APPLICATION NOTE

Human liver microphysiological system for predicting the drug-induced liver toxicity of differing drug modalities

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Introduction

The liver is one of the organs most susceptible to drug toxicity. Drug-induced liver injury (DILI) is a major cause of drug attrition, with more than 750 FDA-approved drugs known to have a degree of DILI risk[1]. There are several strategies to de-risk DILI in drug discovery and, in recent years, focus has turned to more reliable human *in vitro* 3D liver models to better predict DILI[2]. These models culture primary human liver cells, often in co-cultures, in a physiologically-relevant environment, thus allowing them to stay functional for extended periods of time[3].

Here, we assessed whether an *in vitro* human liver microphysiological system (MPS), also known as Liver-on-a-chip, could be used to accurately predict DILI risk for a broad set of compounds, of which thirteen are known severely and mildly hepatotoxic pharmaceutical drugs and two antisense oligonucleotides (ASOs). The liver MPS has previously been shown to maintain highly functional 3D liver microtissues, under flow perfusion, for up to four weeks, potentially making it highly suited to assessing both acute and chronic DILI[4].

Here, we demonstrate the potential of CN Bio's liver MPS to accurately identify drugs with varying DILI risk via three case studies. The first study examines a pair of pharmaceutical drugs, tolcapone and entacapone, that are currently used for the management of Parkinson's disease, both classified as catechol-O-methyltransferase (COMT) inhibitors. Tolcapone was the first COMT inhibitor approved by the FDA and was introduced to the US market in 1998. However, it was withdrawn soon after its approval due the death of several patients with severe liver complications, indicating that its DILI risk was missed during the preclinical safety testing. Tolcapone was subsequently re-introduced to the market in 2009 to be used only as last resort treatment for Parkinson's disease[5]. Entacapone, Tolcapone's structural analogue, is the second COMT inhibitor approved for Parkinson's disease and has low risk of hepatotoxicity. It is therefore considered as relatively safe but can occasionally cause some liver damage[6]. *In vitro* cellular assays showed that both compounds can induce mitochondrial

dysfunction in a dose-dependent manner or via inhibition of efflux transporters[7] but only tolcapone was associated with immune-mediated DILI (iDILI) causing a sharp increase in T regulatory cells[8] whereas entacapone did not exert an effect on this type of cells.

The second case study presents data for two antidiabetic thiazolidinediones: troglitazone and pioglitazone. Troglitazone, classified as of severe-DILI concern, was licensed for use in 1997 to treat type 2 diabetes but was withdrawn by the FDA only three years later due to the frequency of liver injury, including acute liver failure, reported in the post-marketing surveillance. Moreover, to date, published animal studies failed to predict troglitazone's potential to cause severe liver injury. The toxicity of this compound was also not detected in standard *in vitro* 2D hepatic assays[9]. Pioglitazone is a compound known to be of low-DILI concern; instances of acute liver injury due to pioglitazone are rare but have been occasionally reported post-marketing in patients with obesity or heart failure [10,11]. During its development, pioglitazone did not exert any hepatotoxicity in classic *in vitro* 2D primary hepatocytes cultures or in more advanced 3D models[12,13].

The third case study explores the ability of CN Bio's Liver MPS to predict the DILI risk of two (non-approved) ASOs: LNA32 and LNA43, shown to be safe and severely hepatotoxic in *in vivo* rodent models respectively[14,15]. ASOs are engineered molecules that contain locked nucleic acid (LNA) designed to induce target RNA cleavage. Many studies show there is strong association between liver toxicity and the sequence content of the LNA gapmer of ASOs[16], and that the liver is one of the accumulation target organs where ASO-mediated toxicity is often observed[14]. *In vitro* biotransformation studies at an early stage of drug development are paramount for the prediction of potential hepatotoxicity of ASOs. For modalities such as ASOs, which have a human-specific mode of action, animal models are less suited and therefore it is imperative to use the most human-translatable *in vitro* models available for their preclinical evaluation to rule out false reporting due to interspecies differences.

Here, for each compound, a range of functional liver-specific endpoints were analyzed (including clinical biomarkers) across a dose-response using multi-chip plates to generate EC:50 curves, which is more challenging for single-chip MPS. Those functional liver-specific endpoints were analyzed

to create a distinct mechanistic “signature of hepatotoxicity” from the liver MPS to demonstrate its capacity to assess human DILI risk of novel pharmacological agents. To improve the translational relevance of this model, functional liver-specific endpoints were analyzed (including clinical biomarkers such as serum albumin and alanine aminotransferase, ALT).

