

Perfluoropolyether-Based Gut-Liver-on-a-Chip for the Evaluation of First-Pass Metabolism and Oral Bioavailability of Drugs

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Abstract

In the evolving field of drug discovery and development, multi-organs-on-a-chip and microphysiological systems, are gaining popularity owing to their ability to emulate in vivo biological environments. Among the various gut-liver-on-a-chip systems for studying oral drug absorption, the chip developed in this study stands out with two distinct features: incorporation of perfluoropolyether (PFPE) to effectively mitigate drug sorption and a unique enterohepatic single-pass system, which simplifies the analysis of first-pass metabolism and oral bioavailability. By introducing a bolus drug injection into the liver compartment, hepatic extraction alone could be evaluated, further enhancing our estimation of intestinal availability. In a study on midazolam (MDZ), PFPE-based chips showed more than 20-times the appearance of intact MDZ in the liver compartment effluent compared to PDMS-based counterparts. Notably, saturation of hepatic metabolism at higher concentrations was confirmed by observations when the dose was reduced from 200 μ M to 10 μ M. This result was further emphasized when the metabolism was significantly inhibited by the coadministration of ketoconazole. Our chip, which is designed to minimize the dead volume between the gut and liver compartments, is adept at sensitively observing the saturation of metabolism and the effect of inhibitors. Using genome-edited CYP3A4/UGT1A1-expressing Caco-2 cells, the estimates for intestinal and hepatic

availabilities were 0.96 and 0.82, respectively; these values are higher than the known human in vivo values. Although the metabolic activity in each compartment can be further improved, this gut-liver-on-a-chip can not only be used to evaluate oral bioavailability, but also to carry out individual assessment of both intestinal and hepatic availability.

1. Introduction

Oral bioavailability, a critical parameter for confirming the therapeutic efficacy of orally administered drugs, relies on the interplay between the absorption processes within the gastrointestinal tract and first-pass metabolism in the liver. The clinical development of numerous drug candidates has been hindered by their poor oral bioavailability. As a result, reliable predictive methods are needed to assess human oral bioavailability during the preclinical phase. Conventionally, such predictive strategies are based on the comprehensive exploration of in silico, in vitro, and in vivo animal models¹⁻¹⁰. Given the ethical considerations surrounding animal welfare, interspecies variations, and the limitations of these models, an integrative approach employing physiologically based pharmacokinetic models and in vitro experimental data, such as cellular permeability and microsomal metabolism, has been frequently adopted^{1-3, 11-14}.

The landscape of ADME/Tox studies is currently being reshaped by the growing interest in microphysiological systems¹⁵⁻²¹, which offer numerous advantages over traditional culture systems such as Transwell. For instance, these systems enable the simulation of tissue structures and fluid dynamics, which closely resemble physiological conditions. We are actively developing and characterizing an organ-on-a-chip that mimics various organs (e.g., the liver²²⁻²⁴,

kidney^{25, 26}, intestine²⁷, and tumors²⁸) to assess ADME/Tox and drug delivery systems. Current research has not only pivoted to replicate the physiological functions of individual organs, but also to explore inter-organ interactions facilitated by microfluidic channels^{19, 20, 29-34}. The gut-liver-on-a-chip has been used to examine drug metabolism³⁵⁻⁴⁰ and the intricate network of inflammatory inter-tissue crosstalk⁴¹⁻⁴³. The use of small-volume microfluidic channels to connect cells provides the distinct advantage of reducing the dilution of secreted humoral factors in the culture medium, which influences cellular function. Moreover, it is equally critical to minimize the loss of lipophilic medium components, secreted end products, and drug molecules owing to sorption onto the chip platform. Despite its widespread use due to ease of processing, polydimethylsiloxane (PDMS) is associated with extensive chemical sorption⁴⁴⁻⁴⁶. In contrast, perfluoropolyether (PFPE)-based elastomers, as demonstrated in our recent investigations, might be a more suitable material for the fabrication of an organ-on-a-chip²².

