APPLICATION NOTE

Evaluating a human DILI assay kit's ability to unlock complex mechanisms of toxicity

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Introduction

Drug-induced liver injury (DILI) remains the most common cause of acute liver failure and is a leading cause of attrition of compounds in late drug development. DILI events are typically classified into three categories. Intrinsic, where adverse effects are dose-dependent and occur shortly after administration; indirect, which shows some dose dependence and often relates to the drug's pharmacodynamics; and finally idiosyncratic, which has minimal dose dependence and is largely unpredictable, driven by specific patient phenotypes.

Traditional *in vitro* approaches for assessing DILI range from 2D cell-line or primary hepatocyte-based assays to 3D spheroid models. These are efficient tools for detecting bad actors early in the pipeline, as they can be used in high throughput screening. However, these cultures are relatively short-lived and have limited physiological relevance, which makes detecting more complex, indirect and idiosyncratic events challenging. Complementary *in vivo* models provide a system-level understanding; however, the biology, physiology and immunology of animals is inherently different to humans. This can result in adverse events being missed, or the misclassification of candidates that are safe in humans.

DILI is often caused by the alteration of several pathways and therefore a more complete pharmacodynamic understanding of the drug's effects is required to understand the mechanism behind the cause. The liver's hepatocytes represent the driving force of drug metabolism and several key metabolic pathways controlled by the liver can be adversely affected. Interspecies differences in metabolism can result in dissimilar metabolite generation *in vivo* - some of which may be toxic. Traditional *in vitro* 2D hepatocyte assays lack the metabolic capacity to adequately bridge this knowledge gap. Furthermore some therapeutics may cause inhibition or further activation of metabolizing enzymes, therefore careful evaluation of drug-drug interactions is also important utilizing a human relevant system.

Another common cause of DILI in humans is mitochondrial toxicity. This is often caused by therapeutics or their metabolites interfering with proteins involved in the respiratory cycle, resulting in the generation of reactive oxygen species (ROS) (Allison *et al*., 2023). Production of ROS can also be

upregulated through inflammatory pathways triggered by hepatocyte innate or adaptive immune cell activation. The local and systemic immune cell population interaction with the liver is a critical pinpoint of potential hepatotoxicity and is an important consideration when developing new modality drugs.

The inhibition of mitochondrial function may also induce lipid accumulation in hepatocytes. Steatosis is reported in approximately a quarter of DILI cases (Kleiner *et al*., 2014). It can also be caused by increased uptake of free fatty acids, lipogenesis or insulin resistance. This can often be misdiagnosed as Metabolic dysfunction-associated steatotic liver disease (MASLD); however, an important distinction is the marked increase in serum aminotransferase, which is linked to DILI (Garcia-Cortes *et al*., 2023).

A key function of the liver is the regulation of bile production. Hepatotoxicity is frequently associated with injuries of the bile duct which can lead to accumulation of bile and hepatocellular stress (Yang *et al*., 2013). The inhibition of bile salt export pump (BSEP) transporters, which allow unidirectional transport of bile acids in hepatocytes, is a common mechanism of cholestasis.

Here, we demonstrate how the PhysioMimix DILI assay kit: Human 24 and PhysioMimix Core System captures more complex mechanisms of human DILI than traditional preclinical approaches to reduce the risk of unforeseen event detection in the clinic. The evaluation was performed using the tool compounds troglitazone and amiodarone. Troglitazone is a well-known example of a drug withdrawn post-marketing due to fatal DILI events. A range of mechanisms have since been associated with its hepatotoxicity, including oxidative stress, reactive metabolite generation, mitochondrial dysfunction, cholestasis and steatosis. Amiodarone is still in clinical use for advanced heart disease, although liver toxicity has been identified in 1% of treated patients. Damage to lysosomal and mitochondrial function, followed by micro-vesicular steatosis are considered the key mechanisms of hepatotoxicity. Using the PhysioMimix DILI assay kit: Human 24, clinically relevant signatures of hepatotoxicity were captured using multiple soluble and tissue markers, demonstrating the assay's value when integrated into preclinical toxicology workflows to ease reliance on animals and decrease hepatotoxicity risks in the clinic.

Aims

Demonstrate the superior sensitivity and translatability of the PhysioMimix DILI assay kit: Human 24 over traditional *in vitro* methods by identifying complex DILI events and providing deeper mechanistic insights that explain the drug's effects.

