

APPLICATION NOTE

Connecting the gut and liver: a human relevant multi-organ microphysiological system for preclinical profiling of oral bioavailability

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Introduction



Absorption, distribution, metabolism, and excretion (ADME) are four key processes that indicate the behavior of a drug following administration, and therefore play a key role in defining a compound's pharmacokinetic (PK) properties and bioavailability. Oral bioavailability is defined as the fraction of a drug that reaches systemic circulation following absorption across the intestinal wall and first pass metabolism in the liver. ADME and bioavailability are central in determining the safety and toxicology profiles of compounds and are therefore crucial measurements at the preclinical stage of drug development.

Currently, a combination of simple *in vitro* assays that either model the gut (Caco-2 cell line) or the liver (liver microsomes and suspension hepatocytes), and *in vivo* animal models is used to profile oral bioavailability; however, significant limitations exist with both approaches. Caco-2 cells, which have been the workhorse for assessing *in vitro* intestinal permeability, cannot account for liver metabolism, plus the cell line has absent or low levels of enzyme and transporter expression. Liver microsomes and suspension hepatocytes are used for *in vitro* drug metabolism screening studies, but do not consider intestinal absorption. Collectively, these limit the accuracy of their estimations. Furthermore, in a seminal study investigating 184 compounds, animal models were found to have a weak correlation with bioavailability in humans ($R^2=0.34$)¹. A new human relevant approach, which combines oral absorption and hepatic metabolism, is therefore required to more accurately estimate drug bioavailability.

In the last decade, microphysiological systems (MPS), also known as organ-on-a-chip (OOC), have shown their potential to improve the human translatability of ADME studies. They are designed to recapitulate the structural and functional biomarkers of cells and tissues in a more physiologically relevant manner through the culture of primary human cells on perfused 3D scaffolds. Efforts to improve the *in vitro* to *in vivo* translation of drug efficacy and safety data has led to the emergence of more complex MPS where multiple organs, such as gut and liver, are fluidically linked together to simulate processes such as drug absorption and first pass metabolism².

Aim



Here, we introduce a dual-organ MPS that links our established primary human liver MPS with a primary model of the human intestine. For the intestinal barrier, primary cells are isolated from the human jejunum and expanded on a biomimetic scaffold (RepliGut®). To link the primary gut and liver tissues together, we developed a chemically defined media that supports both organ models in the dual-organ MPS. This media enables both the maintenance of hepatic metabolic functionality and intestinal barrier integrity. Using well-studied drug compounds, we aimed to demonstrate the improved predictive capacity of this primary Gut/Liver MPS for profiling the ADME behavior of oral drugs compared to an equivalent Caco-2 Gut/Liver MPS.

Methods



Here, we describe a dual-organ MPS that links a primary human intestine (RepliGut® Planar-Jejunum, Altis Biosystems) with a primary human liver-on-a-chip (CN Bio). The dual-organ MPS is cultured using the PhysioMimix® Multi-organ System and its bespoke “Multi-chip” Dual-organ consumable plate (Figure 1A). The dual-organ plate consists of six wells, each with two compartments (i) a Transwell® compartment and (ii) a liver compartment. Fluidic flow can be independently controlled in each compartment (intestine and liver), and in the interconnecting channel between the organs (Figure 4A). The intestinal barrier was established through the expansion of human jejunum stem/progenitor cells on a Transwell® coated with a biomimetic scaffold, followed by differentiation into a polarized barrier comprised of all post-mitotic lineages found in the human intestine. Media was changed every 48 hours with either RepliGut® Growth or Maturation media. Intestinal barrier integrity was assessed with transepithelial electrical resistance (TEER) measurements every 48 hours or by determining the permeability of Lucifer yellow across the Transwell at the end of the dual-organ experiment. Expression of markers confirming intestinal origin (Villin and CDX2) and the presence of the tight-junction marker ZO-1 was confirmed by fluorescent microscopy. Mucus production was confirmed by histological staining of the RepliGut® Planar-Jejunum

cross-section with Alcian blue and by in-well immunofluorescent staining for the Muc-2 protein. Expression of metabolic and transporter genes were evaluated using RNA isolated at day 15 of Caco-2 culture or day 7 post differentiation of RepliGut® Planar-jejenum. Gene expressions of metabolic enzymes and transporters were measured by qPCR using TaqMan™ Gene Expression Assays, with relative expression determined using $\Delta\Delta CT$ analysis.