In this study, we designed a PFPE-based gut-liver-on-a-chip to facilitate the exploration of first-pass metabolism and oral bioavailability of drugs. The system was composed of a co-culture of genome-edited Caco-2 cells, tagged as CYP3A4-POR-UGT1A1-KI Caco-2 cells (Caco-2-3PU) owing to the engineered expression of CYP3A4, P450 oxidoreductase, and UGT1A1⁴⁷, along with human primary hepatocytes. This sophisticated gut-liver-on-a-chip model enabled us to examine drug metabolism during intestinal absorption and in the liver. Drugs permeating the epithelial layer are transported to the liver compartment via subepithelial microchannel fluid flow. By quantifying the drug concentrations in the liver compartment effluent, we evaluated both first-pass metabolism and bioavailability. Moreover, by incorporating pulse dosing experiments into the liver compartment inlet, we could separately evaluate the intestinal and hepatic metabolic processes. Using the assessment and analysis techniques developed in this

study, we explored the potential pharmacokinetic drug-drug interactions following oral co-administration of perpetrator and victim drugs using the gut-liver-on-a-chip.

2. Materials and Methods

2.1. Materials

Midazolam (MDZ), 1'-hydroxymidazolam (1'-OH MDZ), and ketoconazole were purchased from FUJIFILM Wako Pure Chemicals (Osaka, Japan), Cayman Chemical (Ann Arbor, MI), and Tokyo Research Chemicals (Tokyo, Japan), respectively. Dulbecco's modified Eagle's medium (DMEM), a penicillin-streptomycin mixed solution, and phosphate-buffered saline (PBS) were obtained from Nacalai Tesque (Kyoto, Japan). Other consumables, including William's E medium, fetal bovine serum (FBS), nonessential amino acids, K8200 OptiPlate hepatocyte medium, and CM-4000 cell maintenance supplement, were purchased from Thermo Fisher Scientific Inc (Waltham, MA). Additionally, Matrigel and rat-tail collagen type I were purchased from Corning (Glendale, AZ).

2.2. Fabrication of the Gut-Liver-on-a-Chip

Our gut-liver-on-a-chip was conceptualized and fabricated using a PFPE elastomer (SIFEL X-71-8115A/B Shin-Etsu Chemical, Tokyo, Japan). A schematic diagram of the chip mold arrangement is shown in Figure 1A. The microchannels had a width of 1 mm and height of 260 μm , and fabricated using photolithography on a silicon wafer with SU-8 2100 (MicroChem, Westborough, MA). According to previously reported protocols, the master mold was replicated with epoxy resins (Epo-Tek OG142-87, Epoxy Technology, Billerica, MA), and the features were transferred to the PFPE layers through a soft lithography process. A PFPE layer, perforated with six 5-mm diameter holes at guide points, was face-to-face bonded with another PFPE layer

sandwiching the porous polyethylene terephthalate (PETE) membranes, and then baked at 120 °C for 4 h. Polytetrafluoroethylene tubes (TOMBO No. 9003-PFA-SG, Nichias, Tokyo, Japan) with a 4 mm ID, 5 mm OD, and ~8 mm cut length were inserted into each hole to secure the height of the medium reservoirs. A schematic diagram and photograph of the fabricated chip are presented in Figure 1B.

For comparison, PDMS-based chips were also fabricated. The soft lithography process mirrored that used for PFPE-based chips, except for variations in the curing temperature and time, which were set at 60 °C and 6 h, respectively.