Materials & Methods

Primary Cell Culture

The experiment was performed using the PhysioMimix DILI assay: Human 24 kit and the PhysioMimix Core System. Cryopreserved Primary Human Hepatocytes (PHHs) and Human Kupffer Cells (HKCs) were obtained from LifeNet Health Life Sciences. Pre-validated co-cultures of PHHs and HKCs were seeded at a 10:1 ratio into PhysioMimix Multi-chip Liver-12+ (MPS-LC12+) plates using Liver Seeding Media. Following the standard DILI assay protocol, cells were cultured using the PhysioMimix Organ-on-a-chip (OOC) System for eight days in Liver Maintenance Media. On day four, following quality control checks, Liver microphysiological system (MPS) microtissues were treated with each test compound, once daily for four days. A physiologically relevant concentration was chosen for each tool compound to reflect 1.5 times the Minimal Important Difference (MID) Clinical Concentration. Vehicle control was 0.1% DMSO. Treated and untreated wells were randomized throughout the Multi-chip Liver-12+ plates, and each condition was tested in triplicate.

Functional Assessment of Liver Microtissues

Quantification of LDH Production and Urea Synthesis

Quality control checks of Liver MPS microtissues were performed on day four post-seeding by sampling the culture media. Cytotoxicity

was quantified from samples using the Cytotox 96® Non-Radioactive Cytotoxicity Assay (Promega, G1780) to assess LDH release pre and post drug treatment. Urea release in samples was measured using the QuantiChrom™ Urea Assay Kit (BioAssay Systems, DIUR-100). Subsequent measurements were taken on day six, and day eight, following the supplier protocol.

Quantification of IL-6 and Bile Acid Secretion

Enzyme-linked immunosorbent assay (ELISA) technique was used to measure Interleukin 6 (IL-6) secretion from treated Liver MPS microtissues by sampling the culture media. Human IL-6 DuoSet ELISA (R&D Systems, DY206) and DuoSet ELISA Ancillary Reagent Kit 2 (R&D Systems, DY008B) were used to quantify IL-6 in media samples every 48 hours. A colorimetric Bile Acid Assay Kit (Abcam, ab239702) was used to quantify the concentration of total bile acids secreted by Liver MPS microtissues into the culture media.

Immunocytochemistry

Mitochondrial Labelling

Prior to fixing, MitoTracker™ Red CMXRos 579/599 nm (Invitrogen™, M7512) was used for mitochondrial staining of Liver MPS microtissues. MitoTracker™ stock solution was diluted to a final concentration of 100 nM. The staining solution was prewarmed to +37°C and incubated with the scaffolds for 30 mins. Post-staining the scaffolds were washed with Phosphate Buffered Saline (PBS) until fixing.

Immunostaining

Scaffolds intended for immunostaining were removed from Liver-12+ plates and washed with PBS prior to fixing with 4% Paraformaldehyde (PFA) for 15 minutes. Intracellular lipids were stained using the Nile Red Staining Kit (Abcam, ab228553), Phalloidin FITC Reagent (Abcam, ab235137, 1:200) was used to stain F-actin. Hoechst 33342, Trihydrochloride, Trihydrate - 10 mg/mL Solution in Water (Invitrogen™, H3570, 1:4000) was used as the nuclear stain. C-reactive protein (CRP) was stained to quantify intracellular inflammation using CRP Monoclonal Antibody (1G1) (Invitrogen™, MA5-

17061, 1:500) with Secondary Donkey Anti-Mouse IgG H&L (Alexa Fluor® 488) preabsorbed (Abcam, ab150109).

Images were acquired using the Nikon A1-R point-scanning confocal microscope at 10x magnification. The fluorescence intensity of each stain was quantified by using a custom Image J macro and all values were normalized to Hoechst 33342.

Data Analysis

Statistical analysis and graph generation were performed using GraphPad Prism 10. Data are reported as the mean and standard deviation (SD) for each experiment. Statistical analysis was performed using one-way ANOVA on ranks. In all cases, * represents $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ and ****, $P < 0.0001$ by the range test.