For the liver, primary human hepatocytes (PHH) were seeded in the liver compartment of the PhysioMimix Dual-organ plate, on a porous 3D collagen-coated scaffold (Figure 1C). On day 4, following PHH seeding and the formation of microtissues, differentiated RepliGut cultures were added to the PhysioMimix Dual-organ plate to establish the Gut/Liver co-culture. A chemically defined media was used to maintain functionality of the gut and liver tissues for at least 48 hours, during which compounds were added to study their ADME profile. In this study, we compared the performance of the primary RepliGut/liver MPS versus a Caco-2 Gut/liver MPS. The Caco-2 Gut/liver MPS was established by adding Transwells with differentiated Caco-2 monolayers (at 15-17 days post seeding) to the PhysioMimix Dual-organ plate on day 4, following PHH seeding and the formation of microtissues.

We used 7-hydroxycoumarin (7-HC), a fluorescent compound that undergoes Phase II metabolism by glucuronidation, in a proof-of-concept study to demonstrate absorption through the intestinal barrier and subsequent first pass metabolism in the co-culture model. The PhysioMimix Dual-organ plate allows for flexibility in compound dosing with either Gut/Liver in co-culture or gut only (no PHH) and liver only (no gut barrier, with compounds dosed into a blank Transwell with no cells).

In this study, two compounds (Temocapril and Midazolam) whose human ADME properties were not predicted by existing models, were investigated. Compounds were added at day 4 post seeding of PHH and addition of the gut tissues to Dual-organ plates by either oral (drug added to the apical surface of the Transwell) or intravenous (IV) dosing (liver only, drug mixed into co-culture media). Samples of media were taken at 0, 1, 4, 6, 24, and 48 hours, and analyzed by liquid chromatography–mass spectrometry (LC-MS) to determine the concentration of parent compounds in the liver compartment. An estimation of area under the curve (AUC) of both oral and IV concentration profiles was made using GraphPad Prism.

Results and Discussion



Using the PhysioMimix Multi-organ System and its Multi-chip Dual-organ plate (Figure 1), we established a primary Gut/Liver MPS to overcome the human-relevance limitations of current models in profiling oral drug bioavailability. In the RepliGut model (Figure 2A), jejunum stem and progenitor cells were expanded to confluence on a biomimetic scaffold before undergoing differentiation, which resulted in increasing barrier strength (Figure 2B). Gene expression confirmed downregulation of proliferative cell genes and upregulation of differentiated enterocyte genes relative to cells in the proliferative phase (Figure 2B). In the differentiation phase, the RepliGut model expressed tissue-specific markers confirming intestinal origin as well as markers of tight junctions and mucins, confirming multi-cellular lineages (Figure 2C). We confirmed this via histology with observations of a distinct and continuous layer of mucus at day 7 post-differentiation. By comparison, mucus production was absent in the epithelial cell line, Caco-2 model (Figure 2D). To assess the RepliGut model's potential to improve predictivity over the Caco-2 model, we investigated the expression of major metabolic enzyme and transporter genes that are important in drug metabolism and active drug transport. Gene expression was found to be improved compared to the Caco-2 cell line (Figure 2E).

One of the challenges with the co-culture of two, or more, tissues together is establishing the conditions that maintain functionality of both tissue types. We designed a chemically defined media, which is added to the Transwell basolateral and liver compartments of the Dual-organ plate (Figure 3A). This media maintained hepatic cell health and functionality in co-culture for at least 48 hours, measured by LDH release, CYP3A4 activity and albumin production (Figure 3 B-D). Intestinal barrier integrity was also maintained for the 48 hours of co-culture, as measured by TEER (Figure 3E) and a Lucifer yellow permeability assay (Figure 3F), after the addition of the RepliGut model to the dual-organ plate. Functionality was further demonstrated by 7-HC dosing (Figure 4A) which undergoes metabolism through glucuronidation (Figure 4B), with both intestinal and hepatic tissues contributing to its clearance (Figure 4C).