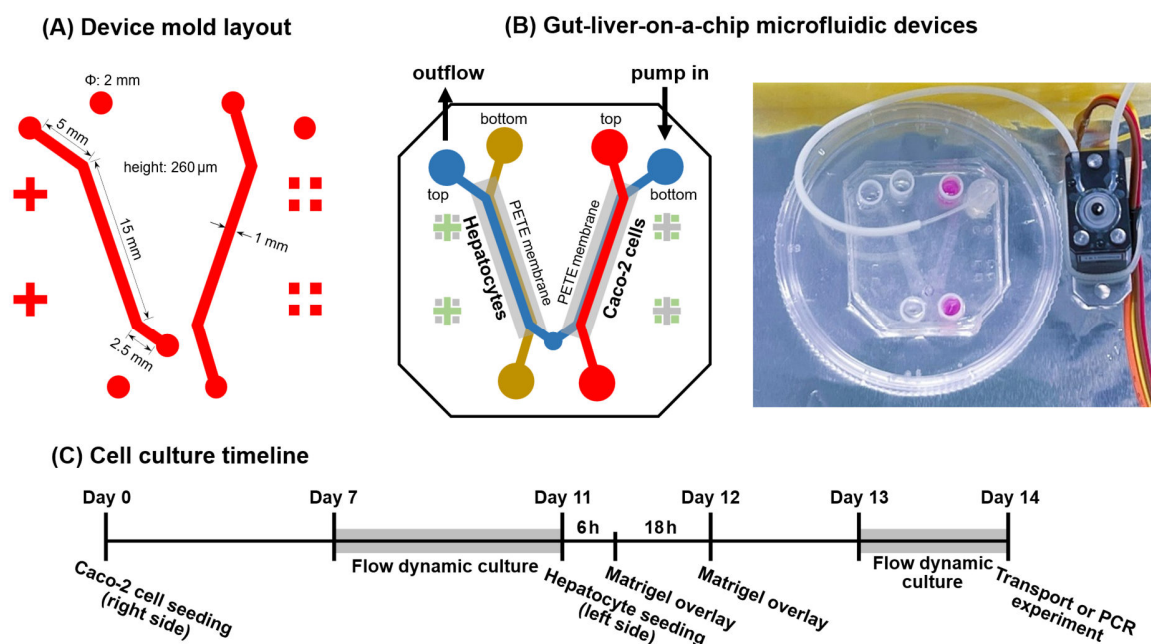


Figure 1. Mold layout (A) and appearance (B) of the current gut-liver-on-a-chip and cell culture timeline (C). When two blocks, created from the mold shown in Figure 1A, are placed facing each other with a PETE film in between and bonded, the chip shown on the left side of Figure 1B is created. The bottom channel on the right side is designed to be connected with the top channel on the left side. Caco-2 cells are injected into the top channel (red, in Figure 1B) of the right side on day 0 and cultured for 14 days. On day 11, human hepatocytes are injected from the mid-junction towards the left channel and cultured. If the drug is administered to the gut-top channel, it will

pass through the Caco-2 cell layer and be transported from the bottom channel by the liquid flow towards the hepatocytes. This simulates the process of a drug being absorbed in the intestine and subsequently passing through the liver.

2.3. Cell Culture on the Gut-Liver-on-a-Chip

Caco-2-3PU and wild type Caco-2 cells (Caco-2-WT) were maintained in high-glucose DMEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% nonessential amino acids in a humidified 37 °C and 5% CO₂ incubator.

The gut microchannels were primed with 15 µL of the coating solution, which comprised 40 µL Matrigel and 40 µg rat tail collagen type I in 0.8 mL PBS. A cell suspension (100,000 cells in 20 µL) was delicately introduced into the gut-top microchannel and incubated for 60 min at 37 °C under 5% CO₂. The culture medium (100 µL) was then added to each interconnected reservoir, and the chip was returned to the incubator. The medium was refreshed every second day. During days 7 to 11, the chip was placed on a seesaw rocking shaker (OrganoFlow[®], Mimetas, Oegstgeest, The Netherlands), set to operate in 30-min cycles with a peak slope of 7°.

On day 11, after the gut-liver coupling channel was rinsed twice with pre-warmed PBS, 15 µL of the coating solution was injected toward the liver compartment. Two hours later, primary human hepatocytes (Lot. HC1-30, XenoTech, Kansas City, KS) were thawed according to the supplier's protocol and suspended in K8200 OptiPlate hepatocyte medium. These cells (75,000 cells in 15 µL) were slowly administered from the channel mid-junction toward the liver compartment, and then incubated for 1 h. For each of the gut-liver coupling channel and liver-bottom channel, 100 µL of the hepatocyte plating medium was added to one of the interconnected reservoirs. After 6 h, the OptiPlate hepatocyte medium was substituted with William's E medium containing a CM-4000 cell maintenance supplement. After 18 h (day 12),

the William's E medium was replaced with culture medium containing 0.25 mg/mL Matrigel for sandwich culture. The chip was returned to the seesaw-rocking shaker on day 13, marking the initiation of dynamic cell culture, set for a 3-min cycle with a maximum slope of 12°.

