CN-BIO

2021 WEBINAR SERIES

Human Liver Microphysiological System for Studying Acute and Chronic Drug-Induced Liver Toxicity

Q&A

A full run down of questions & answers from our September 30th webinar

Abbreviations

5-CDF - 5 (and 6)-Carboxy-2',7'-Dichlorofluorescein

AST - Aspartate Transaminase

ALT - Alanine Transaminase

COC - Cyclic Olefin Copolymer

DILI - Drug-Induced Liver Injury

ERAs - Endothelin receptor antagonists

NPCs - Hepatic Non-Parenchymal Cells

HKCs - Human Kupffer Cells

HLA - Human Leukocyte Antigen

MOS - Margin of Safety

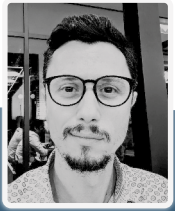
NAFLD - Non-Alcoholic Fatty Liver Disorder

NASH - Non-Alcoholic Steatohepatitis

OOC - Organ-on-a-Chip

PHHs - Primary Human Hepatocytes

Q&A participants



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Another question?

Drop an email to one of our experts - **sales@cn-bio.com**

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Questions

Q1

Q: Can you run a Drug-Induced Liver Injury (DILI) assessment study for longer than 8 days?

A: From Dr Ovidiu Novac, Senior Scientist, Biology, CN Bio:

Yes, we can run a Drug-Induced Liver Injury (DILI) assessment study for much longer than 8 days. The protocol I used to derive the dataset in the webinar requires 96 hours of drug exposure. This protocol is the standard one used by our **Toxicity Testing Service**. The **PhysioMimix™ OOC** is, however, capable of maintaining highly functional and metabolically active liver microtissues for up to 4 weeks **(Rowe, C. *et al.* 2018)**. We can therefore tailor this assay to investigate chronic drug exposure if desired.

To learn more, watch our "Testing on Humans" on-demand webinar: **https://webinars.cn-bio.com/testing-on-humans**

Q2

Q: What is the quantity of liver cells per well?

A: From Dr Ovidiu Novac, Senior Scientist, Biology, CN Bio:

In this particular assay, we seeded primary human hepatocytes (PHHs) with human Kupffer cells (HKCs) at a ratio of 10:1 per well, with 400,000 PHHs and 40,000 HKCs respectively.

Q3

Q: Do you use several donors to measure the donor-donor variability in DILI events?

A: From Dr Ovidiu Novac, Senior Scientist, Biology, CN Bio:

The same PHH and HKC donors were used for each compound in this study. This approach was taken to ensure data reproducibility across replicates and enable the DILI liabilities of compounds to be ranked against each other.

It is, however, possible to set your experiment up in a slightly different way to look at donor-donor variabilities, or even genetic susceptibilities to DILI. Internally, we have several validated lots of donors, from different vendors, that can be used for this kind of application. Our in-house validation process establishes which cell lots are highly functional (versus our QC parameters) and form

good 3D microtissues when cultured using the PhysioMimix OOC System. These 3D-validated cells are used for our own R&D projects as well as by our Contract Research Services and are part of our 3D validated cell supply catalogue - which helps our customers to fast-track their route to reliable and robust human translatable data.

To learn more about our 3D validated cells, please visit **https://cn-bio.com/3d-validated-cells/** or contact **sales@cn-bio.com**.

Q4

Q: Is it possible to use a pool of donors in your model?

A: From Dr Ovidiu Novac, Senior Scientist, Biology, CN Bio:

It is possible to test pools of donors in the PhysioMimix OOC systems. However, pools of donors supplied by vendors are highly unreliable and often fail to form functional and 3D microtissues with high metabolic activity. This is mostly due to the fact that the quality of the individual donors varies greatly, making it challenging to generate good quality liver tissue.

What we have done, and recommend, is to first validate several single donors (as described in Question 3), then generate your own in-house pool to investigate donor-donor variability. In our experience, this approach is more successful than using mixed pools of donors supplied by vendors.

Although used to study drug metabolism, here's an example of where in-house mixed pools were used that could easily be applied to DILI assessment (**Tsamandouras N. *et al.*, 2017**,).