To demonstrate the improved predictive capability of the primary RepliGut/Liver MPS versus an equivalent Caco-2 Gut/Liver MPS, we investigated the ADME properties of two drugs where both animal models and the Caco-2 cell line failed to accurately profile their ADME behavior and bioavailability in human. The first drug, Temocapril, is a pro-drug designed to be resistant to intestinal hydrolysis that is metabolized to Temocaprilat by carboxylesterase 1 (CES1) (Figure 5A). The isoenzyme pattern in human is well-characterized with the liver and intestine expressing CES1 and CES2 respectively³. In Caco-2 cells, there is a miss-match as CES1 is predominantly expressed, resulting in an overestimation of drug clearance (Figure 5B-C). In contrast, the more human representative ratios of both CES1 & CES2 expression by the RepliGut model make it more relevant for pro-drug studies (Figure 5G). Temocapril clearance was profiled with oral and IV dosing (Figure 5E). Rapid clearance was observed by the liver within 24 hours following IV dosing (Figure 5F). In line with intestinal CES expression, the primary RepliGut/Liver MPS correctly reported resistance to intestinal hydrolysis of Temocapril and less Temopcarilat was produced at 48 hours in the gut apical compartment, following oral dosing.

Finally, we assessed the bioavailability of Midazolam in both Gut/Liver MPS models, a drug known to predominately undergo intestinal clearance by the CYP3A4 enzyme⁴. Midazolam was rapidly cleared by the liver following IV dosing, reflecting its high intrinsic hepatic clearance rate (19 mL/min/Kg) (Figure 6A). We observed greater clearance of Midazolam by the primary RepliGut/Liver MPS, compared to the Caco-2 Gut/Liver MPS (Figure 6B). The primary RepliGut/Liver MPS also delivered an oral bioavailability estimation that more closely represented human clinical observations (Figure 6C).

Figure 1. Set-up of a standard Gut/Liver MPS in the PhysioMimix Dual-organ plate.

A) Set-up of the PhysioMimix Multi-organ System and its Multi-chip Dual-organ plate, showcasing the location of the gut and liver compartments within the plate. Each plate can culture up to six Gut/Liver models. **B)** Schematic diagram of a RepliGut® model cultured on a biomimetic scaffold within a Transwell insert, forming an intestinal epithelial barrier. **C)** Schematic representation of fluidic flow within the liver compartment of the Dual-organ plate.

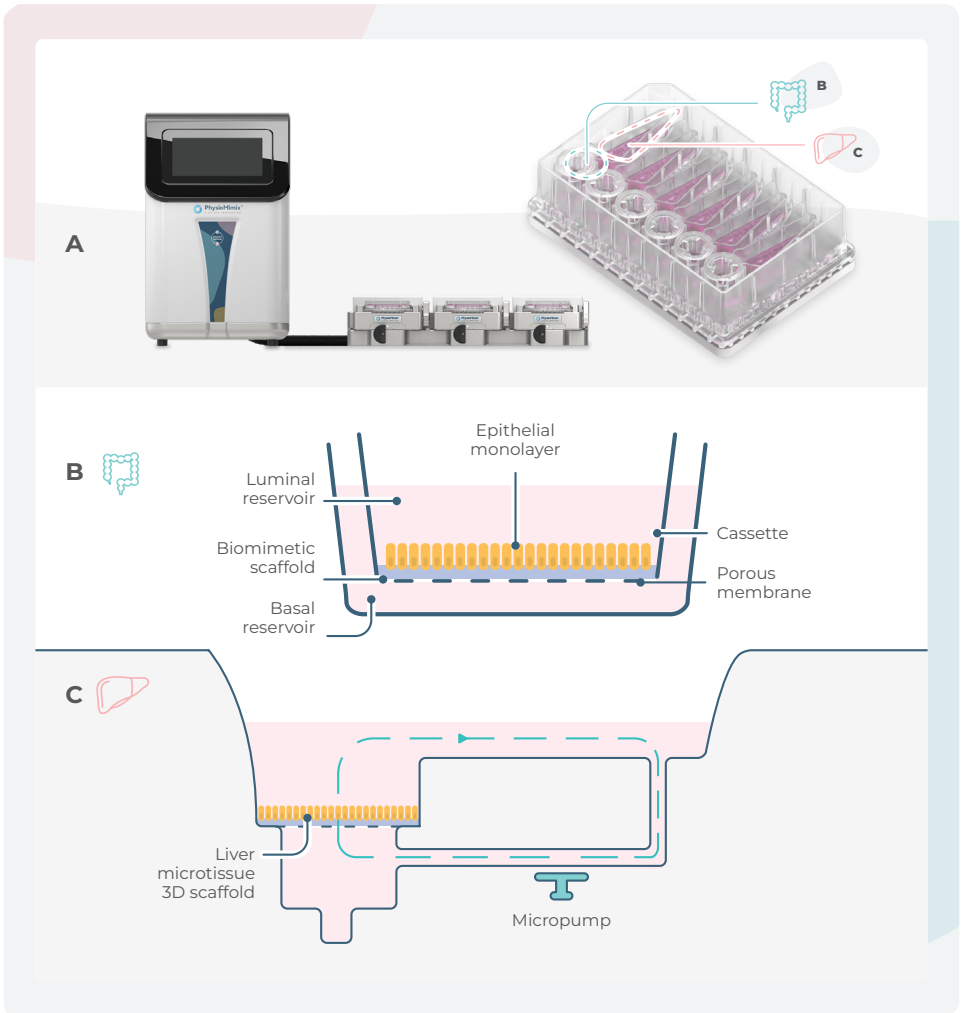
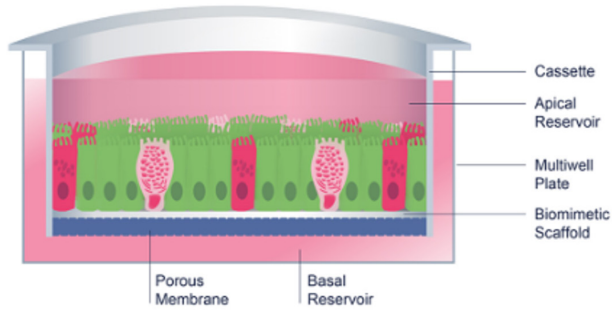