A supplementary set of experiments was performed to elucidate the impact of co-culturing with intestinal cells on hepatic metabolism. Briefly, hepatocytes were cultured on a gut-liver-on-a-chip. To prevent leakage of the medium into the gut-top microchannel, a specialized chip equipped with thin PFPE-coated PETE membrane was used. In the absence of Caco-2 cells, hepatocytes were inoculated and maintained on an equivalent timeline as performed in previous experiments.

2.4. Evaluation of Oral Bioavailability using Gut-Liver-on-a-Chip

Oral bioavailability investigations were performed on day 14, using the gut-liver-on-a-chip. The initial culture medium in the chip were replaced with the corresponding basal medium. In particular, DMEM basal medium was used in the gut-top channel, while William's E basal medium was used in the remaining two channels. The gut-liver coupling channel underwent a single-pass perfusion with William's E basal medium regulated at a flow rate of 50 $\mu\text{L/h}$ via a peristaltic micropump.

After a 30-min stabilization period, 1 μL of 10 μM MDZ was pulse injected into the mid-junction of the gut-liver coupling channel using a microliter syringe (Hamilton Company, Bonaduz, Switzerland). Liver compartment effluents were collected 1 and 1.5 h post-injection, weighed, and analyzed to quantify the MDZ and 1'-OH MDZ contents using LC/MS/MS, adhering to previously reported conditions²². The hepatic extraction ratio (E_H) and clearance (CL_H) were computed using the following formulas:

$$E_H = \frac{X_{met}}{X_{intact} + X_{met}} \quad (1)$$

$$CL_H = Q \cdot E_H \quad (2)$$

where X_{intact} and X_{met} are the quantities of intact MDZ and 1'-OH MDZ that appeared within 1 h, respectively, and Q is the flow rate; quantities emerging between 1 and 1.5 h were considered negligible.

After an additional 30-min runtime and effluent medium removal, half of the gut-top channel medium was exchanged with an equal volume of medium containing either 20 or 400 μ M MDZ. For one group, the gut-top channel medium contained 50 μ M ketoconazole. The effluent from the liver compartment was collected hourly. Upon completion of the 4 h experiment, the gut-top channel medium was used to determine the quantity of 1'-OH MDZ.

Generally, oral bioavailability (F) of a drug is expressed as:

$$F = F_a \times F_g \times F_H \quad (3)$$

where F_a is the fraction of the absorbed dose; F_g is the fraction of the drug bypassing the gut wall without metabolism, and F_H is the fraction of the unmetabolized drug passing through the liver. Within the context of this gut-liver-on-a-chip system, F_a should be equal to unity, as the gut-top channel operates in a closed system. As the metabolites produced in intestinal cells are secreted into both the gut-top and gut-bottom channels, bioavailability was calculated using the following formula:

$$F = F_g \times F_H = \frac{V_{intact,H}}{V_{intact,H} + V_{met,H} + V_{met,G}} \quad (4)$$

where $V_{intact,H}$ and $V_{met,H}$ are the effluent appearance rates for intact MDZ and 1'-OH MDZ, respectively, and $V_{met,G}$ is the average appearance rate of the metabolite within the gut-top channel medium over a 4-h period. Since F_H is equal to $1 - E_H$, F_g can be obtained using Eqs. 1 and 4.

2.5. Influence of Co-Culture on Hepatocyte Gene Expression

The potential impact of co-culture with Caco-2-WT or Caco-2-3PU cells on hepatocyte gene expression was assessed using real-time polymerase chain reaction (RT-PCR). On day 14, the PETE membrane in the liver compartment was carefully retrieved from the chip after removal of the culture media and a subsequent double wash with prewarmed PBS. The extracted membrane was immersed and agitated in 70 μ L of lysis buffer from the RNeasy Micro Kit (Qiagen, Hilden, Germany). Total RNA was extracted, purified, and reverse-transcribed into cDNA using an RT Reagent Kit (Takara Bio, Shiga, Japan). Real-time quantitative PCR was conducted on a Step-one Sequence Detection system, following a protocol reported previously²².