Q5

Q: You have shown a very subtle DILI effect from Levofloxacin, do you think this could be due to the donors used in this study or do you expect to see a similar profile for this drug in other donors?

A: From Dr Ovidiu Novac, Senior Scientist, Biology, CN Bio:

Sure, a different toxicity profile might have been captured for levofloxacin if we had tested it on a different donor. Investigating this possibility would be a great next step for this project. Should differences arise, it would certainly highlight the importance of considering donor-to-donor variability using an organ-on-a-chip (OOC) approach prior to entering the clinic.

Q6

Q: Have you optimized an assay for detecting hepatocyte regeneration?

A: From Dr Ovidiu Novac, Senior Scientist, Biology, CN Bio:

We currently have several new *in vitro* models in our development pipeline, but not specifically targeting hepatocyte regeneration. This is something we are considering developing in the near future.

Q7

Q: What are your plans for advancing the model for high content drug screening?

A: From Dr Audrey Dubourg, Product Manager, CN Bio:

The answer to your question depends on the angle from which you are asking - throughput, content element, or compatibility with high content screening imaging devices.

Starting with throughput, our current set-up allows for up to 6 plates, or 72 wells to be screened at the same time using one PhysioMimix OOC Single-Organ System. For a fully perfused OOC system, this is high throughput when compared to competing chip-based approaches.

There is, however, a pull to improve the physiological relevance of assays at all stages of drug discovery to prevent a rubbish in/rubbish out scenario. As such, in response to market demand, higher throughput plates for determining DILI liability are in our developmental plan. However, when miniaturizing models into higher throughput formats there are compromises that need to be made which are often at the detriment of physiological relevance and assay sensitivity (see Questions 23, 25 & 26). There is therefore a fine balance to be struck to stay true to what we at CN Bio are trying to achieve.

In our opinion, the PhysioMimix OOC already offers high content data output. Samples can be taken over time in longitudinal studies to provide biochemical assessments that build up a picture over time. The large amount of tissue recovered at the end of the experiment can be examined in proteomics or genomic studies to look at gene regulation, to inform a comprehensive toxicology profile. This ability to highly multiplex enables researchers to go beyond simply identifying the presence of acute or chronic liver toxicity, or translating the results gained *in vitro* using lab-grown organs into a clinical setting. It also enables them to investigate the cause.

Finally, although our solution does not allow for live cell imaging, due to the current set up of the microfluidics that enable fluidic flow, it is still possible to perform various imaging techniques (from basic brightfield to confocal immunofluorescence or TEM) on the microtissue when taking down the experiment. We do not have plans to enable compatibility with high content screening imaging devices.

Q8

Q: Is 300 µM a relevant concentration for troglitazone?

A: From Dr Ovidiu Novac, Senior Scientist, Biology, CN Bio:

Yes, we believe so. It correlates with the concentrations used for *in vivo* treatment regimens and is comparable to the range of tested concentrations (up to 800 µM troglitazone) used in an FDA study characterizing the reliability and reproducibility of our PhysioMimix OOC for use in drug toxicity, metabolism and accumulation applications (**Rubiano *et al.*, 2021**).

Q9

Q: At which day do you start observing bile canaliculi, and do you have data regarding transporter substrate clearance?

A: From Dr Ovidiu Novac, Senior Scientist, Biology, CN Bio:

In a study by **Ortega-Prieto *et al.*, 2018**, the stable secretion of DPPIV/CD26, a marker of bile canaliculi was detected after 6 days of culture. Furthermore 5-CDF staining of their cultures demonstrated that hepatic bile canaliculi are functional and maintained for extended periods of time. Also, ultrastructural analysis of PHH microtissues grown for 20 days revealed structural features resembling liver sinusoidal architecture, including bile canaliculi and tight junction formation. We have not worked on specific transporter substrate clearance, but is something that that can be assessed in our model. We have data generated on metabolism and hepatic clearance research in our liver model (**Tsamandouras *et al.*, 2017**; **Rowe *et al.*, 2018**).

Q10

Q: Is it possible to subcontract a study on a compound in your system? How much would this cost (for a standard trial)?

