawing Medium	CHRM*	-	-	-	-	-	-

*Cryopreserved Hepatocyte Recovery Medium

**Volume of Hydrocortisone (Sigma-Aldrich cat# H6909, 200 μ M) to result in 100 nM. Volume varies depending on initial Hydrocortisone concentration.

Vendor for all other components: ThermoFisher Scientific

Cocktail A (3.6%) results in 1% Pen-Strep, 4 μ g/mL Insulin, 2 mM GlutaMAX, and 15 mM HEPES.

Cocktail B (4%) results in 0.5% Pen-Strep, 6.25 μ g/mL Insulin, 6.25 μ g/mL Transferrin, 6.25 ng/mL Sodium Selenite, 1.25 mg/mL Bovine Serum Albumin, 5.35 μ g/mL Linoleic Acid, 2 mM GlutaMAX, and 15 mM HEPES.

Table S2. Gene symbols and respective gene description. Statistical significance (t-test determined p-value < 0.05) in the calculated fold change was observed for these genes between the test group of outlier samples and the control group.

Gene	Protein	Fold Change
CYP17A1	cytochrome P450 family 17 subfamily A member 1	Up +10.12
CYP4F2	cytochrome P450 family 4 subfamily F member 2	Up +9.92
MAOA	monoamine oxidase A	Up +3.90
CYP2R1	cytochrome P450 family 2 subfamily R member 1	Up +1.34
CYP4A11	cytochrome P450 family 4 subfamily A member 11	Up +1.33
ALDH4A1	aldehyde dehydrogenase 4 family member A1	Up +1.29
ALDH1A1	aldehyde dehydrogenase 1 family member A1	Up +1.25
CYP1A2	cytochrome P450 family 1 subfamily A member 2	Up +1.19
ADH7	alcohol dehydrogenase 7 (class IV), mu or sigma polypeptide	Down -1.21
ALDH6A1	aldehyde dehydrogenase 6 family member A1	Down -1.25
FMO3	flavin containing dimethylaniline monooxygenase 3	Down -1.26
CYP24A1	cytochrome P450 family 24 subfamily A member 1	Down -1.28
CYP3A43	cytochrome P450 family 3 subfamily A member 43	Down -1.31
CYP4F12	cytochrome P450 family 4 subfamily F member 12	Down -1.31
HSD17B10	hydroxysteroid 17-beta dehydrogenase 10	Down -1.31
UCHL3	ubiquitin C-terminal hydrolase L3	Down -1.33
CYP2C18	cytochrome P450 family 2 subfamily C member 18	Down -1.54
ADH5	alcohol dehydrogenase 5 (class III), chi polypeptide	Down -1.64
XDH	xanthine dehydrogenase	Down -1.89

Table S3. Quantity of PHHs and media volume. Liver MPS, wells with spheroids and sandwich cultures.

Platform	liver MPS	spheroids	sandwich culture
Media Volume (μL)	1600	75	100
Number of Seeded Cells	600000	5000	60000
Cell: Volume Ratio (Cells/ μL)	375	66.7	600

Table S4. Comparable features of used platforms to culture PHHs: liver MPS, spheroids, sandwich cultures. Listed features include brief references of relevance to the presented study. Symbols (+, ++, +++) indicate different levels of magnitude of each feature that was observed in the use of each platform from low (+), medium (++), and high (+++). The sign (-) corresponds to situation where feature is poorly developed or not available.

features		liver MPS		spheroids		sandwich cultures
hepatic function	+++	▪ high and prolonged CYP3A4 activity and albumin production	++	▪ prolonged CYP3A4 activity and albumin production	+	▪ high hepatic function within the first days after seeding
throughput	+	▪ 12 wells per plate	+++	▪ < 96 wells per plate ▪ potential for automation of assays	+++	▪ 96 wells per plate ▪ potential for automation of assays
multicell	+++	▪ co-culture of PHHs with PHKCs and demonstrated potential for other cell types	+++	▪ demonstrated potential for co-culture of PHHs with other cell types	-	▪ optimal for monocultures of PHHs
assay perfusate	+++	▪ ~1.4 mL of perfusate	+	▪ ~60 µL of perfusate	+	▪ ~100 µL of perfusate
assay lysate	+++	▪ 600,000 PHHs per well	+	▪ 5,000 PHHs per well	++	▪ 60,000 PHHs per well
microscopy	+	▪ after disassembling plate	+++	▪ compatible with live cell microscopy	+++	▪ compatible with live cell microscopy
instrumentation footprint	+++	▪ pneumatic control ▪ traditional cell culture	+	▪ traditional cell culture	+	▪ traditional cell culture
training	+++	▪ thawing PHHs ▪ plate assembly, priming, media changes, disassembly	++	▪ thawing PHHs ▪ pipetting skills to avoid disrupting spheroids	++	▪ thawing PHHs ▪ handling Geltrex/Matrigel
cost	+++	▪ PHHs, plate, tools, instrumentation	+	▪ PHHs	++	▪ PHHs

Execution of Experiments

Detection of trovafloxacin toxic effects to test variability of liver MPS results between sites and different batches of PHKCs

Reproducibility of results between sites and with distinct batches of primary Kupffer cells (PHKCs) was evaluated by detecting the toxicity of trovafloxacin, as described in **Figure S1**. This experimental setup was repeated in two distinct sites (A and B) using the same cells, system preparation procedures, treatment protocols and measurement schedules. Site A was CN Bio Innovations in Cambridge, United Kingdom and site B was FDA laboratories in Silver Spring, Maryland, USA. An additional experiment was conducted at site B using a different lot of PHKCs. The additional experiment in site B was also done with a liver MPS format controlled by a PhysioMimix platform as described in the **Detailed Materials and Methods** section and in **Figure S5**. The same lot number of primary human hepatocytes (PHHs) was used in all experiments. The compounds were co-dosed with lipopolysaccharides (LPS) to activate PHKCs (**Figures S8 and S9**). Cell death was assessed from quantifying an increased production of lactate dehydrogenase (LDH) and CYP3A4 activity normalized to day 6 activity was also assayed to generally assess cellular hepatic function and confirm known inflammatory effects of PHKCs activated with LPS in inhibiting metabolism in PHHs.¹

Comparing the sensitivity of the liver MPS to hepatotoxicants relative to spheroids and sandwich cultures

A varied range of concentrations of hepatotoxicants (troglitazone, tamoxifen and digoxin) were added to cells after four days of shifting from plating medium to serum-free maintenance medium to evaluate if the sensitivity of PHHs to hepatotoxicants depended on the way cells were cultured, as previously suggested in similar comparisons between platforms.^{2, 3} After incubating toxicants for two days in the liver MPS, spheroids and sandwich cultures, CYP3A4 activity normalized to day 4 activity, albumin production, and LDH were assayed (**Figure S2**). Different batches of liver MPSs were used for these experiments as exemplified in **Figure S10**. The data resulting from these experiments was fit to a sigmoid curve to estimate the concentration of the used compounds that induced a half-maximal response: EC₅₀.

Assessment of liver MPS hepatic function over time

PHHs were maintained for 29 days in the liver MPS after shifting from plating medium to serum-free maintenance medium and routinely measured CYP3A4 activity and albumin production (**Figure S3**). Cells from the same batch of PHHs were also cultured as spheroids and sandwich cultures and the same assays were routinely performed with these cell culture settings until observing loss of cellular function. For spheroids, plating medium was changed five days after seeding cells, while plating medium was changed one day after seeding cells for sandwich cultures.³ Measured CYP3A4 activity and albumin production were normalized by total media volume and by the total protein assayed after lysing cells or number of seeded cells to compare results between platforms.

Quantification of phase II metabolism, intracellular drug accumulation and non-specific binding of compounds to the liver MPS

Troglitazone was exposed for two days to cells cultured in the liver MPS at concentrations of 50 μM and 100 μM , and troglitazone and its glucuronide and sulfate metabolites were quantified before adding the medium to cells and after two days of incubation. In another set of liver MPS wells, chloroquine was added at a concentration of 31.5 μM to quantify its intracellular accumulation after two days. This antimalarial drug is known to extensively accumulate in several tissues, including the liver.^{4, 5} This experimental setup is described in **Figure S4**. Related to demonstrating the potential for estimating drug metabolism and accumulation with the liver MPS, a common concern regarding the quantification of compounds and metabolites in MPSs relates to non-specific physical-chemical interactions between compounds and device materials. Such interactions may lead to variations in compound concentration due to adsorption or absorption to MPS materials. An experiment reported by Tsamandouras and colleagues⁶ was repeated to evaluate the possibility of variations in compound concentration due to non-specific interactions with liver MPS materials. For this purpose, the following six compounds of similar molecular weight but with different physical-chemistry were used: ibuprofen, propranolol, diclofenac, prednisolone, lidocaine and phenacetin. Each compound was dissolved in cell culture medium at concentrations of 1 μM , 0.1 μM and 10 nM, and added to a dedicated well in the liver MPS plate operating without cells in a cell culture incubator to later measure the extent that was adsorbed to the MPS materials with liquid chromatography-mass spectrometry (**Detailed Materials and Methods** section below).