2.6. Statistical Analyses

The data are presented as mean $\pm$ SE of at least three independent experiments. Statistical significance was assessed using one-way analysis of variance (ANOVA), followed by a post-hoc Tukey-Kramer test.

3. Results

3.1. Comparison between PDMS and PFPE

In this preliminary experiment, we evaluated the suitability of PFPE for the fabrication of a gut-liver-on-a-chip. MDZ, a widely recognized substrate of CYP3A4, was used in this study. Figures 2A and 2B present the kinetic profiles of intact MDZ and its metabolite, 1'-OH MDZ, in the liver compartment effluent following the administration of 200 μ M MDZ to the gut-top channel. Notably, the appearance rate of MDZ in PFPE-based chips was markedly higher, i.e., 21.5-fold than that in PDMS-based chips. We further assessed the metabolite-to-parent ratio, which denotes the ratio of 1'-OH MDZ to intact MDZ. The observed values were 0.043 for the

PFPE-based chips and 0.098 for the PDMS-based chips, indicating a lower ratio for the PFPE-based chips.

Figure 2C shows the concentration of MDZ remaining in the gut-top channel medium at the end of the experiment. A marked reduction in the concentration of MDZ in the gut-top donor medium was observed in the PDMS-based chips, a phenomenon that could not be entirely attributed to transepithelial absorption when mass balance is considered (Table 1). This result suggests pronounced sorption of MDZ by the PDMS resins, an effect not observed with the PFPE resins. Overall, this finding highlights the utility of PFPE over PDMS in drug absorption and metabolism studies using the gut-liver-on-a-chip.

The observed low metabolite-to-parent ratio in the PFPE-based chips compared to the PDMS-based chips may be attributable to the saturation of the metabolic process. This finding could also be attributed to the substantially higher concentration of intact MDZ observed in the PFPE-based chips. To mitigate this effect, subsequent studies using the PFPE-based chips were conducted at a reduced MDZ dosing concentration of 10 μM .

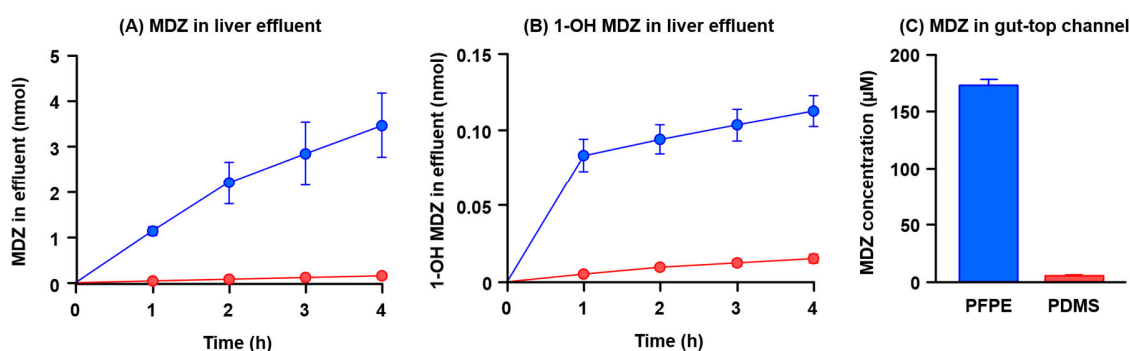


Figure 2. Effect of the fabrication materials, namely PFPE and PDMS, on the kinetic profiles of midazolam (MDZ) in the gut-liver-on-a-chip. Primary human hepatocytes (PHHs) were co-cultured with wild type Caco-2 cells (Caco-2-WT) on the chip. Figures 2A and 2B show the time-course of MDZ and 1'-hydroxymidazolam (1'-OH MDZ) appearing in the liver

compartment effluent, represented by blue for PFPE and red for PDMS, respectively. Figure 2C shows the concentration of MDZ remaining in the gut-top donor medium at the end of the experiments. All data are presented as mean $\pm$ SE (n = 4).