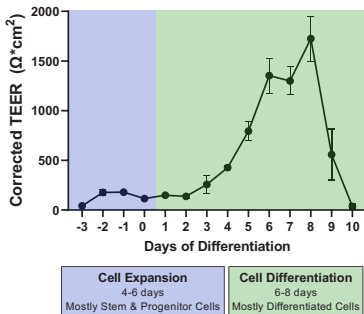
Figure 2. RepliGut primary model of the intestine is more human relevant compared to the standard Caco-2 cell line model.

A) Crypt epithelium stem/ progenitor cells are isolated from jejunum samples and expanded on a biomimetic scaffold in static conditions. **B)** Barrier integrity measured by TEER and relative gene expressions, in the expansion and differentiation stages of the RepliGut model. **C)** Immunofluorescence images of the RepliGut model. The barrier is stained for DAPI (dark blue), the tight junction protein, ZO-1 (light blue), and other intestinal markers including MUC2 (yellow), villin (purple) and CDX2 (green). The image scale bars equate to 100 μm for CDX2 and 200 μm for the other three markers. **D)** Histology sections of the RepliGut and Caco-2 models. Monolayer cross-sections are stained with hematoxylin and eosin to visualize nuclei and with Alcian blue to visualize the mucus layer. **E)** qPCR to demonstrate relative gene expressions of key transporters and metabolic enzymes in the RepliGut and Caco-2 models.