Quantification of LDH production and CYP3A4 activity for quality control of the liver MPS

Before performing experiments with liver MPSs, their quality was systematically evaluated by assaying LDH production and CYP3A4 activity at day four after seeding cells (**Figure S6**). We also systematically acquired microscopy images of cells within scaffolds at the end of experiments, after maintaining systems for several days. It was hypothesized that functional quality of liver MPS wells could be estimated from combined non-destructive measurements of LDH and CYP3A4 activity early after assembling the system and seeding cells. For demonstrating the effectiveness of this workflow, a liver MPS plate was assembled and seeded with PHHs to measure LDH and CYP3A4 activity for the 12 wells within the plate (A1-A6 and B1-B6) at day four. At day eight, scaffolds were extracted from the MPS plate and imaged prior to isolating RNA from cells for profiling the expression of 84 human genes that encode for proteins that play roles in drug transport, phase I and phase II metabolism.

General Considerations and Recommendations for Using Liver MPSs

Collectively, the results from this study, in agreement with published data,^{7, 8} suggest that liver MPSs can yield reproducible results when used in applications related to assessing drug toxicity, metabolism and distribution if system preparation, treatment with compounds and measurement schedules are comprehensively planned prior to experiments. For this purpose, drug adsorption, stability of metabolites and stability of specific hepatic endpoints should be assessed in MPS experiments or evaluations. Most importantly, the use of quality control criteria to yield functional microtissues (**Figure 5, Figure S6, Figure S7, Figure S8, Figure S9** and **Detailed Materials and Methods** section below) were central in enabling reproducibility of results by excluding functionally impaired outlier wells. Moreover, the liver MPS was observed to prolong hepatic function relative to other culture platforms, which could underly differences in results between platforms. Based on the experience from this study, the following are recommended for implementing MPS assays and characterizing their performance:

1. Establish quality control criteria that ensure proper assembly and preparation of functional systems. Formation of bubbles, variations in fluid flow, and inhomogeneity of protein coatings can result from improper assembly/ calibration of system components and affect reproducibility of results if not screened prior to experiments. Fowler and colleagues⁷ described in detail how system operation matters for applications in adsorption, distribution, metabolism, and excretion (ADME) studies. Based on throughput, operation details and other experimental practicalities, Baudy and colleagues⁸ recommended how different types of systems can be used for specific applications.
2. Test cellular properties to enable the intended system use. As shown here for testing inflammatory effects of trovafloxacin on the liver MPS, quality control of PHKCs (**Figures S8 and S9**) enabled the assurance that the used cellular batches were quiescent and viable, unless activated by LPS, which activated IL-6. For other contexts of use, the required cellular components should also be previously tested to mechanistically perform as intended. Baudy and colleagues⁸ recommended different tiers of characterization for liver systems that depend on context of use and provide information on recommended baseline functional properties. Fowler and colleagues⁷ provided additional baseline recommendations for ADME applications of liver systems.
3. Characterize the adsorption of compounds to the materials of the MPS. To ensure that the cells or tissues are exposed to specific drug formulations, users should characterize to what extent compounds can be lost by being adsorbed by the MPS materials (**Figure S12**). Fowler and colleagues⁷ provided additional information on addressing this need.
4. Perform stability tests for compounds and metabolites to be used in the selected cell culture medium. In addition to being adsorbed, compounds or their metabolites can be degraded in cell culture medium at 37 °C (**Figure S11B**), which will affect the results of assays with MPSs, specifically if being used for studying long-term drug exposures.
5. Compare the stability of the intended performance of an MPS in relation to state of the art culture platforms. For mechanistic evaluation of tissue function represented by specific endpoints, as done here by measuring CYP3A4 activity and albumin production, studies should evaluate how an MPS performs differently from traditional platforms (**Figure 3**). This is particularly relevant if tissue-specific functional robustness affects a drug mechanism of action or metabolism. Fowler and colleagues⁷ have alluded the need for the stability of metabolism

and transport of hepatocytes in MPSs and Baudy and colleagues⁸ detailed baseline functional needs for the performance of liver systems.

6. Use compound test sets with known clinical drug effects to evaluate the performance of assays with MPSs developed for specific contexts of use. Fowler and colleagues⁷ recommended compounds for evaluating the suitability of systems for ADME applications. Baudy and colleagues⁸ also proposed compounds for characterizing the use of liver MPSs for the assessment of drug safety and other lists have been proposed for other contexts of use and organ systems.
7. Consider modifying an MPS to perform specific assays. Despite the extent of assays that can be done with MPSs, assays that depend on real-time image-based methods, automated collection of perfusate, sensitivity limits, and stability of metabolites can be difficult to perform without modifying a system to accommodate experimental needs. Fowler and colleagues⁷ elaborated on the importance of dosing and sampling when developing ADME studies with MPSs.

Detailed Materials and Methods

Liver Microphysiological System (MPS)

Liver MPS devices and the associated components that set and regulate its microfluidics were designed to enhance the physiological relevance of *in vitro* cultures of primary human hepatocytes (PHHs),⁹ while also enabling the possibility to co-culture these cells with other types of hepatic cells,¹⁰ such as primary Kupffer cells (PHKCs)¹¹ in this study. Specifically, the 12-well liver MPS used in this study was developed to expose 3D cultures of hepatic cells to fluid flow.^{12, 13} Such was achieved by culturing cells as multicellular microtissues attached to the inside walls of 340 μm diameter pores of scaffolds located in each well of the liver MPS plate (**Figure S5**). Micropumps drove media flow through these pores at controllable rate and direction.¹²⁻¹⁵ Unless indicated otherwise, all components of the liver MPS were provided by CN Bio Innovations, and system assembly and use were performed according to the instructions of the vendor. We used two versions of the same liver MPS in this study: LiverChip® platform with LiverChip® plates and the PhysioMimix™ system with LC12 consumable MPS plates. The LiverChip® plates were assembled under sterile conditions and LC12 MPS plates were provided sterile as a pre-assembled consumable that is ready to be used (**Figure S6**). The operation of the LiverChip® platform¹⁶ relied on inhouse compressed air and vacuum supply lines that connected to a pneumatic hookup to regulate the vacuum and compressed air pressures that drove the micropumps within the microfluidic circuits of the MPS (**Figure S5**). Before operating the system with the pneumatic hookup, vacuum pressure was set to -0.4 bar and compressed air pressure was set to 0.4 bar. Flow rate and direction of media within the microfluidic circuit were regulated by a dock controller (LC-3DK) and adjusted through a touchscreen display (LC-DIS). The LC12 MPS plates have the same design and operation as the LiverChip® plates but were designed to operate with the PhysioMimix™ controller system, which is a one-piece unit that generated vacuum and compressed air and contained all the components that regulate, control, set, and monitor the rate

and direction of media flow in the microfluidic circuits of the LiverChip® (**Figure S5**). The PhysioMimix™ control system also included predefined programs for enabling cell seeding, culture, media exchange and plate priming. Except for the pumping apparatus controlling the operation of each plate system, both plate types were analogous: similar dimensions, same microfluidic design (**Figure S5**), handle similar media volumes, use the same type of scaffolds for cell culture and operate under the same ranges of media flow rate. In addition, the same numbers of cells were used for both types of plates (**Figure S6**). For this reason, similar ranges of cellular function were observed between LiverChip® and LC12 plates after seeding PHHs (**Figure S16**).

Liver MPS Plate Assembly

Most of the assembly steps of the LiverChip® MPS plate were done in a biosafety cabinet to maintain the sterility of its different components. We assembled this plate two days before seeding cells (**Figure S6**). A sterile thin polyurethane membrane was carefully placed on top of the acrylic-based pneumatic plate, while ensuring that it was correctly aligned over the screw holes and the metal dowel pins on the pneumatic plate. We then placed the autoclavable polysulfone culture plate on top of the pneumatic plate holding the membrane, while ensuring that the membrane was not displaced during this step and placed a sterile lid over the culture plate. After turning over the plate and assuring that all components were well aligned with each other, and that the membrane did not cover any of the screw holes, we added a screw with a washer to each one of the 43 threaded holes. Outside of the biosafety cabinet, on a clean and decontaminated surface, we tightened the screws placed on the respective holes of the pneumatic plate with a calibrated electronic torque driver (Kolver, Thiene VI, Italy) set to 0.26 N·m. After using the electronic torque driver, we further secured the screws with a manual torque driver (Gedore, Skipton, United Kingdom) set to 0.30 N·m. We then transferred the plate back to the biosafety cabinet to inspect for any evidence of improper assembly. Wrinkles on the membrane, lack of tight contact between the pneumatic plate and the culture plate, or evidence of damage were used as criteria to evaluate the quality of the plate before continuing the next preparation steps.