Aims

1. Demonstrate the capability of a human liver MPS to generate clinically relevant, high-content data (six endpoint EC:50 curves) from a broad set of thirteen compounds from severely to mildly hepatotoxic.
2. Demonstrate the capability of a human liver MPS to accurately assess the DILI risk of new modalities by generating clinically relevant, high-content date (six endpoint EC:50 curves) for two ASOs.
3. Determine whether the liver MPS can more accurately predict human DILI versus conventional preclinical approaches.

Materials & Methods

Cryopreserved Primary Human Hepatocytes (PHHs) were obtained from BioIVT and Human Kupffer Cells (HKCs) from Lonza. Cocultures of PHHs (0.4×10^6 cells/well) and HKCs (0.04×10^6 cells/well) were seeded into PhysioMimix™ Multi-chip Liver-12 plates. Cells were cultured using PhysioMimix Core Microphysiological System, for eight days in Liver Maintenance Media.

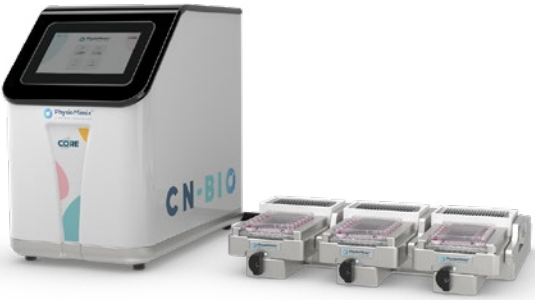
At Day four, liver microtissues were dosed with seven increasing concentrations of each test compound. Microtissue dosing was repeated every 48 hrs. for four days. Vehicle control consisted of Liver Maintenance Media containing 0.1% DMSO. Treated and untreated wells were randomized throughout the plates, and each condition was tested in triplicate.

Albumin production was measured by ELISA (R&D systems), LDH release using the Cytotox96 assay (Promega), urea synthesis using QuantiChrom™ kit (Universal Biologicals), CYP3A4 activity using P450-Glo™ CYP3A4 Assay (Promega), ALT activity was measured with Alanine Transaminase Activity Assay kit (Abcam), and cell viability was assessed using the CellTiter-Glo® 3D Cell Viability assay (Promega).

Results

Figure 1 – PhysioMimix Core enables the generation of 3D human liver microtissues, functional for up to four weeks. A) The liver *in vitro* model is generated by the PhysioMimix Core Microphysiological System, which uses open well-plates designed for the culture of primary liver cells in 3D in an engineered scaffold. **B)** Schematic representation of a PhysioMimix Multi-chip Liver-12 plate, which features an open-well design for the coculture of PHHs and HKCs in 3D on an engineered scaffold. **C)** Cross-section of a well indicating the scaffold and fluidic flow perfusion of 3D liver microtissues by micropumps. **D)** Phase contrast microscopy (10x and 20x) and immunofluorescence (IF) labeling of 3D liver microtissues generated by coculturing PHHs and HKCs in the Liver MPS for assessing DILI. To visualize the HKCs, prior to seeding HKCs were transduced with an adenoviral vector expressing eGFP. Representative photomicrographs are shown. The transduction and imaging were performed as a standalone experiment to demonstrate cell localization. HKCs cells are pre-validated in-house prior to use in experimental cell culture, and must have low levels of post-thaw activation; this is assessed by measuring biomarkers IL-6 and TNF-α.

A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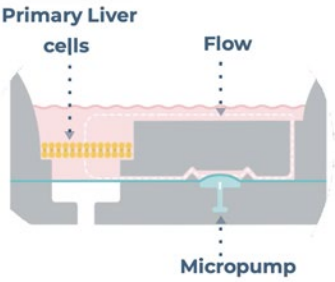
Primary Liver cells
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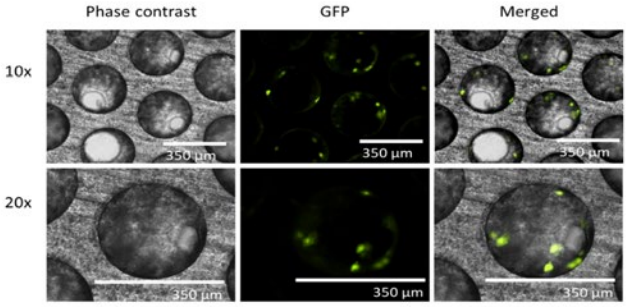
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Figure 2 – Experimental timeline of a standard DILI assay in the PhysioMimix Multi-chip Liver-12 plate.