A: From Dr Ovidiu Novac, Senior Scientist, Biology, CN Bio:

Yes, this is possible. The cost of our **Toxicity Testing Service** depends on the number of compounds that are submitted for testing, and the number of concentrations required per assessed compound. A standard trial is run on one PhysioMimix **MPS-LC12** plate, and includes one control compound plus either three concentrations of one test compound, or three test compounds at one fixed concentration (all in triplicate).

For more information about different packages available, please contact: **sales@cn-bio.com**

Q11

Q: Have you ever established an *in vitro-in vivo* correlation between your *in vitro* system and *in vivo* liver injury incidence ?

A: From Dr Ovidiu Novac, Senior Scientist, Biology, CN Bio:

For each compound tested we have determined the ratio of the EC_{50} to total plasma C_{max} or exposure-corrected cytotoxicity and plotted it as the "margin of safety" (MOS). MOS values were determined for all tested compounds at 96 hours of exposure. MOS was assayed for albumin, urea and ATP assays. We have used fixed thresholds of 50x MOS values to assess the sensitivity and specificity of the assay by comparing DILI positive/negative status as determined by EC_{50} or MOS threshold with known DILI status for each compound. Following data analysis, we observed a clear trend between MOS values and DILI severity and a strong *in vitro-in vivo* correlation.

Q12

Q: Have you got an explanation for the fact that some *in vitro* parameters (urea, ATP...) are not as sensitive as the albumin? What is the mechanism?

A: From Dr Ovidiu Novac, Senior Scientist, Biology, CN Bio:

Some compounds might exert toxicity at a hepatocellular functional level but not impact the cells' viability (ATP) or impair other functions (e.g. urea synthesis).

This type of toxicity can only be captured using sensitive assays such as albumin because albumin production is one of the most essential functions of primary hepatocytes.

Q13

Q: Have you ever used cryopreserved human hepatocytes?

A: From Dr Audrey Dubourg, Product Manager, CN Bio:

All the PHHs used in our R&D studies and our Contract Research Services are supplied to us cryo-preserved and thawed prior to use. We source primary cells from various suppliers such as Lonza, BioIVT and ThermoFisher. The 3D validated cells that we provide are also supplied to our customers cryo-preserved.

To learn more about our 3D validated cells, please visit **https://cn-bio.com/3d-validated-cells/** or contact **sales@cn-bio.com**.

Q14

Q: Is there protein in the media?

A: From Dr Ovidiu Novac, Senior Scientist, Biology, CN Bio:

We only add protein into the seeding media for the first 24 hours of culture, after which the media is replaced by protein-free maintenance media which is used until the end of the study.

Q15

Q: Have you also examined rat hepatocytes in this system?

A: From Dr Audrey Dubourg, Product Manager, CN Bio:

Years ago, when working with the prototype version of the PhysioMimix OOC, we successfully used rat hepatocytes to develop a rodent liver model (**Domansky *et al*, 2010**). More recently, however, our interest has focused on the human-relevant liver-on-a-chip model and its potential to improve DILI predictions ahead of the clinic to help reduce attrition rates.

Q16

Q: Have you tested iPSC-derived hepatocytes in your system?

A: From Dr Ovidiu Novac, Senior Scientist, Biology, CN Bio:

We have designed and optimized a liver model based on iPSC-derived hepatocytes and compared it to its static spheroid/2D standard counterparts. Our flow perfused model showed improved cell health and function in a 2-week culture period, however, we have yet to investigate its suitability for DILI screening.

Q17

Q: Do you follow CYP3A4 activity evolution or just its expression?

A: From Dr Ovidiu Novac, Senior Scientist, Biology, CN Bio:

To measure the CYP activity in our model, we use a standard P450-GloCYP3A4 assay using Luciferin which we adapted for 3D tissues. This enables us to follow the evolution of the activity along with the expression over time. We start measuring CYP3A4 activity from day 4 post-seeding. Thereafter, we monitor its activity throughout the study until the end of the experiment. The CYP3A4 kit that we use is optimized for use with 3D cellular cultures, and has minimal impact on liver microtissues and is easy to run.