In contrast, LC12 MPS plates were received from CN Bio Innovations as a preassembled supply, ready to prime with cell culture medium and equilibrate before seeding cells.

Priming, Mounting Scaffolds, and Microfluidic Equilibration

Immediately after assembling LiverChip® MPS plates, we connected them to a 1-unit acrylic dock inside the biosafety cabinet, which connected the microfluidic system in the pneumatic plate to the compressed air and vacuum lines that regulated the micropumps of each well (**Figure S5**). After placing the plate on the acrylic dock, we added 450 μ L of plating medium (**Table S1**), prewarmed at 37 °C, to the reservoir end of each well and set media to flow in the upward direction at 2 μ L/s for 2.5 minutes (**Figure S6**). We then filled the entirety of the inner surface of the reservoir and culture well of each MPS well with 1.4 mL of prewarmed plating medium and transferred the plate to a docking station in a cell culture incubator. Media was set to flow up at 1 μ L/s overnight. On the same day of assembling and priming the plate, we also incubated collagen-coated polystyrene scaffolds (12 per assembled plate) in prewarmed plating medium and eliminated air bubbles by carefully pipetting up and down medium against them with a 1 mL pipette. Scaffolds were then left in incubation overnight.

The following day, we transferred the LiverChip® MPS plate to the biosafety cabinet to remove bubbles that could have accumulated in the culture well and to place a pre-incubated scaffold in each well. When present, bubbles were removed from each culture well by pipetting the medium up and down against the well bottom with a 1 mL pipette. After completely removing bubbles, we submerged a filter in each of the culture wells using sterile metallic forceps and ensured that it was set in the bottom of the well. Then, we added a pre-incubated scaffold to the bottom of each culture well, while ensuring that they were fully submerged by the plating medium. After inspecting if scaffolds were flat and on top of the filters, we submerged a retaining ring in each culture well and placed it on top of each scaffold. We then pushed the retaining ring firmly into place with a retaining ring pusher to lock each scaffold in the bottom of its corresponding culture well. This step finalized the assembly process of each LiverChip® MPS plate, which was then transferred back to the acrylic dock in the cell culture incubator, and medium was set to flow up overnight at 1 $\mu\text{L/s}$ before seeding cells.

For the LC12 MPS plates, priming was done one day prior to seeding cells, following a similar procedure to the one used with the LiverChip® MPS plate, but using the PhysioMimix™ system and skipping the previously described assembly steps. In the biosafety cabinet, we removed the sterile plate from its package and mounted it on the movable PhysioMimix™ MPS driver. After this step, the plate was kept mounted on the MPS driver during the entirety of the experiment. The PhysioMimix™ MPS driver was designed to mediate the variations in pressure that regulate the micropumps of each well upon connection to the PhysioMimix™. These variations in pressure generated the flow rate and direction that was set on the PhysioMimix™ touchscreen. To prime the plate, we added 500 μL of plating medium prewarmed at 37 °C to the reservoir end of each well and transferred the MPS driver bearing the plate to a cell culture incubator with a docking station for the MPS Driver. After properly placing the MPS driver on the corresponding docking station, we selected the ‘prime’ program in the touchscreen and pressed play, setting media to flow up at 2.5 $\mu\text{L/s}$ for 3 minutes. We then transferred the MPS driver to the biosafety cabinet to add 1.1 mL of prewarmed medium, while ensuring that the whole well was covered. We returned the MPS driver to the docking station in the incubator and selected the ‘incubate’ program, which was designed to set media to flow at a rate of 1 $\mu\text{L/s}$ in up flow. We maintained the plate under these conditions overnight.

Overall, for both the LiverChip® and the LC12 MPS plates, overnight flow of media ensured equilibration of microfluidic circuits that set the operation of the liver MPS micropumps. Before seeding cells, we inspected for bubbles within the scaffolds or any evidence of contamination and used these criteria for quality control of this step. Plates with wrinkles or bubbles in the thin polyurethane membrane or wells with bubbles were excluded from experiments and not used for cell seeding.

Cell Thawing

In this study, we used monocultures of PHHs (Transporter Qualified, lot number HU8264, Thermo Fisher Scientific Inc., Waltham, MA) or co-cultures of the same PHHs and PHKCs (lot number BIX, BioIVT, West Sussex, United Kingdom or lot number HK8323, Thermo Fisher Scientific). The ability of PHHs to express functional active uptake transport proteins was assessed by the vendor for transporter qualification of these cells. Cryopreserved PHHs were thawed following the instructions of the vendor: frozen vials were quickly thawed until a sliver of ice was left in the vial.

Upon thawing each vial from cryopreservation, PHHs were transferred to cryopreserved hepatocyte recovery medium (**Table S1**), which was previously pre-heated in a water bath maintained at 37 °C. The recovery medium was provided in a 50 mL conical tube, which was then centrifuged for 10 minutes at 100 g at room temperature upon transferring cells. Then, after aspirating the supernatant without disturbing the pellet of cells, we resuspended PHHs in 1.5 mL of plating medium per vial of PHHs by gently rocking back and forth the 50 mL conical tube until we observed a homogeneous cell suspension. The plating medium we used to seed PHHs as monocultures was different in composition from the plating medium that we used to seed PHHs as co-cultures (**Table S1**). After observing a homogeneous cell solution, we assayed for cell viability with the trypan blue exclusion method using a disposable hemocytometer (Incyto, C-Chip DHC-N01, INCYTO, Hongcheondanggok-gil, Republic of Korea). Viability was consistently above 85% (**Figure S7**). While counting cells and preparing the liver MPS or other cell culture platforms for seeding, we kept the tube containing the resuspended PHHs on ice. Following vendor instructions, we seeded 600,000 cells per liver MPS culture well in a volume of plating medium within 100-200 μ L.

To thaw PHKCs, we vertically submerged the cell-containing cryovial in a water bath at 37 °C, while ensuring the cap of the vial stayed dry. Once we observed a thin spindle of ice inside the vial, we transferred the cells into a 15 mL conical tube containing 10 mL of ice-cold advanced DMEM (Thermo Fisher Scientific). We then pipetted 1 mL of this thawing medium into the emptied vial to rinse its interior and transferred it back to the tube. After tightening the cap on the tube, cells were suspended by carefully rocking the tube manually for a few seconds and we then centrifuged the tube for 5 minutes at 500 g. The media was aspirated, while not disturbing the cell pellet and 500 μ L of ice-cold plating medium that we used for PHH- PHKC co-cultures (**Table S1**) was added. Cells were resuspended by carefully pipetting up and down. After counting cells as we did with PHHs, and before seeding them in the liver MPS wells (60,000 cells per well), we added more medium to obtain a seeding volume in the range of 40-80 μ L per well. During cell counting and preparation of the plates for seeding, the tube with resuspended PHKCs was kept on ice.

Seeding PHHs as monocultures or in co-culture with PHKCs

In addition to maintaining PHHs in the culture wells of the LiverChip® MPS plates or LC12 MPS plates, as monocultures or in co-culture with PHKCs, we also cultured PHHs in 2D as sandwich cultures¹⁷ and as 3D as spheroids¹⁸ to compare cellular effects of culturing cells in the liver MPS with the cellular performance of these culture formats.

Monocultures of PHHs in the liver MPS

Overall, we followed an identical procedure for seeding PHHs with both the LiverChip® MPS platform and the PhysioMimix™ system. However, given the differences between the equipment used for operating the two systems, we followed different procedures to perform steps with similar purposes. After resuspending PHHs and keeping them in a 50 mL conical tube on ice, we changed the two-day old medium of the liver MPS plate and prepared it to seed 600,000 cells per culture well. For the lot number of PHHs we used and the resuspension protocol we followed, 600,000 cells consistently corresponded to volumes between 100 μ L and 200 μ L. Calculating this volume before changing the media in the liver MPS plate was necessary for ensuring that a volume of

medium with seeded cells per culture well did not exceed a value of 500 μL for the LiverChip® MPS plates and 400 μL for the LC12 MPS plates.