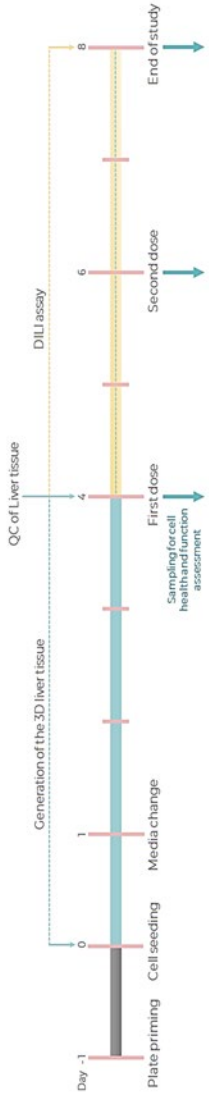


Figure 3 – The Liver MPS produces highly reproducible data and consistent microtissues. A) 3D Liver microtissue quality control (QC) metrics at Day four (Mean ± standard deviation (SD), N = 360); Functionality assessment at the end of the drug dosing (96 hrs) – **B)** Albumin and Urea, and **C)** CYP3A4 and ATP. Data was collected from 12 individual experiments; for each experiment three vehicle control replicates were used (data shown are Mean ± SD, N = 36).

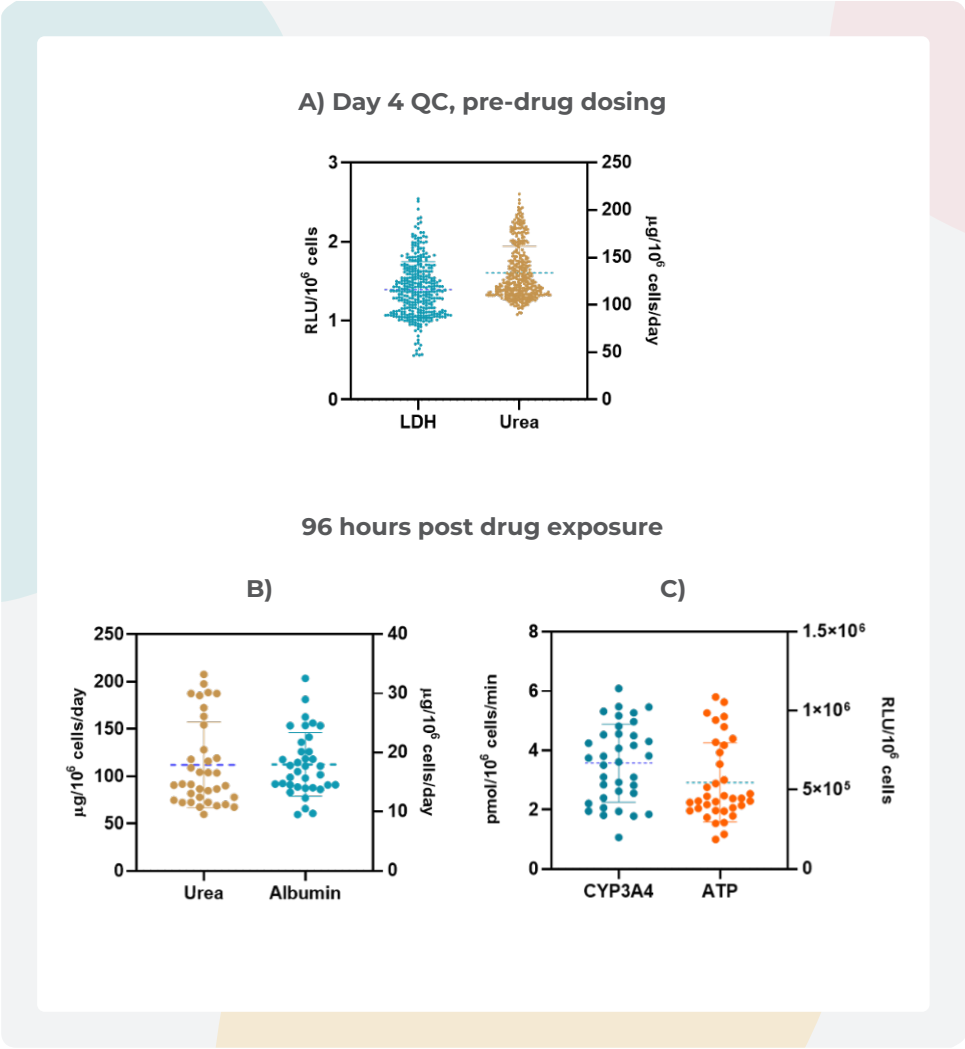