3.2. Investigation of Intestinal Metabolism and Simultaneous Perpetrator Presence

Although Caco-2 cells are known for their limited CYP metabolic activities, the introduction of genome-edited Caco-2 cells, i.e., Caco-2-3PU⁴⁷, enabled the creation of a gut-liver-on-a-chip exhibiting metabolic activities in both the gut and liver compartments. On the other hand, ketoconazole, a well-known CYP3A4 inhibitor, was utilized to emulate oral drug-drug interactions.

Table 1. Appearance of midazolam (MDZ) and its metabolite (1'-OH MDZ) in the liver compartment effluent of the gut-liver-on-a-chip.

No.	Gut cell	Dose conc ^{a)} (μ M)	Material	Perpetrator	Appearance rate in the effluent ^{b)}			Gut-top conc at 4 h		Total Recovery (%)
					MDZ (pmol/h)	1'-OH MDZ (pmol/h)	1'-OH MDZ ratio (%) ^{c)}	MDZ (μ M)	1'-OH MDZ (μ M)	
1	Caco-2-WT	200	PDMS	—	39.7 $\pm$ 5.6	3.82 $\pm$ 0.66	8.9 $\pm$ 1.5 [§]	4.56 $\pm$ 0.77	N.D. ^{d)}	3.1 $\pm$ 0.6
2	Caco-2-WT	200	PFPE	—	863.2 $\pm$ 201.4	24.46 $\pm$ 2.06	3.2 $\pm$ 0.6	173.0 $\pm$ 5.6	N.D. ^{d)}	104.4 $\pm$ 11.6
3	Caco-2-WT	10	PFPE	—	53.9 $\pm$ 2.7	6.63 $\pm$ 0.16	11.0 $\pm$ 0.5 ^{§,¶}	4.42 $\pm$ 0.20	N.D. ^{d)}	69.4 $\pm$ 3.9
4	Caco-2-3PU	10	PFPE	—	48.1 $\pm$ 7.0	9.37 $\pm$ 1.19	16.5 $\pm$ 1.1	4.87 $\pm$ 0.09	0.013 $\pm$ 0.01	72.7 $\pm$ 7.8
5	Caco-2-3PU	10	PFPE	ketoconazole	46.9 $\pm$ 5.2	0.76 $\pm$ 0.04	17.0 $\pm$ 0.2 [¶]	5.18 $\pm$ 0.58	N.D. ^{d)}	71.4.4 $\pm$ 13.4

a) Initial dose concentration of MDZ administered to the gut-top medium.

b) The appearance rates of MDZ and 1'-OH MDZ in the liver compartment effluent correspond to the slopes of the kinetic profiles shown in Figures 1 and 2.

c) Ratio of 1'-OH MDZ to the sum of MDZ and 1'-OH MDZ in the liver compartment effluent. The superscripts, § and ¶, indicate significant differences from conditions 2 and 4, respectively (p<0.05).

d) Not detected.

e) The limits of quantitation for MDZ and 1'-OH MDZ were 2 nM.