Results

Figure 1: The PhysioMimix DILI assay kit: Human 24 enables the generation of human relevant 3D Liver MPS microtissues. A) The *in vitro* liver model is generated by the PhysioMimix Core. 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 MPS microtissues by micropumps. PHHs and HKCs cells are pre-validated in-house prior to use in MPS culture.

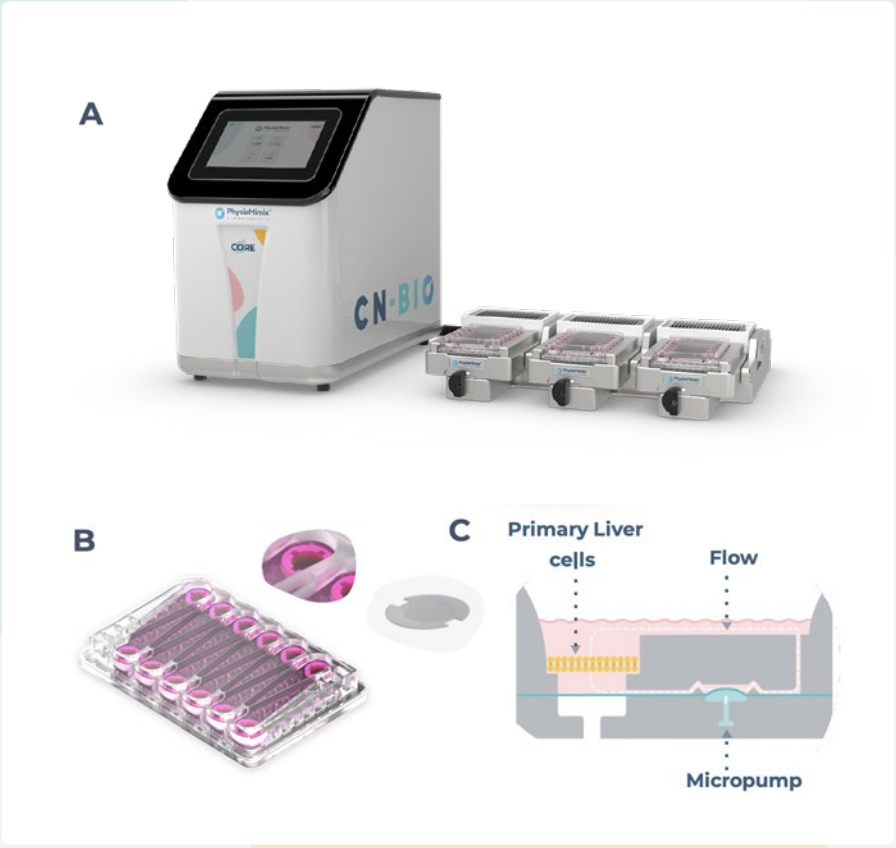
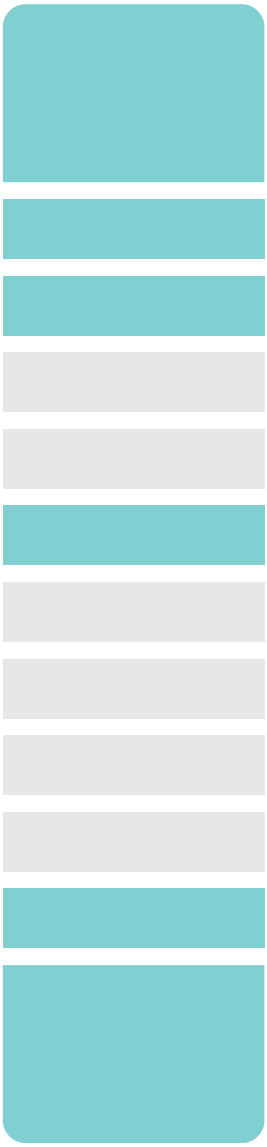


Figure 2: Experimental timeline



1 day before:

Prepare Liver Seeding Media, prime LC12+ plates

Day 0: Seed cells Liver Seeding media

Day 1: Switch to Liver Maintenance media

Day 4: QC tissues, media sampling, media change, drug dosing

Drug dosing every day

Day 8: End-point analysis

Data analysis

Figure 3: DILI assay kit: Human 24 profiling of amiodarone and troglitazone using cell health and functionality biomarkers. Liver MPS microtissues were exposed to vehicle control (teal), amiodarone (orange) and troglitazone (pink) for 96 hrs. Endpoint measurements for cell health (LDH) and functionality (Urea) at 0, 48 and 96 hr timepoints were derived by repeat media sampling from the same Liver MPS culture. Data shown for LDH release and urea synthesis are mean ± SD, N = 3, and all from 96 hr samples.