Figure 4 – Liver MPS accurately generates DILI profiles of tolcapone (high-DILI-concern) and entacapone (low-DILI-concern), using multiple hepatotoxic endpoints. Liver microtissues in the MPS were exposed to tolcapone (blue) and entacapone (brown) for 96 hrs. Endpoint measurements were all derived from the same liver MPS culture. Data shown are mean ± SD, N = 3, and all from 96 hrs samples, apart from LDH release which was measured at 48 hrs.

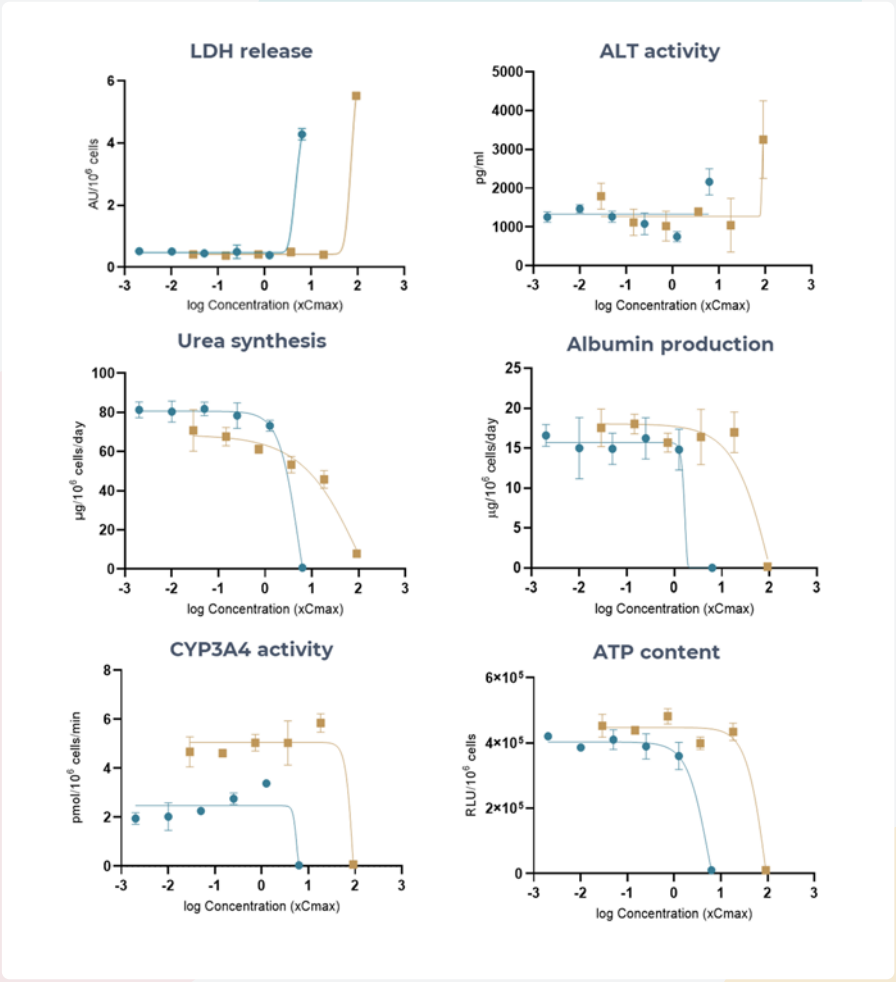


Table 1 – Toxicity of tolcapone and entacapone in 3D human liver MPS microtissues.