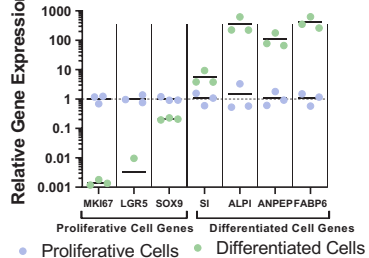
A



B



Relative Gene Expression



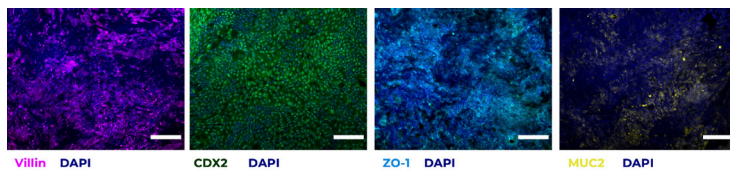
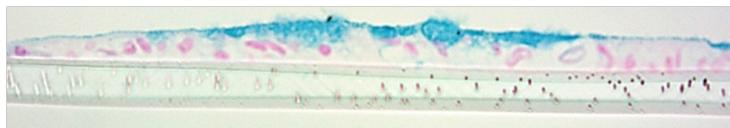
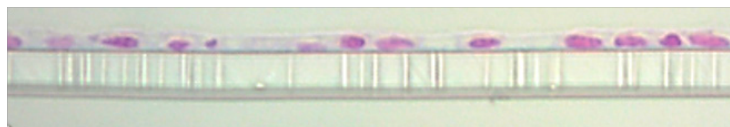
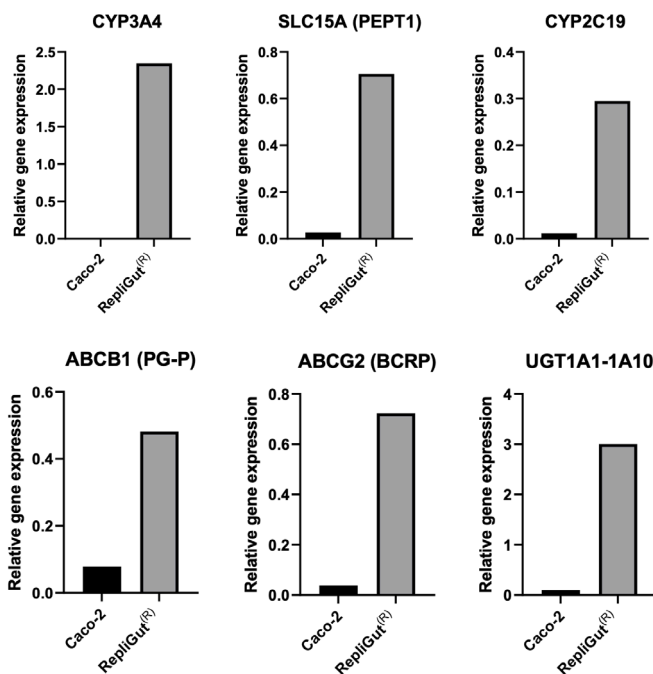
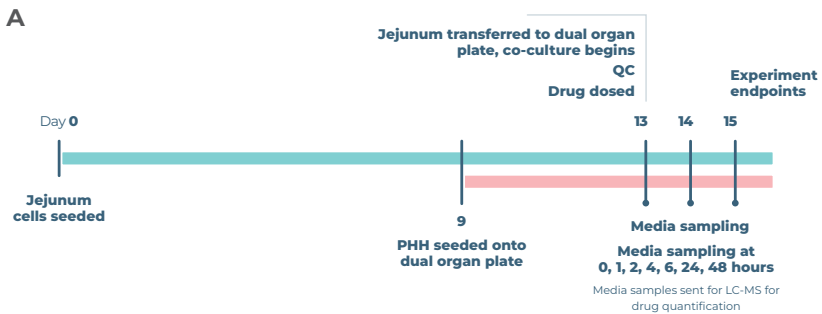
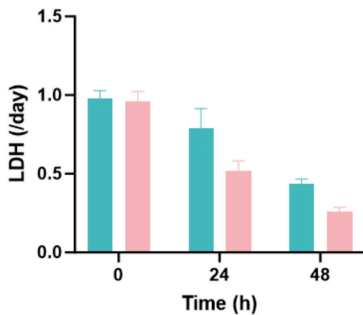
C**D****RepliGut®****Caco-2 only****E**

Figure 3. Liver and gut functionality markers are maintained throughout the primary cell Gut/Liver co-culture.

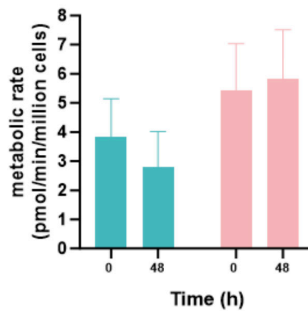
A) Experiment timeline to establish the primary RepliGut/Liver MPS. RepliGut was first cultured independently, in static, for 13 days before being transferred to the Transwell compartment of the PhysioMimix Dual-organ plate, 4 days after PHH seeding. The co-culture was maintained for 48 hours in a chemically defined media. Markers of liver cell health and functionality were measured at 0, 24 and 48 hours of co-culture, with a liver only MPS cultured in parallel as a control. We investigated **B)** LDH release **C)** CYP3A4 activity and **D)** Albumin production. Barrier integrity of RepliGut in the Gut/Liver MPS was assessed by **E)** TEER and **F)** permeability to Lucifer yellow and compared to independent RepliGut cultured in static, for its full duration in standard RepliGut[®] Maturation media.