First, two-day old plating medium was aspirated from the reservoir and from the culture well. For LiverChip® MPS plates, medium was aspirated down to the level of the retaining ring. For LC12 MPS plates, medium was aspirated down to the level of the deeper notch of its retaining ring. We then added 400 μL of fresh plating medium to each well and pumped it down for three minutes and 30 seconds at 1 $\mu\text{L}/\text{s}$, while aspirating the medium being pumped out from micropump channel into the narrow end of the reservoir chamber. For LC12 plates, we transferred the MPS driver with the plate to the incubator after adding 400 μL of fresh plating medium and connected it to PhysioMimix™ to run the media exchange program, which was programmed to automatically stop after three minutes of pumping in downward direction at 1 $\mu\text{L}/\text{s}$. We then transferred the MPS driver back to the biosafety cabinet and aspirated the medium from the reservoirs and culture wells. At this point in the procedure, the two-day old plating medium was completely removed from the wells of any of the plate types being used and we added fresh plating medium to the bottom of each culture well before seeding suspended PHHs as defined in the following equation:

$$Volume_{\text{plating medium}} = Volume_{\text{culture well}} - Volume_{600,000 \text{ cells}}$$

Culture well volumes were 500 μL for LiverChip® plates and 400 μL for LC12 plates. After adding fresh plating medium to the bottom of each culture well, we ensured that PHHs were fully resuspended and added the volume of medium with 600,000 PHHs by carefully pipetting cells evenly across the scaffold held within each well. We then initiated media pumping in downward direction at 1 $\mu\text{L}/\text{s}$ for LiverChip® plates and carefully added 900 μL of plating medium to the reservoir side of each well, while ensuring that the whole surface was covered with medium. At this point, we transferred the plate to the incubator where we initiated the automatic eight hours seeding routine at a flow rate of 1 $\mu\text{L}/\text{s}$ in downward direction. For LC12 plates, we connected the MPS driver to the PhysioMimix docking station in the cell culture incubator and ran the seed program, which was programmed to automatically pause after two minutes of pumping in downward direction at 1 $\mu\text{L}/\text{s}$. After this pause, we transferred the MPS driver back to the biosafety cabinet to carefully add 1000 μL of plating medium to the reservoir of each well, while ensuring that the whole surface was covered with medium. The MPS driver was then transferred back to the incubator to connect the PhysioMimix™, and the seed program was initiated again. After two minutes of operation, the program paused, and we clicked ‘play’ again to initiate the eight hours seeding routine.

After eight hours, the media flow in LiverChip® MPS plates or LC12 plates were automatically switched to the up direction, while maintaining the rate at 1 $\mu\text{L}/\text{s}$, and cells were kept in these conditions of media flow until the end of each experiment. After one day of seeding PHHs, all wells were changed from seeding medium to maintenance medium (**Table S1**).

To change media in the liver MPS plates, we first aspirated the plating medium from the reservoirs and culture wells down to the level of the retaining ring of each well for LiverChip® MPS plates and down to the level of the deeper notch of the retaining ring for plate LC12 MPS plates. We then added 400 μL of maintenance medium to each well. For LiverChip® plates, we pumped medium at 1 $\mu\text{L}/\text{s}$ in downward direction for three and a half minutes and aspirated the old plating medium that was pushed to the reservoirs from the micropump channels. Before carefully adding 1.4 mL of fresh maintenance medium to each reservoir and adjacent culture well, we also aspirated excessive medium from the culture well, down to the level of the retaining ring. For LC12 plates,

we connected the MPS driver to the PhysioMimix™ docking station in the cell culture incubator and ran the media exchange program, which pumped medium in downward direction for three minutes. After transferring the MPS driver back to the biosafety cabinet, we aspirated the medium from the reservoir and culture well down to the level of the deeper notch of the retaining ring and added 1.4 mL of fresh maintenance medium to each reservoir and adjacent culture well. After media changes, plates were transferred back to their respective incubators, and media was pumped at 1 $\mu\text{L/s}$ in the upward direction until the next media change.

PHHs co-cultured with PHKCs

To seed co-cultures of PHHs and PHKCs in the liver MPS culture plates, we mainly followed the same seeding protocol described in the previous section for seeding PHHs, while modifying some steps to accommodate the addition of plating medium with suspended PHKCs. As detailed in the previous section, we removed the old plating medium that had been used for priming and equilibrating the microfluidic system of the plates and added the following volume of plating medium to the bottom of each culture well:

$$Volume_{\text{plating medium}} = Volume_{\text{culture well}} - Volume_{600,000 \text{ PHHs}} - Volume_{60,000 \text{ cells PHKCs}}$$

Culture well volumes were 500 μL for LiverChip® plates and 400 μL for LC12 plates. After ensuring that cells were resuspended, we added a volume of medium with 600,000 resuspended PHHs to each culture well, by carefully pipetting it evenly across the collagen-coated scaffold held within each well and repeated this process for adding a solution of medium with 60,000 PHKCs. Depending on the plate type, we then pumped medium at 1 $\mu\text{L/s}$ in downward direction while carefully adding 900 μL of plating medium to the well reservoir (LiverChip® plates) or ran a seed cycle with the PhysioMimix and added 1000 μL of plating medium to the reservoir (LC12 plates). After these steps, plates were submitted to an eight-hour cycle of downward flow, before transitioning to a steady state of upward media flow at 1 $\mu\text{L/s}$, as described in detail in the previous section. Media changes were also performed as already described for monocultures of PHHs in the liver MPS while using different types of maintenance media. We first used a maintenance medium for culturing cells between the first and the third day post-seeding and used another maintenance medium beyond the third day of culture (**Table S1**).

The medium for seeding and maintaining co-cultures of PHHs with PHKCs did not contain dexamethasone (**Table S1**), as dexamethasone is known to suppress inflammatory activity of PHKCs.¹⁹

2D cultures of PHHs

We cultured PHHs in 2D as sandwich cultures¹⁷ in tissue culture 96-well plates, which were pre-coated with collagen (Thermo Fisher Scientific). Cells were seeded in 100 μL of plating medium at 37 °C to each well of the 96 well-plate. The plate was then placed in the cell culture incubator to be kept in a sterile environment at 37 °C and 5% CO₂. After ensuring that thawed PHHs were homogeneously resuspended in plating medium, we transferred the necessary volume to seed 60,000 PHHs to each well and kept plate with cells in the incubator. We prepared plating medium with 0.35 mg/mL of Geltrex (Thermo Fisher Scientific) on ice. Six hours after seeding PHHs, we removed the plating medium, washed once with 100 μL of phosphate-buffered saline (PBS - Thermo Fisher Scientific) and added 100 μL of plating medium with Geltrex to each well

containing PHHs in culture. We then transferred the plates back to the incubator and replaced cell culture medium the following day with maintenance medium at 37 °C (**Table S1**).

Spheroid 3D cultures of PHHs

We seeded PHHs in wells of 96-well plates that were designed to enable the formation of cellular spheroids (Corning, NY part number 4515) through the establishment of intercellular interactions, in opposition to adhering to extracellular proteins coated on the bottom of each well.¹⁸ 5,000 cells were seeded in each well in 75 µL of plating medium at 37 °C and 96-well plates were centrifuged at 100 g for two minutes and left in the cell culture incubator for 5 days to enable spheroid formation. We then removed 50 µL of the three-day old plating medium from each well and added 55 µL of fresh maintenance medium, accounting for some evaporation. The total volume of media at the end of experiments was around 75 µL, confirming that adding additional 5 µL every media change was necessary to maintain a constant volume.

Changing the culture media

PHHs cultured in any of the used cell culture platforms (liver MPS, 2D sandwich cultures or 3D spheroids) were fed every other day with maintenance medium (Mondays, Wednesdays and Fridays) after the first media change, following the procedures described in the previous section for each platform.

Quality control of cell culture platforms

Cellular viability of PHHs and PHKCs was measured after thawing cells, and all events where less than 86% of PHHs were viable were flagged to test if higher levels of cell death during thawing resulted in decreased hepatic performance of cells later in culture. Before using wells of liver MPS plates, 2D sandwich cultures or 3D spheroids for any experiment (e.g. to evaluate drug cellular effects or variations in their hepatic function), we tested the quality of each well with seeded cells by measuring cellular production of lactate dehydrogenase (LDH) and cytochrome P450 (CYP3A4) activity. We performed these tests at day 4 after cell seeding for liver MPS plates and 2D sandwich cultures, at day 6 for liver MPS plates with co-cultures, and at day 7 for 3D spheroids.