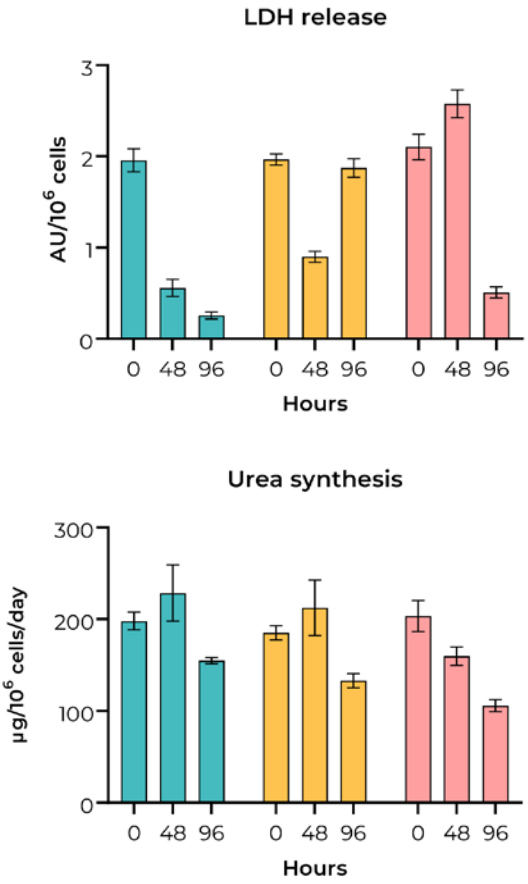


Figure 4: The DILI assay kit: Human 24 captures the anti-inflammatory effects of amiodarone and troglitazone. Liver MPS microtissues were exposed to vehicle control (teal), amiodarone (orange) and troglitazone (pink) for 96 hrs. Endpoint measurements for IL-6 at 0, 48 and 96 hr timepoints were derived by repeat media sampling from the same Liver MPS. Data shown are mean ± SD, N = 3, and all from 96 hr samples. Image analysis quantification of CRP was performed from the confocal images of stained scaffolds and normalized to Hoechst. Statistical analysis was performed using one-way ANOVA on ranks. In all cases *** represents P < 0.001 and **** P < 0.0001 by the range test.

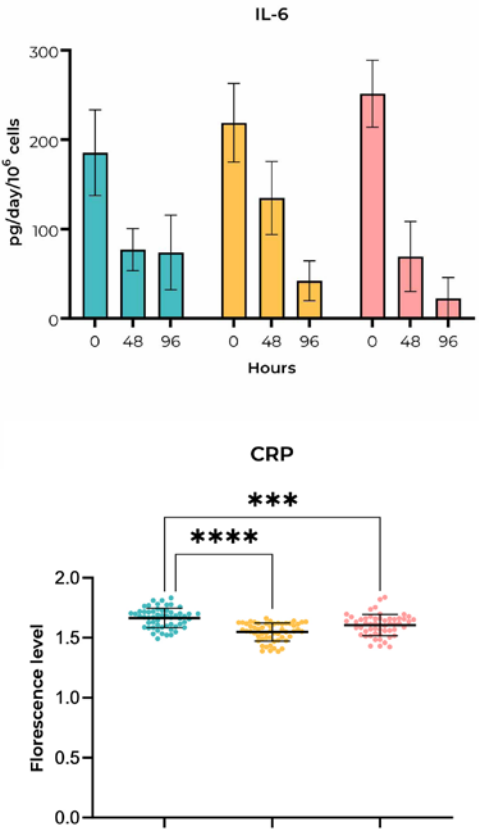


Figure 5: The DILI assay kit: Human 24 captures the cholestatic effect of troglitazone. Bile acid secreted by Liver MPS microtissues exposed to vehicle control (teal), amiodarone (orange) and troglitazone (pink) was quantified at 96 hrs. Data shown are expressed as fold-change, mean ± SD, N = 3 from 96 hr samples. Statistical analysis was performed using one-way ANOVA on ranks. In all cases, * represents P < 0.05 by the range test.