B LDH

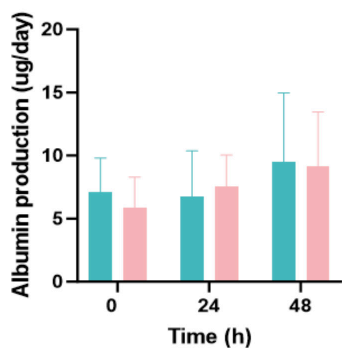


C CYP3A4 Metabolism



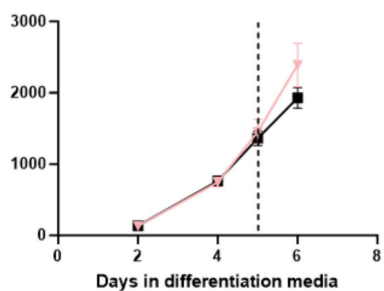
Legend: Liver only (teal), Gut/Liver, RepliGut[®] (pink)

D Albumin



Legend:
Liver only (teal)
Gut/Liver, RepliGut® (pink)

E Barrier Integrity



Legend:
Static, RepliGut® (black squares)
Gut/Liver, RepliGut® (pink squares)

F Permeability to Lucifer Yellow

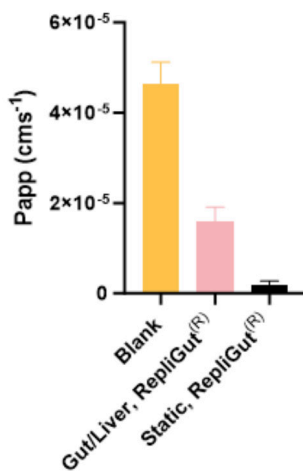


Figure 4. First pass metabolism can be modeled by the primary cell Gut/Liver MPS.

A) Schematic representation of 7-HC dosing in the Gut/Liver MPS model. 1mM 7-HC was dosed in the apical side of the Transwell compartment, containing the RepliGut barrier, within the Dual-organ plate. 7-HC was then transported across the barrier into the media circulating between the gut and liver tissues. The gut only control was prepared by inserting the RepliGut model into the Dual-organ plate Transwell compartment that was connected to a blank liver compartment (without PHH). Likewise, the liver only control was prepared by dosing 7-HC into a blank Transwell insert (without gut cells), in the Transwell compartment of the Dual-organ plate. **B)** Pathway of 7-HC metabolism. 7-HC undergoes phase II metabolism by UGT enzymes to form the non-fluorescent metabolite 7-hydroxycoumarin glucuronide. **C)** Changes in 7-HC concentration in the circulating media over time.

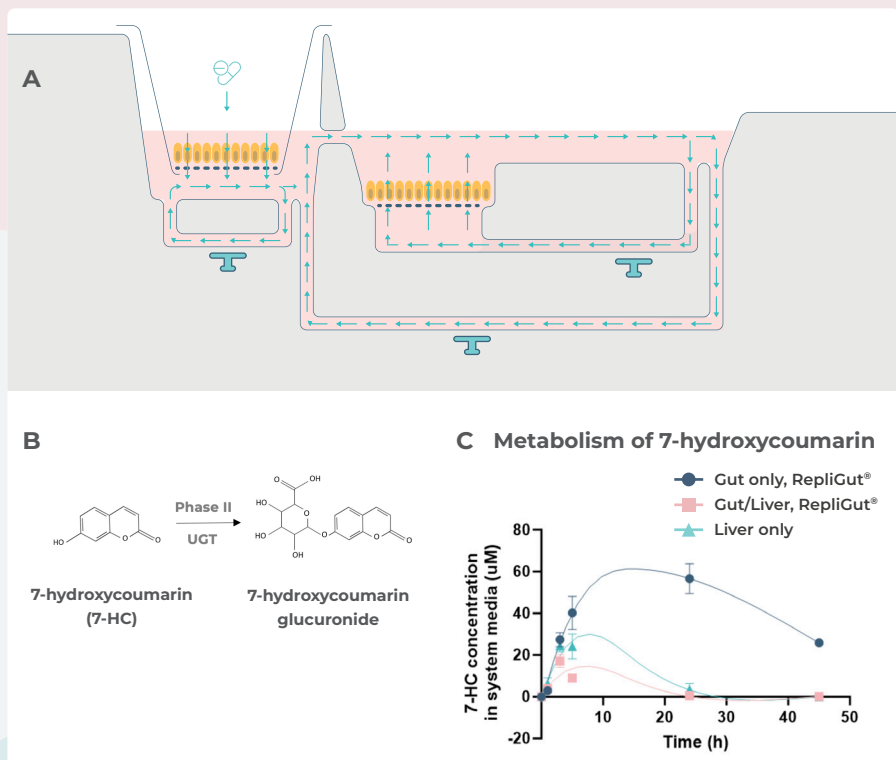
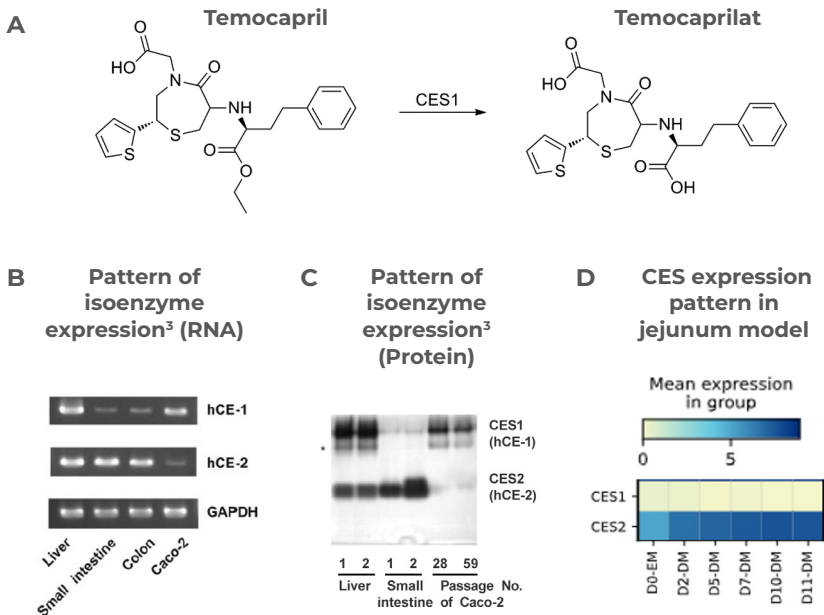
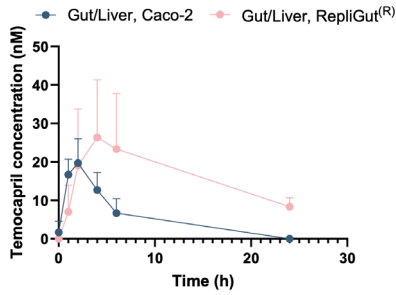