Increased cellular production of LDH is associated with cell death, tissue damage or cellular metabolic changes,²⁰⁻²² and we assayed it to screen for potential variations in these cellular properties within wells in the same type of cell culture platform. CYP3A4 activity is a key player in hepatic drug metabolism,^{23, 24} and it was measured to assess potential differences in hepatic function between cellular preparations. After experiments and during the disassembly of the liver MPS plates as described ahead, we also acquired microscopy images of the scaffolds harboring the cellular microtissues with an inverted microscope (Nikon Eclipse Ti2, Minato City, Tokyo, Japan), using the brightfield channel (4× objective: 30 mm diameter, 17.2 working distance, 0.13 numerical aperture) to qualitatively evaluate the number of cells in each scaffold.

LDH assay

Before performing LDH assays (CytoTox 96® Non-Radioactive Cytotoxicity Assay, Promega, Madison, WI), the provided substrate mix was reconstituted by adding 12 mL of the assay buffer into one vial of the substrate mix and repeated this step when more reconstituted substrate was necessary. For long-term storage, we kept the reconstituted substrate in -20 °C. On the day of each assay, the substrate and the stop solutions were brought to room temperature and three 50 µL samples of supernatant were collected from each well of the liver MPS plate and transferred to clear 96 well-plate wells (ThermoFisher Scientific). When possible for all assays, we performed the same measurement three times to evaluate the technical variability of each measurement. Included with the assay, we also used an LDH technical positive control, which we prepared by diluting it 1:5000 in maintenance medium. Triplicate measurements were also prepared for the LDH technical positive control and for the fresh maintenance medium (**Table S1**) that we used as a technical negative control. After preparing all samples for measurements in triplicates, a volume of 50 µL of the reconstituted substrate mix was added to each well with the samples and the microplate was incubated in the dark at room temperature for 30 minutes. Then, we added 50 µL of stop solution to each well and measured the absorbance at 490 nm with a plate reader (TECAN, SPARK 10M).

LDH measurements were not repeated three times for assaying 2D sandwich cultures and 3D spheroids because these cultures were maintained with volumes of medium below 150 µL. We also performed the LDH assay for detecting drug-induced cell death.

Cytochrome P450 (CYP3A4) activity

We used a commercially available luminescent assay to quantify the CYP3A4 activity (P450-Glo™ CYP3A4 assay and screening system, Promega) of PHHs cultured in the platforms in this study. This assay consisted of quantifying the luminescence of a luciferin-based product that is metabolized by CYP3A4 from a parent proluciferin-based substrate. To prepare this assay, we reconstituted the luciferin detection reagent with reconstitution buffer by mixing and swirling several times, followed by equilibration at room temperature for one hour, as recommended by the vendor. Upon reconstitution, the luciferin detection reagent was stored at -20 °C and thawed to room temperature before using it again.

To perform the assay, we first prepared enough volume of maintenance medium at 37 °C for media changes, containing a 1:1000 dilution of the luminogenic substrate medium. Then, we collected the spent medium from each well (liver MPS, 2D sandwich cultures or 3D spheroids, to be used for LDH or albumin assays), as previously described, and replaced it with the luminogenic substrate medium, while saving 500 µL to later analyze as a blank solution. Cells were incubated in substrate medium for 60 minutes and we then transferred 50 µL of this medium to wells of a clear 96 well-plate for further processing before measuring luminescence. When analyzing liver MPS microtissues, we collected three samples of medium from each liver MPS well to ensure for low technical variability of the assay. We also made sure to leave at least 2 empty rows and columns between the wells with samples to avoid interference between sample readings. Plates with samples were then incubated on a plate shaker at 400 rpm for 20 minutes in the dark before reading luminescence with a plate reader.

To correlate luminescence signals of samples to luminogenic product concentration, we also measured the luminescence of samples with a range of known concentrations of beetle luciferin potassium salt, from 4 nM to 2 μ M, and plotted a standard curve.

Quantification of human albumin

We quantified human albumin with an ELISA kit (AssayPro, LLC., St. Charles, MO) in cell culture medium collected from the liver MPS plates or from plates containing cultures of hepatocytes in 2D or 3D. For this purpose, we collected 150 μ L of medium from the liver MPS wells or 50-100 μ L from wells of plates used for sandwich or spheroids into Eppendorf tubes that can contain up to 1.5 mL of liquid and immediately spun them down at 1500 rpm for 10 minutes at 4 °C. Tubes were then kept at -80 °C until enough samples were collected (96 including triplicates for each measurement and albumin standards) to continue this assay, which relied on measuring each sample in a corresponding well of a 96-well human albumin microplate, included in the assay. After reaching a critical number of samples to analyze, tubes were thawed, and each sample was diluted and partially transferred to a well of the human albumin microplate. Milli-Q water was used to dilute the diluent buffer and the wash buffer from their initial concentrations of 10x and 20x, respectively. Once diluted, we prepared working solutions from the diluent buffer (1x) for the biotinylated human albumin antibody, the SP conjugate, human albumin standards, supernatant samples, and negative control media without albumin.

To obtain a standard curve where albumin concentration of a measured sample could be determined from absorbance measurements, we made different standard solutions of human albumin from a stock solution of 200 ng/mL. This solution was first thawed for 10 minutes and then gently agitated before diluting it further with diluent buffer into various concentrations: 100, 50, 25, 12.5, 6.25, 3.125, 1.56 ng/mL. At this point, we also diluted the different samples of supernatant medium in diluent buffer. For liver MPS media, we used a dilution ratio of 1:500. For sandwich cultures and spheroids, we respectively used dilution ratios of 1:250 and 1:50 in diluent buffer. Triplicates of 50 μ L volumes of diluted samples and standards were pipetted into the wells of the microplate to ensure that technical variability of measurements was negligible when compared with inter-sample variability. The wells of the microplate were then covered with sealing tape and incubated at room temperature for 1 hour. After incubation, the samples were aspirated, and we rinsed the wells six times with 300 μ L of wash buffer (1x). After this wash cycle, 50 μ L of the biotinylated human albumin (1x) were added to each well, which was followed by an incubation step for 30 minutes before washing again six times with wash buffer. 50 μ L of streptavidin-peroxidase conjugate solution was then added to each well and incubated for 30 minutes before another wash cycle with wash buffer. Then, 50 μ L of chromogen substrate solution were added to each well and incubated for 15 minutes. To stop the reaction, 50 μ L of stop solution were added to the existing 50 μ L of chromogen substrate, and the plate was gently swirled prior to measuring the absorbance of the content of its wells at 450 nm with an exposure of 300 ms.

Quantification of interleukin-6 (IL-6)

Human IL-6 secreted from PHKCs in response to inflammatory stimuli induced by LPS was quantified with an ELISA kit (R&D Systems, Minneapolis, MN) from the cell culture medium aspirated from liver MPSs. Prior to executing the assay, 25x wash buffer and 10x reagent buffer were diluted in deionized water to working concentrations and capture antibody, detection

antibody and human standard were reconstituted following the recommendations of the vendor. The capture antibody was first diluted to the working concentration of 2.0 µg/mL in PBS without carrier protein and 100 µL of this solution coated each well of a dedicated 96-well microplate, which was then coated overnight at room temperature after sealing with tape. The samples of medium were removed from the freezer the following day and allowed to thaw at room temperature and placed in the fridge once thawed. The capture antibody was washed from each well three times with 400 µL of wash buffer and the plate was blotted on paper towels after the final wash to ensure absolute removal of liquid. Wells were then blocked by adding 300 µL of reagent diluent to each well, which were subsequently sealed and left at room temperature for one hour. During this time, thawed medium samples were centrifuged at 3000g for three minutes to exclude any cell debris. Reagent diluent was washed away from the wells by washing three times with 400 µL of wash buffer and blotting the plate on paper towels to remove all liquid. 100 µL of each medium sample were added to a respective well in the plate, where standards were analyzed in duplicate and samples assayed singly. After sealing the plate and incubating it for two hours at room temperature, samples in wells were washed away three times with 400 µL of wash buffer and the plate was blotted on paper towels to remove all liquid. Following vendor recommendations, the working solution of streptavidin-HRP was prepared and 100 µL of this solution were added to each well and incubated for 20 minutes at room temperature. After washing three times with 400 µL of wash buffer and blotting the plate on paper towels, 100 µL of substrate solution (1:1 mix of color reagent A and color reagent B) were added to each well and incubated for 20 minutes on a plate shaker and protected from light. 50 µL of stop solution were then added to each well, while ensuring thorough mixing and bursting any formed bubbles. Absorption of plates was analyzed with the plate reader at 450 nm and IL-6 concentration was quantified from the standard curve.