Liver microtissues in the MPS were exposed to both compounds for 96 hrs. LDH data was measured at 48 hrs post-dose. Data shown are generated from seven-point dose responses, with N = 3 per concentration. Maximum test concentration for tolcapone = 6.3 x C_{max} and for entacapone = 91.5 x C_{max}

Assay	Tolcapone		Entacapone	
	EC50 (xC_{max})	R^2	EC50 (xC_{max})	R^2
LDH	4.9	0.9	73.8	0.9
ALT	3.3	0.6	87.1	0.7
Albumin	0.3	0.9	62.5	0.9
Urea	4.5	0.9	39.3	0.9
CYP3A4	5.5	0.7	82.5	0.9
ATP	7.2	0.9	75.7	0.9

Figure 5 – Liver MPS accurately generates DILI profiles of troglitazone (high-DILI-concern) and pioglitazone (low-DILI-concern), using multiple hepatotoxic endpoints. Liver microtissues in the MPS were exposed to troglitazone (blue) and pioglitazone (brown) for 96 hrs. Endpoint measurements were all derived from the same liver MPS culture. Data shown are mean ± SD, N = 3, and all from 96 hrs samples.

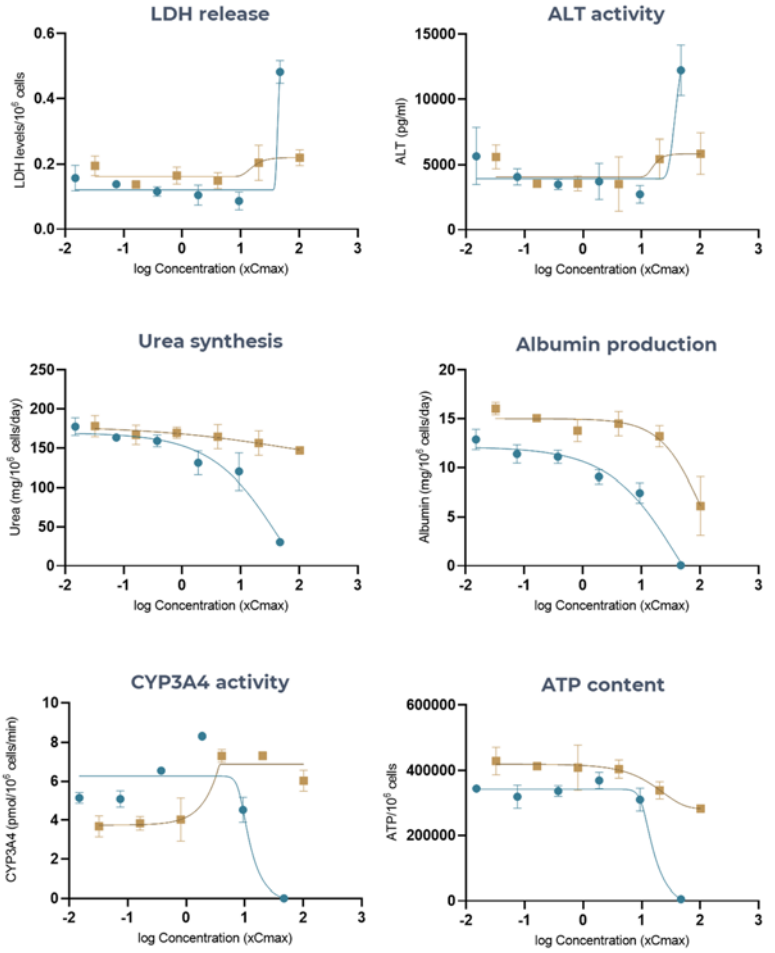


Table 2 – Toxicity of troglitazone and pioglitazone in 3D human liver MPS microtissues.

Liver microtissues in the MPS were exposed to both compounds for 96 hrs. Data shown are generated from seven-point dose responses, with N = 3 per concentration. Maximum test concentration for troglitazone = 47 x C_{max}, maximum test concentration for pioglitazone = 100 x C_{max}.