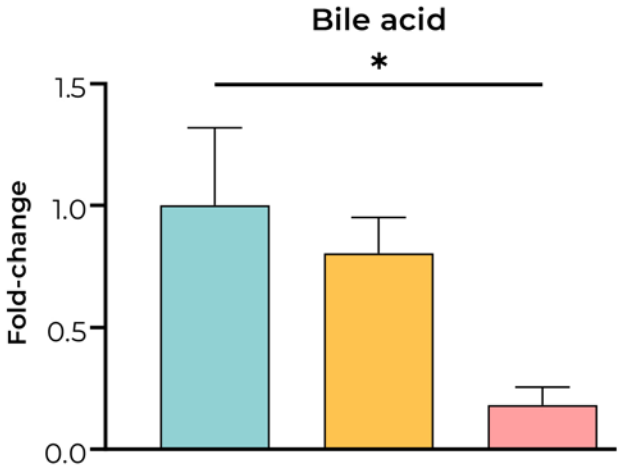


Figure 6: The DILI assay kit: Human 24 captures mitochondrial toxicity in tissues treated with amiodarone and troglitazone. Liver MPS microtissues were exposed to vehicle control (teal), amiodarone (orange) and troglitazone (pink) for 96 hrs. Data shown are mean ± SD, N = 3 from 96 hr samples. Image analysis quantification of mitochondrial toxicity was performed from the confocal images of stained scaffolds with MitoTracker and normalized to Hoechst. Statistical analysis was performed using one-way ANOVA on ranks. In all cases, * represents P < 0.05 by the range test.

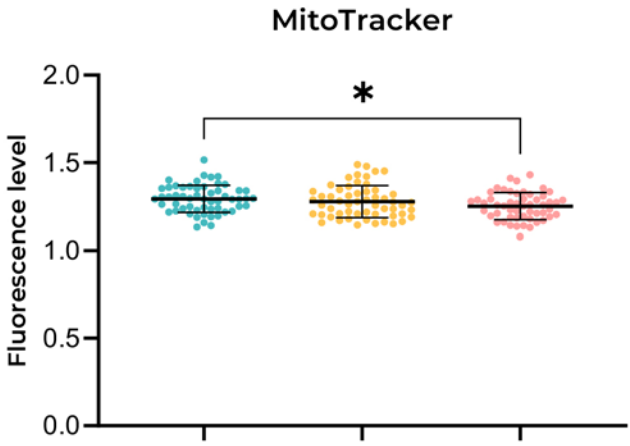


Figure 7: The DILI assay kit: Human 24 captures early steatosis in tissues treated with amiodarone and troglitazone. Liver MPS microtissues were exposed to vehicle control (teal), amiodarone (orange) and troglitazone (pink) for 96 hrs. Data shown are mean ± SD, N = 3 from 96 hr samples. Image analysis quantification of steatosis was performed from the confocal images of scaffolds stained for Nile red and normalized to Hoechst. Statistical analysis was performed using one-way ANOVA on ranks. In all cases ** represents P < 0.01 and *** P < 0.001 by the range test.