For more information about the kit, please visit **Promega's website**

Q18

Q: Which polymer is used for the chip?

A: From Dr Audrey Dubourg, Product Manager, CN Bio:

Our PhysioMimix consumable plates are made of cell culture plastic with Cyclic olefin copolymer (COC) membranes. We opted for COC, which is currently the most inert material available for cell culture, instead of polydimethylsiloxane (PDMS), to reduce the risk of non-specific binding when performing a drug dosing assay such as DILI (**van Midwoud *et al.*, 2012**).

Q19

Q: How much training is required for a customer to adopt the technology?

A: From Dr Audrey Dubourg, Product Manager, CN Bio:

There are 2 aspects to consider: first setting up the system and secondly training the end-user. At CN Bio, we have spent a lot of time ensuring that our systems and consumables are as easy as possible to set-up and to use. This means that our end-users can concentrate on generating human-relevant, data-driven decisions.

- Installing the system is very simple and only takes a few minutes to set-up. Four cables (3 pneumatic cables and 1 electrical cable) provide power and link the instrument's controller to the docking station inside the incubator. There is no requirement for a CO_2 connection or any other cumbersome set-up. The system is operated via a simple touchscreen on the controller for rapid adoption.

- To further remove adoption barriers, we created open-well plates (rather than chips) that enable cell culture scientists to quickly transition from standard microplate-based set-ups to our technology. We provide virtual or onsite training (3 days) for new users and our support packages provide access to detailed protocols (SOPs). Together with our 3D validated cell service (which enables users to select primary cells from our catalogue of lots validated for use with PhysioMimix OOC systems), we ensure that end-users can successfully generate 3D *in vitro* tissue right from the start of their journey with us. Despite the complexity of the microtissues that the PhysioMimix OOC Systems culture, our new users always find the process surprisingly simple!

To learn more about our Training and support programs, please contact us: **sales@cn-bio.com**

Q20

Q: Have you assessed the effect of shear stress on hepatocytes directly exposed to flow, vs the ones located "inside" the 3D structure?

A: From Dr Dharaminder Singh, Principal Bioengineer, Product Development, CN Bio:

By adapting the scaffold design and system microfluidics, we explored different microenvironments within the PhysioMimix MPS-LC12 culture plate to ascertain the levels of shear stress and forces, flow rate, oxygen gradients and more within our liver cultures. We utilized a combination of experimental data and computational fluid dynamics to determine the most physiologically relevant settings for the **PhysioMimix MPS-LC12** plates, which in turn led to improved cell morphology and functionality for up to 4 weeks (**Rowe *et al.* 2018**).

For more information, please watch this on-demand webinar: **https://webinars.cn-bio.com/the-rhythm-of-life**

Q21

Q: In relation to the 3D setting, is the establishment of hepatic zonation something you have looked into?

A: From Dr Ovidiu Novac, Senior Scientist, Biology, CN Bio:

We have previously demonstrated that there is an oxygen gradient across the scaffolds within our MPS-LC12 plates, therefore the

cells across the scaffold will experience slightly different oxygen concentrations (**Domansky *et al.*, 2010**). The scaffold, however, is 250 μM thick so its depth is not enough to mimic hepatic zonation. The system can be run under different oxygen concentrations in the cell culture incubator to allow different areas of the liver to be modelled.

Q22

Q: How different is your system from other similar technologies?

A: From Dr Audrey Dubourg, Product Manager, CN Bio:

Organ-on-a-chip technologies all vary in shape, complexity, throughput, the applications they can be used for and the depth of endpoint analysis that can be achieved.

Some offer simple gravity-driven fluidics in a cell culture plate to achieve higher throughput but poor correlation versus human blood flow. Others offer a better correlation but complex *modus operandi*, very low throughput and content. Our solution has been designed to combine the best of both worlds.

It enables true replication of physiological shear stress, blood flow and cell-cell interaction while also offering medium throughput.

It complements higher throughput OOC solutions that offer a yes/no approach by providing large volumes of media (up to 1 mL) and tissue from which to perform highly multiplexed endpoint analysis (e.g., soluble biomarkers, RNA-seq or other other-omics) to inform a comprehensive toxicology profile and fathom out "how".