Figure 5. Case study 1, Temocapril. Resistance of Temocapril to intestinal clearance observed in the primary cell Gut/Liver MPS, correlated with isoenzyme expression in the human intestine.

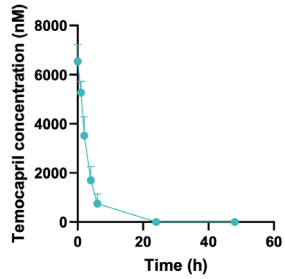
Temocapril was added as either an oral dose (100 μM) in the apical side of the Transwell or as an IV dose (10 μM) and directly mixed with circulating media in the liver compartment. Two configurations of the Gut/Liver MPS were studied: Caco-2/Liver and RepliGut/Liver. Temocapril is **A)** primarily metabolized by carboxylesterase (CES) 1 isoenzyme into the active metabolite Temocaprilat. Pattern of CES1 isoenzyme **B)** RNA and **C)** Protein expressions in human liver, human small intestine, and the Caco-2 cell line³. **D)** Pattern of CES gene expression in the Jejunum model over time in expansion media (EM) and differentiation media (DM); data courtesy of Scott Magness, UNC Chapel Hill **E)** Concentration of Temocapril in the circulating media over time in the Gut/Liver models, measured by LC-MS. **F)** Concentration of Temocapril over time in the liver only model. **G)** Total molar fraction of Temocapril remaining across all compartments in each model at 48 hours. **H)** Relative amount of the metabolite Temocaprilat in the gut apical compartment in both Gut/Liver models at 0 and 48 hours after drug dosing. Peak area is a qualitative measure of compound concentration.