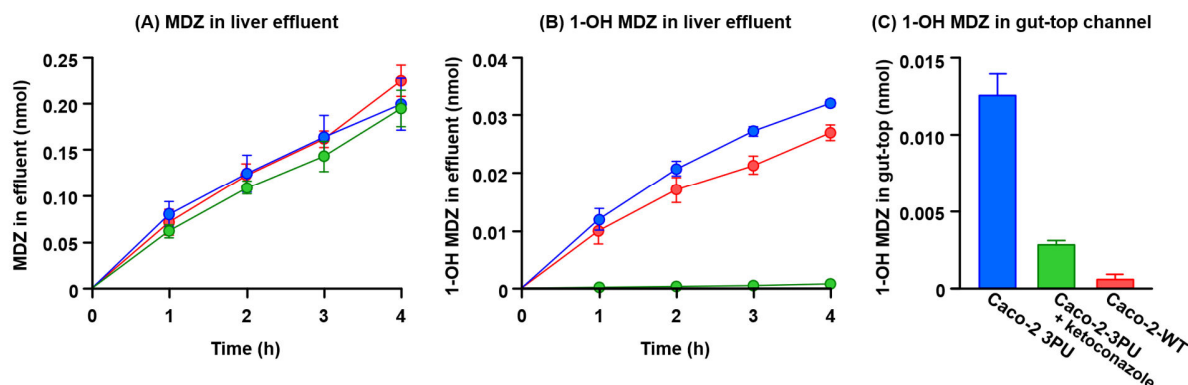


Figure 3. Effect of intestinal CYP3A4 expression and ketoconazole co-administration on the kinetic profiles of midazolam in the gut-liver-on-a-chip. Primary human hepatocytes (PHHs) were co-cultured with CYP3A4-POR-UGT1A1-KI Caco-2 cells (Caco-2-3PU, blue color) or wild type Caco-2 cells (Caco-2-WT, red color). Absorption and metabolism in Caco-2-3PU were also investigated in the presence of 50 μ M ketoconazole in the gut-top donor medium (green color). Figures 3A and 3B represent the time-course of midazolam (MDZ) and 1'-hydroxymidazolam (1'-OH MDZ) appearing in the hepatic outflow. Figure 3C represents the appearance of 1'-OH MDZ in the gut-top medium at the end of the experiments. All data are presented as mean $\pm$ SE ($n = 4$).

Figure 3 shows the kinetic profiles of the quantities of MDZ and 1'-OH MDZ appearing in the liver compartment effluent under three distinct conditions: Caco-2-WT, Caco-2-3PU, and Caco-2-3PU with co-administration of 50 μ M ketoconazole. The appearance rate of MDZ in the effluent was similar across all three groups (Figure 3A). When Caco-2-3PU was employed instead of Caco-2-WT cells, a slight increase in the appearance of 1'-OH MDZ was observed, which was essentially eliminated by the co-administration of ketoconazole (Figure 3B). Table 1 summarizes the appearance rates of MDZ and 1'-OH MDZ in the liver compartment effluent, which correspond to the slopes of the kinetic profiles shown in Figures 1 and 2. When the initial

dose concentration decreased from 200 μM to 10 μM , the relative appearance of 1'-OH MDZ significantly increased, suggesting that hepatic metabolism becomes saturated at higher concentrations.

Interestingly, 1'-OH MDZ was detected in the gut-top medium in Caco-2-3PU cells (Figure 3C), implying that the metabolite was produced in CYP3A4-expressing cells and subsequently secreted into the intestinal luminal side. Notably, the difference in the quantity of 1'-OH MDZ secreted into the gut-top medium between the Caco-2-3PU and Caco-2-WT experiments exceeded the corresponding difference observed in the liver compartment effluent. Of note, the amount of 1'-OH MDZ found in the liver compartment effluent represents the cumulative metabolite production in both the gut and liver compartments. As metabolite production in the liver compartment is unaffected by Caco-2-3PU or Caco-2-WT cells, any observed variation in the liver compartment effluent can be attributed to differences in intestinal metabolism. As the differential quantity of 1'-OH MDZ secreted in the gut-top medium between Caco-2-3PU and Caco-2-WT cells exceeded that in the liver compartment effluent, the metabolites produced in epithelial cells may favor secretion toward the apical side. Nonetheless, these results alone do not rule out changes in the metabolic activity of hepatocytes due to the co-culture of different Caco-2 cells.

3.3. Determination of Intestinal Availability (F_g) and Hepatic Availability (F_H)

To ascertain whether the co-culture of intestinal cells influences the metabolic functions of hepatocytes within the gut-liver-on-a-chip, the absorption and metabolic processes must be kinetically segregated. We postulated that a bolus injection at the inlet of the liver compartment would facilitate the evaluation of the metabolic activity of hepatocytes toward MDZ during a single passage. An experiment employing a bolus injection was conducted prior to the intestinal

absorption experiment described earlier. Figure 4 illustrates the hepatic extraction ratio (E_H) of MDZ or $1 - F_H$, which was calculated from the recovery of MDZ and 1'-OH MDZ in the liver compartment effluent after bolus injection into the mid-junction of the gut-liver coupling channel. Compared to hepatocytes alone, a minor, albeit not statistically significant, reduction in E_H was observed when hepatocytes were co-cultured with Caco-2 cells; however, no discernible difference was found between co-culture with Caco-2-WT and Caco-2-3PU cells. The administration of ketoconazole to the gut-top medium 30 min prior to the bolus injection resulted in E_H approaching zero, indicating substantial penetration of ketoconazole through the Caco-2 cell layers and subsequent inhibition of the metabolic activity of hepatocytes toward MDZ.