Finally, as mentioned in Q19, ease of use is key to the successful adoption of OOC within drug discovery, so to decrease barriers, we purposefully chose a familiar open-well architecture for the consumable plate rather than a futuristic "Chip-based" approach.

Q23

Q: Can you comment on why Dexamethasone shows to be toxic or slightly toxic in your assay?

A: From Dr Ovidiu Novac, Senior Scientist, Biology, CN Bio:

Although dexamethasone is a widely prescribed anti-inflammatory drug and is relatively safe, several reports of DILI have been cited in the clinic at high doses (**LiverTox, Clinical and Research Information on Drug-Induced Liver Injury, 2012**). This observation was recapitulated in our model. Dexamethasone

shows slight toxicity, as detected by functional biomarkers urea and albumin at 48 and 96 hours and by cellular endpoints CYP3A4 and ATP, but only at very high concentrations that are several times above C_{max}. The ratio of the EC_{50} to total plasma C_{max}, or exposure-corrected cytotoxicity, was determined for each compound and plotted as the "margin of safety" (MOS). We used fixed thresholds of 50 x MOS values to assess the sensitivity and specificity of this assay by comparing DILI positive/negative status and, according to the EC_{50} values obtained for all endpoint assays, dexamethasone tested true negative, as described by **Proctor *et al* (2017)**. The binary classification is necessary to identify known clinical hepatotoxicants by using a fixed EC_{50} and MOS values at 50 x threshold. Plotting the 50 x threshold horizontally true positives, false negatives, true negatives and false positives can be visualised in four quadrants (**Proctor *et al*., 2017**)

Q24

Q: How different is your 3D liver model from other 3D spheroid models?

A: From Dr Ovidiu Novac, Senior Scientist, Biology, CN Bio:

Culturing PHHs as spheroidal structures generated by gravitational aggregation in hanging-drops or on ultralow attachment surfaces could offer a high-throughput method for assessing compound liabilities. Also, PHH spheroids have been shown to replicate certain liver pathologies such as steatosis and cholestasis, allowing for the assessment of DILI in a diseased background. A wide variety of models have been developed to include plated micro-patterned co-cultures of hepatocytes with stromal fibroblasts, 3D bio-printed liver tissues and 3D spheroid cultures with or without hepatic non-parenchymal cells (NPCs). However, all these methods still have drawbacks such as a short lifespan, loss of phenotype over time (dedifferentiation) and low metabolic activity.

Traditional *in vitro* PHH models lose key hepatic functions, such as metabolic activity, during short-term culture. Comparatively, the lifespan and functionality of PHHs co-cultured in a more physiologically relevant perfused microenvironment (using the PhysioMimix OOC) are significantly extended. This enables prolonged "chronic" exposure to potential hepatotoxicants to be investigated and more complex biological interactions to be studied over longer periods of time (**Long *et al*., 2016**).

Our collaborators at the FDA monitored the metabolic activity of liver microtissues generated using PhysioMimix versus spheroids

and liver sandwich assays over 30 days and their albumin production over 20 days. PhysioMimix cultures were shown to significantly outperform these other approaches. The FDA has also demonstrated the advantages of PhysioMimix™ in drug safety and metabolism applications over these standard techniques, in this recent publication **Rubiano *et al.*, 2021**.

To improve the translational relevance of any advanced *in vitro* PHH culture, clinically relevant functional endpoints, or toxicity output biomarkers, must be determined to allow data to be compared to *in vivo* or clinical scenarios. These have been notoriously difficult to detect *in vitro,* but our microtissues (made up of 0.4 million primary cells perfused by large volumes of media) increase assay sensitivity to the point where more sophisticated clinical chemistry outputs of liver function can be detected (e.g., aspartate transaminase (AST) and alanine transaminase (ALT).

Finally, these perfused OOC models offer greater flexibility to incorporate circulating immune cells to uncover immune-related toxicity issues, or to induce common liver disorders, such as Non-Alcoholic Fatty Liver Disorder (NAFLD) & Non-Alcoholic Steatohepatitis (NASH), to unlock underlying DILI morbidity susceptibilities.