Assay	Troglitazone		Pioglitazone	
	EC50 (xC_{max})	R^2	EC50 (xC_{max})	R^2
LDH	43.9	0.9	ND	
ALT	40.3	0.8	ND	
Albumin	21	0.9	63.2	0.7
Urea	22.7	0.9	ND	
CYP3A4	11.8	0.8	ND	
ATP	15.5	0.9	16.5	0.7

ND - not detected

Figure 6 – Liver MPS accurately determines the DILI risk of two ASOs (LNA43, LNA32), using multiple hepatotoxic endpoints. Liver microtissues in the MPS were exposed for 96 hrs to LNA43 (blue), known to be severely hepatotoxic *in vivo*, and LNA32 (brown), known to be safe *in vivo*. Endpoint measurements were all derived from the same liver MPS culture. Data shown are mean ± SD, N = 3, and all from 96 hrs samples.

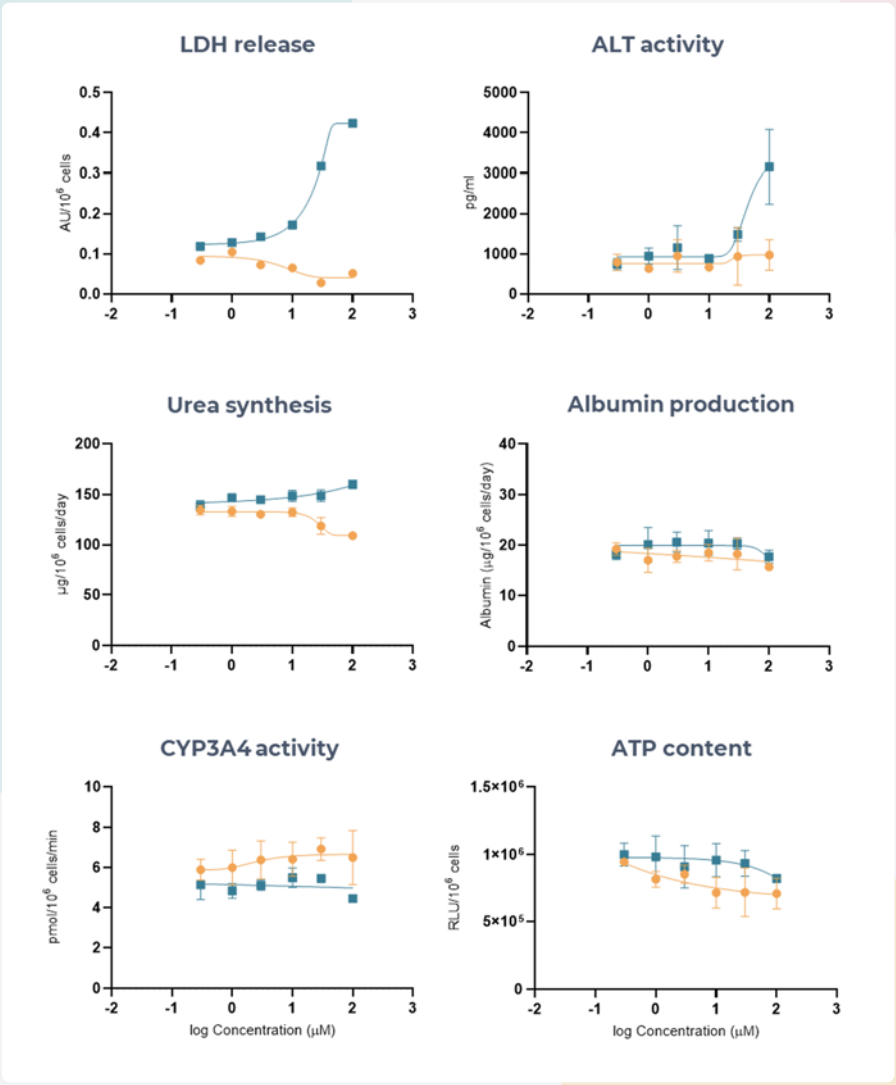
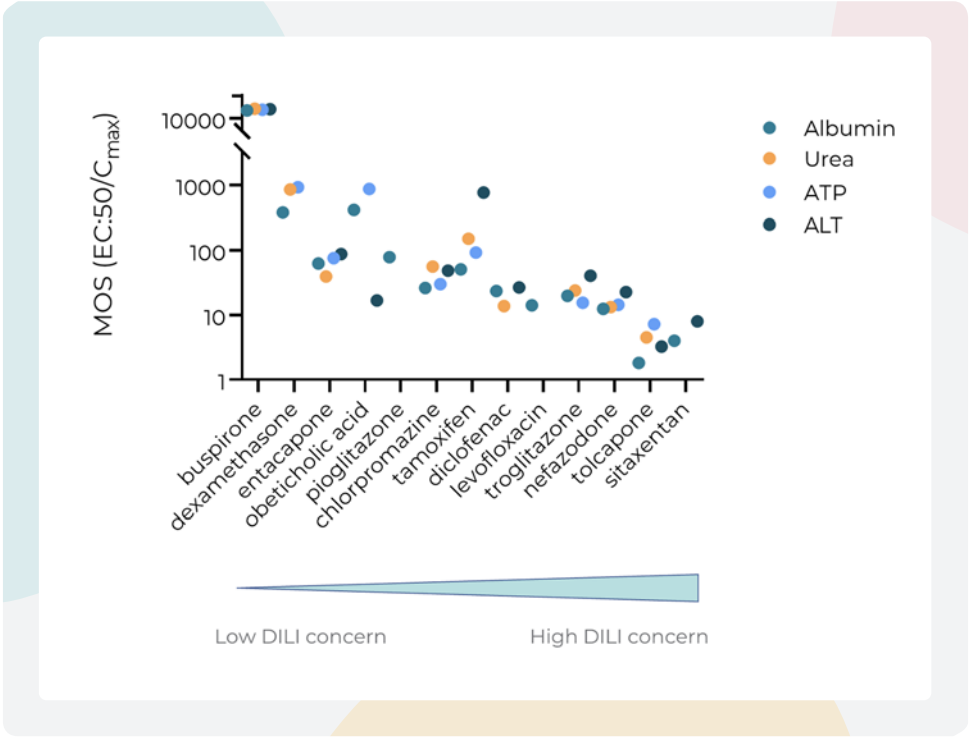