Total protein quantification

The BCA protein assay kit (Thermo Fisher Scientific) was used to measure the total protein content of cells in culture,²⁵ which is a proxy to the total number of cells and therefore to cell concentration. After disassembling the liver MPS plate, each scaffold was transferred from the bottom of the corresponding culture well to a dedicated well of a 24-well plate with 500 µL of PBS to wash and image for quality control purposes. Then, PBS was replaced with 500 µL of cell lysis buffer with the following composition: 0.44% w/v of sodium hydroxide and 2.22% w/v of sodium dodecyl sulfate in distilled water. Scaffolds were then incubated in lysis buffer for two hours at 37 °C to dissolve cellular proteins. After washing 2D and spheroid cultures with PBS and imaging for inspection, we added 100 µL of lysis buffer to each well with 2D sandwich cultures and 50 µL to each well with 3D spheroids. For calibration purposes and derive protein concentration from absorbance measurements, standards were prepared using a 2 mg/mL standard protein solution (stock concentration) provided with the protein assay kit and we diluted it with lysis buffer to obtain concentrations of 1000, 500, 250, 125, 62.5, 31.25 µg/mL.

After incubation with lysis buffer and preparing the standards, we transferred 25 µL of each sample (including standards) to dedicated wells of a 96-well plate. We then added 200 µL of the working reagent, which was prepared in advance by mixing 50 parts of BCA reagent A with one part of BCA reagent B, following vendor instructions. The contents of the plate were mixed with a plate shaker for 30 seconds, and the plate was incubated for 30 min at 37 °C in the dark. After bringing the plate to room temperature, we measured absorbance at 570 nm.

Analysis of troglitazone and diclofenac metabolism with liquid chromatography-mass spectrometry

Troglitazone and diclofenac metabolites and chloroquine were identified and analyzed using UHPLC-MS/MS method. An Agilent 1290 Infinity (Agilent, Santa Clara, CA) UHPLC system coupled to AB Sciex 6500+ QTRAP mass spectrometer (Sciex, Framingham, MA) was used. The mass spectrometer was operated in negative mode with an electrospray ionization (ESI) probe. Detailed description of the methods to identify and analyze metabolites are described below:

Metabolite identification for troglitazone and diclofenac

For metabolite identification, Enhanced Mass Spectrum/ Information-Dependent Acquisition method (EMS/IDA) and Multiple Reaction Monitoring/Information-Dependent Acquisition method (MRM/IDA) was created using Analyst 6.1 software. Same liquid chromatography (LC) method was used for EMS/IDA and MRM/IDA method with a flow rate of 0.500 mL/min with a gradient flow. Ammonium acetate (10 mM) and acetonitrile were used as aqueous (mobile phase A) and organic (mobile phase B) mobile phase, respectively for troglitazone. An Acquity UPLC BEH C18 (2.1x 50 mm) 1.8 μ m column at 40 °C column temperature was used as stationary phase for troglitazone and 2 μ L of the sample was injected onto it. 0.1% formic acid in water and 0.1% formic acid in acetonitrile were used as aqueous (mobile phase A) and organic (mobile phase B) mobile phase, respectively for diclofenac. An Agilent Zorbax SB C-18 (2.1x 50 mm) 3.5 μ m column at 35 °C column temperature was used as stationary phase for diclofenac and 10 μ L of the sample was injected onto it. The gradient method starts at 10% mobile phase B and was held until 0.5 minutes. The mobile phase B gradient was linearly increased up to 90% up to 3 minutes and was held until 4 minutes. The mobile phase B gradient was linearly decreased up to 10% in next 0.5 minutes and held until 5 minutes to reach back to original LC conditions. For EMS/IDA method, 2 experiments were generated within the same period. Experiment 1 scan type was Enhanced MS with Q0 trapping and the parent drug and metabolites were observed in the m/z range of 100.00 – 297.14 m/z and 297.14 – 750.00 m/z with a scan rate of 10,000 Da/sec. Experiment 2 scan type was Enhanced Product Ion with no Q0 trapping and the parent compound and metabolites were observed in the m/z range of 100.00 – 297.14 m/z and 297.14 – 750.00 m/z. Similarly, for MRM/IDA method, 2 experiments were generated within the same period. Experiment 1 scan type was MRM. The software generated a compound specific list of all the possible metabolites and their transitions. The samples were analyzed for these m/z ratios with a dwell time of 3 msec. Experiment 2 scan type was Enhanced Product Ion with no Q0 trapping and the parent compound and metabolites were observed in the m/z range of 100.00 – 297.14 m/z and 297.14 – 750.00 m/z with a scan rate of 10,000 Da/sec. After the samples were run using these methods, metabolites were identified and confirmed using LightSight™ Software (Sciex, Framingham, MA) by uploading the data file in the software. The confirmed metabolites of interest were determined, and UHPLC-MS/MS method was developed for qualitative analysis of all the samples.

Qualitative analysis of troglitazone

For troglitazone sample analysis, troglitazone, troglitazone quinone, troglitazone glucuronide and troglitazone sulfate metabolites were of interest. The same LC conditions were used as described in the metabolite identification section with slight modifications in the gradient. The gradient

method started at 10% mobile phase B and was held until 0.3 minutes. The mobile phase B gradient was linearly increased up to 70% up to 1 minutes and was held until a total of 2 minutes. The mobile phase B gradient was linearly decreased down to 10% in the following 0.3 minutes and held until 2.5 minutes to reach back to original LC conditions. The product ions for the parent and the metabolites were obtained using MRM. The MRM transitions for troglitazone, troglitazone quinone, troglitazone glucuronide and troglitazone sulfate were 440.1 to 397.0 m/z, 456.0 to 413.0 m/z, 616.0 to 440.0 m/z and 520.0 to 440.0 m/z, respectively. The samples were diluted 10x times before analyzing using 50:50 William's E buffer: acetonitrile. For qualitative analysis, peak areas and retention times for all the analytes at specific m/z ratio were determined and compared with the controls.

Qualitative analysis of diclofenac

Analytes of interest for diclofenac sample analysis were diclofenac, diclofenac acyl β -D glucuronide, 4/5' hydroxy diclofenac acyl β -D glucuronide and 4' hydroxy diclofenac sulfate. For the diclofenac method, an Acquity HHS T3 (2.1x 50 mm) 1.8 μ m column at 40 °C column temperature was used as stationary phase for diclofenac and 10 μ L of the sample was injected onto it. Ammonium acetate (10 mM) and acetonitrile were used at a flow rate of 0.500 mL/min as aqueous (mobile phase A) and organic (mobile phase B) mobile phase, respectively. The gradient method starts at 2% mobile phase B and was held until 0.5 minutes. The mobile phase B gradient was linearly increased up to 95% up to 1 minutes and was held until 1.4 minutes. The mobile phase B gradient was linearly decreased down to 2% in next 0.2 minutes and held until 2 minutes to reach back to original LC conditions. The product ions for the parent compound and the metabolites were obtained using MRM. The MRM transitions for diclofenac, diclofenac acyl β -D glucuronide, 4/5' hydroxy diclofenac acyl β -D glucuronide and 4' hydroxy diclofenac sulfate is 295.0 to 251.0 m/z, 471.0 to 295.0 m/z, 488.0 to 295.0 m/z and 375.0 to 331.0 m/z, respectively. The samples were diluted 2x times before analyzing using 50:50 William's E medium: acetonitrile. For qualitative analysis, peak areas and retention times for all the analytes at specific m/z ratio were determined and compared with the controls.

Qualitative analysis of chloroquine

Chloroquine was analyzed from the culture medium and from the cells cultured within liver MPS scaffolds. With cell culture medium, chloroquine study samples were diluted 1:2 in acetonitrile. Each scaffold with cells was lysed in 500 μ L of 100% cold methanol anhydrous (Sigma-Aldrich) and sonicated for 15 seconds. Cell lysates were store at -80 °C and mixed 1:2 in acetonitrile after thawing and before being analyzed. The Agilent 1290 Infinity UHPLC system coupled to Thermo Scientific TSQ Quantum Ultra AM Triple Quadrupole mass spectrometer was used for quantifying chloroquine. Gradient LC method with a flow rate of 0.4 ml/min was used. The gradient starts with 90% of Mobile phase A (5mM ammonium formate with formic acid) and held at the same proportion till 0.4 min. Later, it was linearly changed to 10% in 0.6 min and 90% mobile phase B (acetonitrile) and held consistent until 1.4 min. Then, the initial conditions were brought back with steep change by 1.5 min and equilibrated till end of run (2.0 min). A Phenomenex, Kinetex C-18 (2.1x 50 mm) 1.8 μ m column at 40 °C column temperature was used as stationary phase for chloroquine and 1 μ L of the sample was injected onto it. The mass spectrometer was operated in positive mode with an electrospray ionization (ESI) probe. The ion transitions were monitored in

multiple reaction monitoring (MRM) mode. The MRM transition for chloroquine was 320.2 to 247.2 m/z.