Q25

Q: What are the factors that determine the model's high sensitivity?

A: From Dr Ovidiu Novac, Senior Scientist, Biology, CN Bio:

One of the key factors required for high sensitivity is the cellular expression of key transporters and metabolic enzymes. Secondly, fluidic perfusion helps to drive oxygen and nutrients into cells allowing them to maintain a high level of functionality. Thirdly, to achieve high assay sensitivity requires a large volume of cells, which we have calculated to be 0.4 million/well.

Q26

Q: What is the flow rate in your system?

A: From Dr Audrey Dubourg, Product Manager, CN Bio:

To generate our 3D liver tissue, we use a steady flow rate of 1 µL/s which corresponds to the standard blood flow into a human liver. This flow rate was assessed when we first developed the model with our collaborators at Massachusetts Institute of Technology (**Inman**

***et al.*, 2007**) The flow rate in PhysioMimix systems ranges between 0.5 to 2.5 µL/s so that it can be adapted to the target model being used as not every organ in the body experiences the same force of blood flow. For example, major blood vessels such as arteries experience high forces which change many times a second, while the narrow capillaries within tissues see a much steadier, gentler set of flows and forces. So dependent on the organ or tissue that is being emulated, the PhysioMimix OOC system can provide flow that is strong and pulsatile, or slow and steady.

Q27

Q: Have you examined the concordance of *in vitro* to *in vivo* using the toxicity profile as compared to just a single assay? For example, Sitaxentan produced just albumin reduction compared to troglitazone which produce decreases in all endpoints. Can this be used to discriminate concordance to *in vivo* toxicity?

A: From Dr Ovidiu Novac, Senior Scientist, Biology, CN Bio:

Tested compounds sitaxentan and troglitazone have been withdrawn from the market due to serious adverse effects but have different mechanisms of generating liver toxicity.

Endothelin receptor antagonists (ERAs) (e.g., sitaxentan) have been shown to inhibit human hepatic transporters, which provides a potential mechanism for the increased hepatotoxicity observed for these agents in the clinical setting (**Hartman *et al.*, 2010**). The classical hallmarks of a direct hepatotoxin are that they are dose related, with rapid improvement of liver function on drug withdrawal (**Chin *et al.* 2012**). It has been suggested that ERAs or their metabolites competitively inhibit a bile salt transporter pump, leading to intracellular accumulation of bile salts, but other mechanisms cannot be excluded (**Galie *et al.*, 2011**) .

Troglitazone has been known to cause hepatocellular damage including fulminant hepatitis, hepatic necrosis, and cholestasis (**Boelsterli *et al.*, 2010**). Troglitazone has been proposed to cause hepatotoxicity by inducing oxidative stress and contributing to mitochondrial damage (**Chojkier, 2005**; **Boelsterli *et al.*, 2010**). By implementing the PhysioMimix DILI assay, functional liver-specific endpoints can be analyzed from the culture medium and liver microtissues to create a distinct mechanistic “signature of hepatotoxicity”. Mitochondrial damage following exposure to troglitazone at 48 hours and 96 hours was easily identified by measuring ATP content and by the impaired albumin and urea production, whereas sitaxentan toxicity was captured only by the

functional biomarker albumin. This highlights the importance of using a wide range of biomarkers to detect toxicity in compounds that otherwise might be missed when using only basic cell viability endpoints. As previously stated, this system enables the analysis of clinical biomarkers, such as ALT, which are notoriously difficult to detect *in vitro* allowing improved translation to clinical data.

Q28

Q: Have you looked at autologous donor hepatocytes and Kupffer cells? Is there any difference versus use of non-autologous donor cells?

A: From Dr Ovidiu Novac, Senior Scientist, Biology, CN Bio:

We perform all our studies using non-autologous cells. With good cell preparations of quiescent HKCs, we do not observe auto-activation of cells in the platform. Cells only activate when they are stimulated by exogenous factors. We have also made more complex set-ups with more cell types (e.g., Peripheral Blood Mononuclear Cells (PBMCs)) which are also non-autologous but human leukocyte antigen (HLA) - matched to reduce autoactivation.

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