FIgure 7 – MPS DILI assay accurately determines the liability of a broad set of known severely and mildly hepatotoxic compounds. Exposure-corrected cytotoxicity or margin of safety (MOS = EC:50/Cmax) were determined for four key biomarkers albumin, urea, ATP, and clinical biomarker ALT, following 96 hrs of exposure. Endpoint measurements were all derived from the same liver MPS culture. Data shown are mean ± SD, N = 3. Tested compounds are arranged based on DILI-rank, from low DILI concern (left) to high DILI concern (right).



Conclusion

The acute and chronic exposure of fifteen severely and mildly hepatotoxic compounds were investigated using CN Bio's Liver MPS model. Cellular function and cell health endpoints were used to create a distinct mechanistic "signature of hepatotoxicity" and demonstrate the ability of the MPS to predict DILI risk. The inclusion of ALT, a routinely measured clinical marker, within the panel of endpoints, enables a direct comparison to be made between *in vitro* data and *in vivo* outcomes.

The liver MPS produces highly consistent and functioning liver microtissues. The data presented in **Figure 3A** are generated from the entire study in which the fifteen compounds were tested and shows good levels of reproducibility with low intra- and inter-study variability. After an eight-day culture, multiple health, and hepatic metrics (albumin, urea, CYP3A4, ATP) were also assessed in healthy control microtissues and showed high levels of hepatic functionality and reproducibility (**Figure 3B, C**).

Tolcapone caused a clear acute toxic response, C_{max} driven, that was detected by clinical biomarker ALT and LDH release (4.7 x C_{max}) and a rapid reduction in albumin and urea production at approx. 5 x C_{max} (**Figure 4**). Chronic exposure was captured by all tested endpoints, particularly in albumin at just 0.3 x C_{max}. Cellular endpoints (CYP activity and ATP content) further confirmed tolcapone toxicity, with highly comparable EC:50 values from both assays (**Table 1**).

The model did not detect acute and chronic toxicity effects following treatment with tolcapone's non-toxic structural analogue, entacapone (**Figure 4, Table 1**).

Drug dosing with troglitazone for 96 hrs caused an acute toxic response in the liver microtissues in the MPS, C_{max} driven, detected by ALT and LDH release and a rapid reduction in albumin and urea production, at circa 15 x C_{max} (**Figure 5**). Cellular endpoint (ATP content) and CYP3A4 activity (for assessing metabolic biotransformation), sampled after 96 hrs exposure, further confirmed toxicity caused by troglitazone, and EC:50 values were

highly comparable to other endpoints (**Table 2**). To date, published animal studies failed to predict troglitazone's potential to cause severe liver injury. Furthermore, the toxicity of this compound was also not detected in standard *in vitro* 2D hepatic assays[17].