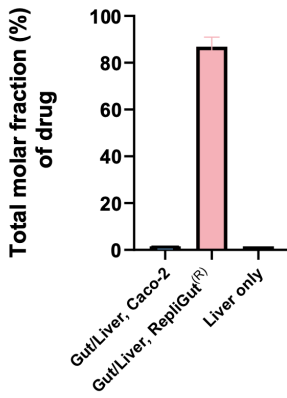
E Oral, Gut/Liver, 100 μM dose



F IV, liver only, 10 μM dose



G Temocapril at 48 hrs in all compartments



H Temocaprilat at 0 and 48 hrs in Gut Apical compartment

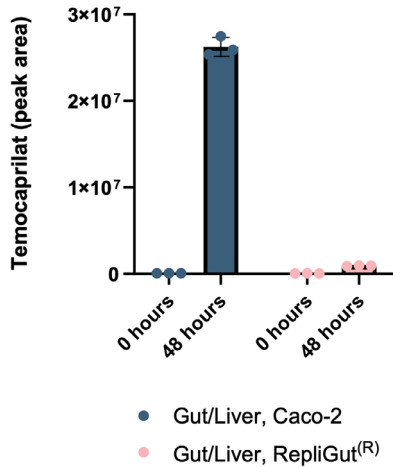
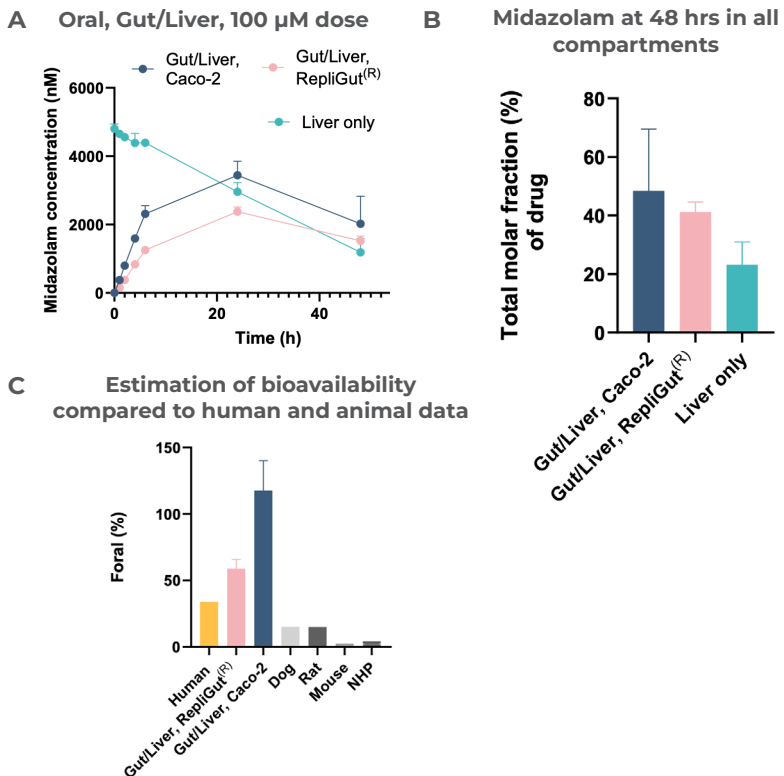


Figure 6. Case study 2, Midazolam. Improved correlation with human bioavailability of Midazolam by the primary cell Gut/Liver MPS.

Midazolam was added as either an oral dose (50 μ M) in the apical side of the Transwell, within the gut compartment, or as an IV dose (5 μ M) directly mixed with circulating media in the liver compartment. Two configurations of the Gut/Liver MPS were studied: Caco-2/Liver and RepliGut/Liver. Media samples were taken over 48 hours and quantified by LC-MS to determine the **A)** concentration of Midazolam in the circulating media following different dosing regimens and the **B)** total molar fraction of Midazolam remaining across all compartments in each model at 48 hours. **C)** We estimated oral bioavailability with both Gut/Liver MPS models by taking the ratio of the area under the curves and dose and compared with human and animal data in published literature¹.



Conclusion



This study demonstrated that the primary RepliGut/Liver MPS more accurately recapitulates the physiological conditions of oral drug dosing in the human. By combining intestinal absorption and hepatic metabolism, the primary RepliGut/Liver MPS generates *in vivo*-like drug concentrations that cannot be replicated using standard preclinical *in vitro* models. The primary RepliGut/Liver MPS offers a more accurate method to study the pharmacokinetics of pro-drugs that undergo CES metabolism compared to an equivalent Caco-2 Gut/Liver MPS, which failed to profile their human bioavailability. The results demonstrate that the primary MPS model offers a viable alternative to circumvent the human-relevance limitations of the Caco-2 cell line for this drug type. By generating more human-relevant data earlier in drug discovery, means that observed issues can be flagged and addressed before costly preclinical *in vivo* studies.

As the primary RepliGut/Liver MPS is entirely made up of primary human cells, there are no interspecies differences to account for, therefore, the model can be utilized to help overcome the poor correlation between animal model ADME predictions and human outcomes. Bridging the gap between *in vitro* assays and *in vivo* studies, the model enables researchers to confidently progress only the most promising drug candidates to support reductions in cost and the number of animals required.

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