Estimation of drug adsorption to the liver MPS materials: analysis of ibuprofen, propranolol, diclofenac, prednisolone, lidocaine and phenacetin by liquid chromatography-mass spectrometry

Adsorption of drugs dissolved in the media to the materials of MPSs is one potential hurdle when modeling drug effects with these systems.^{26, 27} To generally evaluate the tendency of small drugs to be adsorbed into the materials of the liver MPS, we followed a procedure published by Tsamandouras and colleagues,²⁸ where we incubated different compounds of similar molecular weight, but with different physical-chemistry.

Ibuprofen, propranolol, diclofenac, prednisolone, lidocaine and phenacetin were analyzed using UHPLC-MS/MS method. An Agilent 1290 Infinity UHPLC system coupled to AB Sciex 6500+ QTRAP mass spectrometer (Sciex, Framingham, MA) was used for ibuprofen analysis whereas an Agilent 1290 Infinity UHPLC system coupled to Thermo Scientific TSQ Quantum Ultra AM Triple Quadrupole mass spectrometer (Thermo Fisher Scientific) was used for simultaneous analysis of propranolol, diclofenac, prednisolone, lidocaine and phenacetin. Detailed descriptions of the methods are described below:

Qualitative analysis of ibuprofen

Ibuprofen samples were analyzed using the UHPLC-MS/MS method. Isocratic LC method with a flow rate of 0.300 mL/min at 15:85 mobile phase A: mobile phase B composition was used. 0.1% formic acid in water and acetonitrile were used as aqueous (mobile phase A) and organic (mobile phase B) mobile phase, respectively. An Agilent Zorbax SB C-18 (2.1x 50 mm) 3.5 μ m column at 40 °C column temperature was used as stationary phase for ibuprofen and 2 μ L of the sample was injected onto it. The mass spectrometer was operated in negative mode with an electrospray ionization (ESI) probe. The ion transitions were monitored in multiple reaction monitoring (MRM) mode. The MRM transition for ibuprofen was 205.2 to 161.1 m/z. Before analysis, the ibuprofen study samples were diluted using 50:50 William's E Buffer: Acetonitrile.

Qualitative analysis of propranolol, diclofenac, prednisolone, lidocaine and phenacetin

Drug samples were analyzed simultaneously using the UHPLC-MS/MS method. Gradient LC method with a flow rate of 0.500 mL/min was used and 2 μ L of the sample was injected onto the column. 0.1% formic acid in water and 0.1% formic acid in acetonitrile were used as aqueous (mobile phase A) and organic (mobile phase B) mobile phase, respectively and an Agilent Zorbax SB C-18 (2.1x 50 mm) 3.5 μ m column at 40 °C column temperature was used as stationary phase. Method started with an initial gradient of 10% B which was linearly increased up to 40% B up to 1.5 minutes. After 1.5 minutes, the gradient is linearly increased up to 95% B up to 2 minutes and held until 2.4 minutes. The gradient was linearly decreased down to 10% B in the next 0.1 minutes to bring back the gradient to its initial conditions and was held until another 0.3 minutes. The mass spectrometer was operated in positive mode with an electrospray ionization (ESI) probe. The ion transitions for all the analytes were monitored in multiple reaction monitoring (MRM) mode. The MRM transitions for propranolol, diclofenac, prednisolone, lidocaine and phenacetin was 260.1 to

116.0 m/z, 296.0 to 213.9 m/z, 361.1 to 146.9 m/z, 235.1 to 85.9 m/z and 180.1 to 109.9 m/z, respectively. The study samples were diluted with William's E medium and acetonitrile before injecting on the mass spectrometer.

For qualitative analysis of ibuprofen, propranolol, diclofenac, prednisolone, lidocaine and phenacetin, peak areas and retention times for all the analyte samples after incubation were determined and compared with the peak areas and retention times for analyte samples before incubation. Percent change in the peak areas from before to after incubation samples were calculated using the following equation:

$$\% \text{ Change in peak area} = [100 - \{(\text{Peak area of sample after incubation}) / (\text{Peak area of samples before incubation}) \times 100\}]$$

RNA isolation and quantification of gene expression

We isolated Total RNA with an RNA extraction kit (miRNeasy Mini Kit, Qiagen, Germantown, MD) following the handbook protocol (October 2007) with modifications described here. In detail, RNA was harvested from MPS scaffolds that were transferred to a 24 well plate and lysed for 10 minutes directly in 350 μL of Qiazol lysis solution provided with the miRNeasy kit while shaking on a Vortex Genie II (Scientific Industries), on speed 1. Cell lysates were then transferred to sterile 1.5 mL microfuge tubes, and each well was washed with an additional 650 μL of Qiazol and combined with previous lysate for a total lysate volume of 1 mL. The combined lysates of each scaffold were vortexed on high speed for an additional 30 seconds. Lysates were then centrifuged for 10 minutes at 12,000 g and 4 $^{\circ}\text{C}$ to remove any cell debris. The cleared supernatants were then transferred to a new set of sterile 1.5 mL microfuge tubes and 200 μL of chloroform (Fisher Scientific, Pittsburgh, PA) was added to each tube. The tubes were inverted 15 times, followed by vigorous vortexing for 15 seconds, and then allowed to sit on the benchtop for two minutes, followed by centrifugation for 15 minutes at 12,000 g and 4 $^{\circ}\text{C}$ to separate the aqueous and organic phases of the lysates. 550 μL of the upper aqueous phase was carefully removed, avoiding the interface, and transferred to a sterile 2 mL microfuge tube. To precipitate the RNA, 1.5 volumes (825 μL) of absolute ethanol (Sigma-Aldrich, St. Louis, MO) was then added to each tube and mixed by inverting 15 times. 700 μL of each sample was added to a RNeasy Mini spin column, and centrifuged at 8,000 g for 20 seconds, room temperature. Flow-through was discarded, and the remaining RNA precipitate was reapplied to the spin column for a second spin. The optional DNase digestion was performed using RNase-free DNase Kit (Qiagen) as follows: 350 μL of RWT wash buffer, supplied in miRNeasy Kit was applied to each column, and centrifuged for 20 seconds at 8,000 g and at room temperature. 80 μL of DNase/RDD buffer was added to each column and incubated for 15 minutes at room temperature. An additional 350 μL of RWT wash buffer was used to wash the column, followed by three additional washes using 500 μL of RPE buffer (note: this is one additional wash compared to the default kit protocol). To dry the columns, the optional spin was performed at maximum speed for three minutes. To elute the purified total RNA, two 40 μL aliquots of 1X TE was added to each column for a final elution volume of 80 μL .

To determine the RNA concentration and yields of total RNA from each scaffold, 1 μL was run in triplicate on a Nanodrop 8000 instrument (Thermo Fisher Scientific). A260, A280, and A230 values were obtained for qualitative assessment and quantification of each sample. Samples were then diluted to 5 ng/ μL (5000 pg/ μL ; the max range) and run on an Agilent 2100 bioanalyzer

instrument, using an Agilent RNA 6000 Pico Kit Assay to determine the RNA quality and RIN assessment.

RNA was then precipitated using 1/10 vol 5M NaCl (Fisher Scientific), and 2 volumes of absolute ethanol, and incubated at -80 °C for 30 minutes. The samples were then centrifuged at 4 °C for 20 minutes and washed three times with 500 µL of 70% ethanol in milli-Q water. The RNA pellets were air dried for five minutes and then resuspended in 15 µL of 1X TE, and 2 µL was measured on the Nanodrop 8000 instrument, and diluted to 50 ng/µL, which was the suggested starting concentration for RT2-Profiler Assays (Qiagen).

To assess gene expression, RT2 Profiler PCR arrays (Qiagen) for Human Drug Metabolism (330231) were chosen with format PAHS-002ZE-4, for analysis on an ABI ViiA7 384-well block configuration, PCR instrument. This PCR array focuses on a wide variety of common drug transporters, phase I and phase II metabolizing enzymes, and other drug metabolism genes (84 total genes), as well as, assay controls. Default kit protocols were performed without any deviation from the kit handbook (December 2014), format E for our PCR thermal cycler instrument. Following vendor recommendations, RNA isolated from scaffolds with low yields were excluded from the RT2 Profiler assay due to insufficient RNA input for the assay. A no-template-control was also run to serve as a biological negative control for the assay. Data from each experiment was collected using Applied Biosystem's "Quantstudio 6 & 7 Flex Real-Time PCR System Ver1.1" software on a ViiA 7 System PCR instrument, and then exported data from each run was combined into one study file using the Applied Biosystem's "ExpressionSuite Software Ver1.3 for data analysis and hierarchical clustering (**Figure 5D**). Auto threshold settings and auto baseline settings were selected to adjust the Ct values accordingly between the separate runs for the study analysis. Ct values were then exported for further analysis in Microsoft Excel. Values above 35 or with an undetermined call, were substituted with a value of 35 for this analysis as recommended by Qiagen's analysis protocol.