Pioglitazone is a compound known to be of low-DILI concern and did not exert hepatotoxicity in classic 2D primary hepatocytes cultures, and even in some more advanced 3D models[12,13]. Mild hepatotoxic effects were observed at with a moderate reduction in albumin and urea production, at approx. 25 x C_{max} (**Figure 5**). Very minor reduction in ATP content was also observed at high pioglitazone concentrations, but this was not significant (**Figure 5**). The results demonstrate the ability of the liver MPS to detect the toxicity of compounds with mild DILI concern.

Moreover, the liver MPS model distinguished between safe and toxic ASOs (LNA32 – safe *in vivo*, LNA43 – severely hepatotoxic *in vivo*), with acute toxic response detected only by clinical biomarker ALT and LDH release at 96 hrs of exposure (**Figure 6**). Interestingly, the hepatotoxicity of LNA43 was not picked up by more simplistic *in vitro* human cell culture models (data not shown). We hypothesize that the presence of hepatic immune cells (HKCs) in the liver microtissue is key to the assay's increased sensitivity. Whilst our findings recapitulate the published data from *in vivo* trials conducted on mouse and rat models[14,15], we recommend the complementary use of MPS models when working with human-specific modalities to confirm that data from *in vivo* animal studies are in line with predicted human responses. This approach reduces the risk of false positive reporting due to interspecies differences.

The ratio of EC:50 to total plasma C_{max}, or exposure-corrected cytotoxicity, was determined for each compound and plotted as the "margin of safety" (MOS). In **Figure 7**, the MOS values for all tested compounds were plotted at 96 hrs of exposure. Tested compounds are shown on the horizontal axis and are arranged from low DILI severity to the left, to severe DILI concern, to the right. At fixed thresholds of 50x MOS values, the sensitivity and specificity of the assay were assessed by comparing DILI positive/negative status as determined by EC:50 or MOS threshold, with known DILI status for each compound. Data indicates that albumin was by far the most sensitive assay and highlights a clear trend between MOS values and DILI

severity. The data demonstrates that the liver MPS DILI assay has superior sensitivity and specificity over classic 2D primary hepatocytes cultures in detecting drug-induced hepatotoxicity.

The liver MPS accurately predicted human DILI for all compounds tested. Due to its enhanced sensitivity, the model identified hepatotoxicants (pioglitazone, dexamethasone, levofloxacin) that were not detected using 2D and some 3D *in vitro* models[5]. The data and experimental approaches demonstrate how CN Bio's PhysioMimix Core produces highly functional and metabolically active liver microtissues. These liver microtissues can be used reliably and consistently to accurately predict the DILI liability of drugs of differing modalities. We hypothesize that, by recapitulating the immune aspects of the liver that cause DILI, assay sensitivity was increased. The model can be further adapted to include adaptive immune cells such as Peripheral Blood Mononuclear cells (PBMCs) into the recirculating flow that perfuses the MPS model to provide an immunocompetent MPS.

By measuring 6 different endpoint biomarkers, including clinical biomarkers (serum albumin and ALT), the model produced a "signature of hepatotoxicity" for each test article to help to identify compounds with different levels of DILI concern and reveal mechanisms of their toxicity. Incorporation of these methodologies into pharmaceutical workflows will enable for rapid identification of compounds with human DILI liabilities leading to significant economic savings.

Key messages

- ✓ The Liver MPS provides better *in vitro* to *in vivo* translatability by using clinically relevant biomarkers
- ✓ The assay offers advantages over existing *in vitro* approaches by incorporating the immune aspects of the liver that contribute to DILI
- ✓ The Liver MPS predicted the post-marketing DILI liability of Troglitazone

- ✓ The Liver MPS accurately predicts the known DILI liabilities of a broad set of compounds of differing modalities
- ✓ The Liver MPS is especially insightful for the evaluation of drugs with human-specific models of action where animal models are less suited
- ✓ By improving the ability to predict DILI risk in early stages, the Liver MPS can potentially better protect patient's lives and save drug discovery companies huge costs

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Notes

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