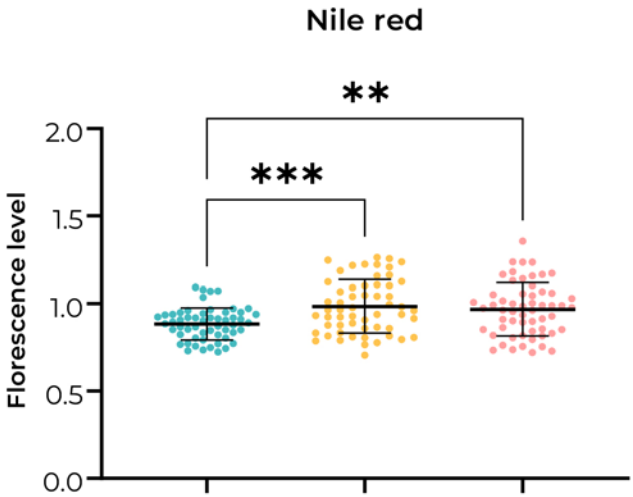
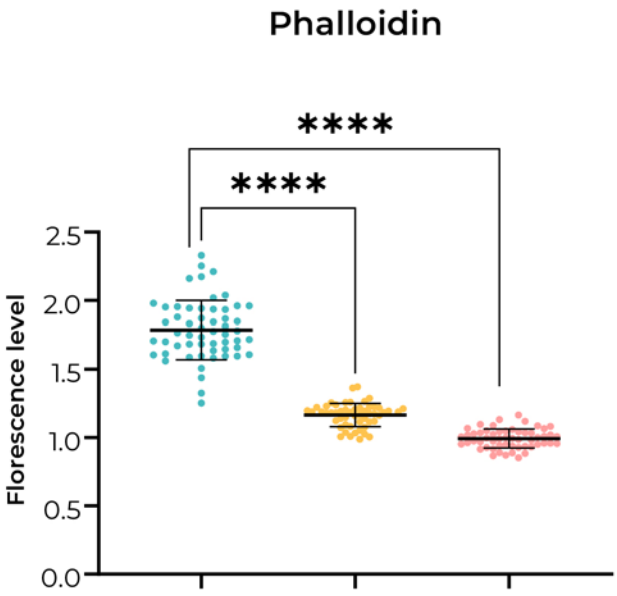


Figure 8: The DILI assay kit: Human 24 captures structural changes in microtissues treated with amiodarone and troglitazone. Liver MPS microtissues were exposed to vehicle control (teal), amiodarone (orange) and troglitazone (pink) for 96 hrs. Data shown are mean ± SD, N = 3 from 96 hr samples. Image analysis quantification of Phalloidin was performed from the confocal images of fixed scaffolds and normalized to Hoechst. Statistical analysis was performed using one-way ANOVA on ranks. In all cases, **** represents P < 0.0001 by the range test.



Conclusion

The acute exposure of two tool compounds of high-DILI-concern was investigated using CN Bio's PhysioMimix DILI assay kit: Human 24 and the PhysioMimix Core System. The kit contains everything required to recreate our co-culture (PHH and HKC) Liver MPS and FDA-recognized DILI assay in your own laboratory (Rubiano *et al*., 2021). Multiple cellular function and cell health markers (soluble, tissue) were assessed to create a distinct mechanistic "signature of hepatotoxicity". This demonstrated the kit's ability to predict DILI risk and understand the mechanism behind the cause.

Liver MPS microtissues, generated using the DILI kit and the PhysioMimix Core system, captured the DILI profiles of amiodarone and troglitazone using cell health and functionality biomarkers (**Figure 3**). LDH release captured a sub-acute cytotoxic response in Liver MPS microtissues treated with troglitazone, with a spike in LDH levels at 48 hrs exposure compared with vehicle control (p-value = 0.0034), and a temporal effect for amiodarone following 96 hrs of exposure, with a spike in LDH at a later time-point (p-value = 0.0034). Urea synthesis, an important functional hepatic marker, significantly dropped following troglitazone treatment at both time points compared to vehicle control (p-value = 0.0162 at 48 hrs, and p-value = 0.0034 at 96 hrs), whereas amiodarone had no toxic effects.

The immune component of the Liver MPS responded positively following exposure to both tested tool compounds, showing a strong anti-inflammatory effect on the IL-6/CRP pathway at 96 hrs of exposure as expected (**Figure 4**). Pro-inflammatory cytokine IL-6 levels, quantified from the cell culture media, showed a reduction across time in all tested conditions, with slightly stronger effect for troglitazone and amiodarone at 96 hrs of exposure compared to vehicle control, but with no statistical significance overall. Conversely, quantification of CRP from confocal images (stained scaffolds normalized to Hoechst) at the end of the experiment, demonstrated a strong anti-inflammatory effect of both tested compounds (p-value = 0.0020 for troglitazone; p-value < 0.0001 for amiodarone) mirroring clinical observations (Hirasawa *et. al*, 2009; Yatagai *et. al*, 2004).