To assess gene expression changes between the individual biological replicate samples from different scaffolds for a specific experimental group, a comparative analysis was performed to evaluate the sample to sample differences between individual biological replicates. This assessment indicated the sample to sample variation, as well as, all gene targets with gene expression signal beneath the assay threshold level of Ct = 35. To select appropriate endogenous controls, each of the 84 candidate-gene Ct values were averaged and the standard deviation was calculated across samples. The endogenous control candidates were then sorted by their standard deviation in ascending order, to determine the least variant genes. To meet the criteria for inclusion as an endogenous control, the target's mean Ct had to fall in the range of 20 – 30. The five least variant targets were selected as endogenous controls. To normalize samples, the average Ct value of all five endogenous control targets was determined for each of the samples and used as the endogenous control value for ΔCt calculations for each gene target and each sample, using the following formula:

$$\Delta Ct_{\text{Sample Target}} = Ct_{\text{Sample Target}} - Ct_{\text{Mean of Endogenous Control Targets}}$$

For fold change calculations and determination of the $\Delta\Delta Ct$ values, the mean of the ΔCt for all 11 samples was used as the calibrator, using the following formula:

$$\Delta\Delta Ct_{\text{Sample Target}} = \Delta Ct_{\text{Sample Target}} - \Delta Ct_{\text{Calibrator}}$$

To calculate the fold change, the following formula was used:

$$\text{Fold Change} = 2^{-\Delta\Delta C_t}$$

For statistical analysis, and plot generation, the Ct data generated from Applied Biosystems Expression Suite software Ver1.1 were uploaded to Qiagen's online data analysis tool, "Geneglobe", for RT2 Profiler PCR Data Analysis, at the following URL: <https://dataanalysis2.qiagen.com/pcr>. The cataloged array, PAHS-068Z RT2 Profiler PCR array: Human Drug Metabolism, was used for proper annotation of the gene targets. The p-value calculation used in Geneglobe is based on parametric, unpaired, two-sample equal variance, two-tailed distribution. Scatter plots, volcano plots, and heat maps were all generated by the Geneglobe tool (**Figures S13 and S14, Table S2**).

Compounds for assaying concentration-dependent effects and adsorption

Except for chloroquine diphosphate (Toronto Research Chemicals, Canada), all compounds were acquired from Sigma-Aldrich and filter sterilized when diluted in cell culture medium and before being added to the liver MPS or cells in culture. Compounds in powder (trovafloxacin, levofloxacin, troglitazone, tamoxifen, chloroquine, lidocaine, phenacetic, propranolol, prednisolone, diclofenac, ibuprofen) were dissolved in dimethyl sulfoxide (DMSO). Digoxin was provided in methanol inside glass ampoules at a concentration of 1.28 mM. Approximately one hour before using compounds, stock solutions were diluted in fresh maintenance medium to obtain solutions with different concentrations. DMSO concentration in the solutions of medium added was consistently below 0.1% V/V. Media was warmed up to 37 °C before being added to the liver MPS or other culture platforms. Upon assaying effects of varying concentrations of the compounds troglitazone, tamoxifen and digoxin on LDH secretion, CYP3A4 activity and albumin production, the resulting data was submitted to a sigmoid curve (QuestGraph™ IC50 Calculator, AAT Bioquest, Inc., Sunnyvale, CA; URL: <https://www.aatbio.com/tools/ec50-calculator>) to estimate the concentration of each compound that induced half-maximal response (EC₅₀) for the different assays.²⁹

Labeling and imaging scaffolds with cells

Once removed from the liver MPS, scaffolds with cells cultured in 3D were individually transferred to wells of a 24 well and washed once in PBS before being fixed in a solution of 4% paraformaldehyde (Electron Microscopy Sciences) in PBS for 15 minutes and washed three times in PBS. Then samples were permeabilized with a solution of 0.1% Triton X-100 (Sigma-Aldrich) in PBS, washed three times in PBS and incubated for 30 minutes in PBS containing Oregon Green 488 Phalloidin (Thermo Fisher Scientific) diluted 1:200 to label actin. After washing three times in PBS, scaffolds were incubated in Hoechst's dye (Thermo Fisher Scientific) diluted 1:5000 in PBS for 5 minutes to label cell nuclei. After washing three times in PBS, scaffolds were imaged with an Axio Observer 7 inverted microscope (Carl Zeiss Microscopy, White Plains, NY) with fluorescent capabilities using an objective with 10X magnification.

Liver MPS disassembly and cleaning

We disassembled LiverChip® MPS plates and cleaned their culture plates, pneumatic plates and screws to later reuse these components. All other parts were disposed of, and LC12 consumable

plates were disposed after use. For LiverChip® plates, we aspirated the cell culture media from the 12 wells before inverting the plates with the lid on and removing the screws with the same electronic torque driver that we also used to assemble these plates.

After separating the different plate components, we immersed the liver MPS culture plate with Alconox solution (Alconox Inc., White Plains, NY) containing 10% sodium hypochlorite for at least six hours. We then thoroughly washed the plate in deionized water and completely immersed the plate in 2% 7X solution (MP Biomedicals, Irvine, CA) in a container and sonicated it for 15 minutes. After thoroughly washing the plate with deionized water, we immersed it in deionized water in a container and sonicated it for 15 minutes. We repeated this step two more times, while changing the water in the container after each sonication period and changing the orientation of the plate inside the container every time. After disposing of the water in the last sonication step, we left the plate on a clean drying table. Once it was completely dried, we place the plate in an autoclavable self-sealing pouch to later autoclave using the plastic cycle (121 °C for 20 minutes). Culture plate was ready to use after being autoclaved, dried and at room temperature.

We placed the metal screws and washers in a 50 mL conical tube and submerged them with the Alconox solution with 10% sodium hypochlorite for two hours. We then washed these under agitation in deionized water three times and left them to dry on a clean environment on top of a paper towel. Once dried, these components were placed together in an autoclavable self-sealing pouch to later autoclave in the plastic cycle and were ready to use after autoclaved and cooled to room temperature.

Pneumatic plates were handled with sterile instruments in a clean environment. The plate surface was wiped with sterile paper towels after lightly spraying a solution of 70% ethanol on it. Stains were cleaned with a sterile cotton Q-tip after being dipped in 70% ethanol. Before drying and storing these plates in a sterilized box, we checked for the integrity of the orange rubbers that cover the pneumatic channels. If damaged, we replaced rubbers following the instructions of the vendor and cleaned the surface one more time with 70% ethanol before storing it in a clean and sterilized container. Microchannels were inspected for any moisture buildup inside of them, and if droplets were identified, the channels would be dried using sterile absorbent paper points.

Experimental replicates and statistical methods for data analysis

Unless indicated otherwise, three biological replicates of the MPSs were used per tested condition in analytical assays (LDH, CYP3A4, albumin, IL-6, total protein and UHPLC-MS/MS). When possible, efforts were made to use biological replicates from more than one MPS batch (**Figure S10**). The material isolated from each biological replicate was split into three parts to be analyzed independently, constituting three technical replicates. Since the volume of cell culture medium used with spheroids and sandwich cultures (**Table S3**) allowed to obtain only one sample from each biological replicate, at least six biological replicates (unless indicated otherwise) were used for each condition when testing these platforms to minimize potential effects of technical variation.

Statistical analyses of data as reported in the captions of figures was performed with GraphPad Prism (GraphPad Software, CA). Tests were performed for evaluating statistical significance of differences between the mean of separate groups of data, such as: i) unpaired student's t-test with Welch's correction and student's t-test (to evaluate significance of differences between two groups); ii) one-way ANOVA test (to compare means of more than two groups). For different experimental conditions (drug concentration or time of culture), linear correlation between distinct

variables (i.e. LDH, CYP3A4, albumin, type of culture platform) was tested with the nonparametric Spearman correlation. Detailed information of samples and statistics on gene expression assay and analysis can be found in the section dedicated to this method, since it used dedicated software.

Supplementary Material References

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