Troglitazone is a well-known BSEP inhibitor and has previously been characterized for its cholestatic effect (Ogimura et. al, 2017). In patients treated with amiodarone, cholestasis has been reported, but only as a rare complication in 1% of patients (Chang *et. al*, 1999, Bratton *et al*., 2019). Bile acid release by liver microtissues into the cell culture media following troglitazone and amiodarone treatment was quantified over 96 hrs of exposure. The data presented in **Figure 5**, expressed as fold-change, showed a strong cholestatic effect in the liver tissues treated with troglitazone (fold-change approximately five times lower than vehicle control, p-value = 0.0185) but no significant cholestatic effect was recorded in the tissues treated with amiodarone, mirroring clinical outcomes.

Impairment of liver mitochondrial function is well characterized for both tested tool compounds. Amiodarone-induced hepatotoxicity includes hepatic cytolysis, cholestasis, and steatosis, secondary to the generation of reactive oxygen species (ROS) (Fromenty, 2023; Felser *et. al*, 2013). Similarly, troglitazone toxicity in the type 2 diabetic population appears to primarily involve mitochondrial dysfunction/disruption (Julie *et al*,. 2008). Quantification of mitochondrial toxicity, from confocal images of stained liver scaffolds with MitoTracker and normalized to Hoechst (**Figure 6**), revealed a decrease in mitochondrial function in liver tissues treated with both tool compounds. However, only troglitazone treatment achieved statistical significance (p-value < 0.05), confirming clinical findings.

Microvesicular steatosis has also been reported in patients treated with troglitazone (Julie *et al*., 2008) and amiodarone (Hubel *et al*., 2021; Fromenty, 2023). Quantification of steatosis, from confocal images of liver scaffolds stained for Nile red and normalized to Hoechst, mirrored clinical outputs for both tested compounds by demonstrating significant steatotic effects (**Figure 7**, p-value = 0.0008, amiodarone; p-value = 0.0050, troglitazone). Furthermore, lipid build-up in Liver MPS microtissues treated with both tool compounds induced morphological changes to the extracellular matrix and cytoskeleton of hepatocytes as indicated by a substantial drop in F-actin filaments compared to vehicle control (**Figure 8**, p-value < 0.0001), in line with Corinaldi *et al*, (2012). This was also accompanied by a strong cholestasis effect for troglitazone.

Previously published data (Novac *et al*., 2023) demonstrated how the Liver MPS correctly identified compounds with human DILI liabilities by measuring a wide range of endpoint biomarkers, including biomarkers of clinical relevance (e.g., albumin and ALT). Here we demonstrate how the PhysioMimix DILI assay kit: Human 24, in combination with sensitive assays to quantify inflammation, oxidative stress, mitochondrial dysfunction, cholestasis and steatosis, enables the underlying mechanisms of toxicity to be understood to produce a "signature of hepatotoxicity". The kit allows more complex and human specific DILI pathways to be profiled, bridging the relevance gap between traditional preclinical approaches and first-in-human (FIH) studies. It's insights can be used to potentially recover flawed candidates (by re-engineering), better inform *in vivo* animal studies to support their responsible use and decrease clinical risk.

Key messages

- The PhysioMimix DILI assay kit: Human 24 is designed for use in preclinical toxicology workflows to prevent cascading consequences, or in investigative toxicology studies to understand DILI discovered in the clinic.
- The assay correctly identified the underlying mechanisms of toxicity of two known hepatotoxicants (troglitazone and amiodarone).
- The assay offers advantages over existing *in vitro* approaches by incorporating the immune aspects of the liver that contribute to immune-mediated DILI .
- The assay provides a viable solution for new drug modalities with human-specific targets and pathways

About the PhysioMimix DILI assay kit: Human 24

The PhysioMimix DILI assay kit: Human 24 bridges the relevance gap between traditional *in vitro* and first-in-human studies. This all-in-one *in vitro* kit is designed for use with the PhysioMimix platform, containing everything required to recreate CN Bio's FDA-recognized DILI assay in your own lab.

The kit utilizes our perfused human MPS (or Liver-on-a-chip), to go beyond intrinsic DILI predictions for a broad range of drug modalities. Long-term, stable hepatocytes have the capacity to rapidly produce toxic metabolites, leading to DILI, especially with compounds requiring metabolic activation for their toxic effects.

Large amounts of recoverable Liver MPS microtissue and culture media enable the profiling of key human DILI pathway, including activation of innate hepatic immune cells. This allows for detailed mechanistic insights into a drug's toxicity profile. Extended culture time (up to 14 days) allows the detection of more latent events, and 24 replicates are provided.

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Notes

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