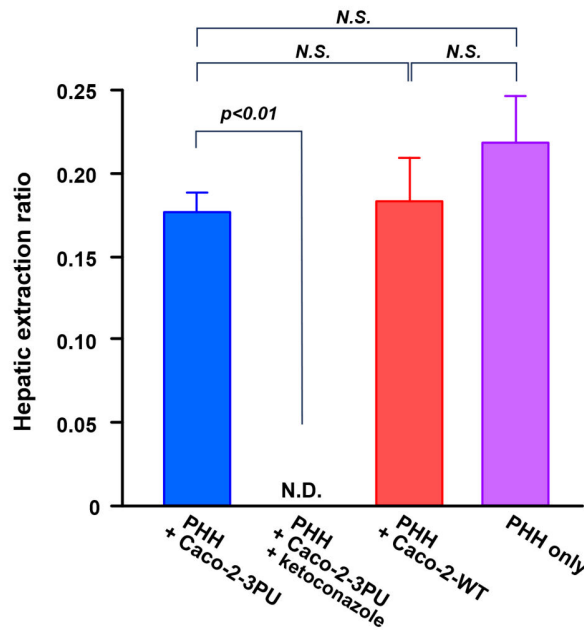


Figure 4. Hepatic extraction of midazolam (MDZ) following a bolus microinjection in the gut-liver-on-a-chip. MDZ was injected into the mid-junction of the gut-liver coupling microchannel of the chip. The hepatic extraction ratio for primary human hepatocytes (PHHs) co-cultured with Caco-2-3PU or Caco-2-WT was compared with that for PHH only. Data are expressed as the mean $\pm$ SE of four samples.

Table 2. Availability of midazolam in first-pass metabolism using the gut-liver-on-a-chip chip.

	Perpetrator	Bioavailability ^{a)} (F)	Intestinal availability ^{a)} (F_g)	Hepatic availability ^{a)} (F_H)
Caco-2-WT	—	0.888±0.012	1.09±0.078	0.817±0.052
Caco-2-3PU	—	0.789±0.035	0.958±0.045	0.824±0.023
Caco-2-3PU	ketoconazole	0.968±0.009	0.968±0.009	1 ^{b)}

a) The availability was calculated using Eqs. 1–4. Of note, F_H equals $1 - E_H$.

b) As ketoconazole treatment prevented the appearance of 1'-OH MDZ after a bolus injection of MDZ in the liver compartment, F_H was estimated to be unity.

The bioavailability (F) of MDZ was estimated from the rate balance at a quasi-steady state, based on the assumption that the steady-state disappearance from the system comprised volumetric elution of MDZ and 1'-OH MDZ from the liver compartment and efflux of 1'-OH MDZ into the gut-top medium. In addition, the fraction absorbed was theoretically assumed to be unity, as the gut-top channel constituted a closed system. Table 2 summarizes the intestinal and hepatic availabilities, and bioavailability of MDZ under various conditions. The F_g value was close to unity, even when metabolite secretion into the gut-top medium was observed with Caco-2-3PU cells. For Caco-2-WT, the F_g value exceeded unity, because the F value was higher than the F_H value. As F_H was deduced from bolus microinjection experiments performed prior to the absorption experiments, this result may be attributed to a decrease in hepatocyte metabolic activity over the duration of the experiment. However, the kinetic profiles of 1'-OH MDZ in the liver compartment effluent were somewhat convex but largely linear over time (Figure 3B), indicating that any potential loss in activity was not pronounced. Therefore, the significantly

smaller F_H than F_g suggests that the liver compartment is primarily responsible for the metabolism of MDZ in the gut-liver-on-a-chip.

3.4. Influence of Co-Culture on Hepatocyte Gene Expression

Real-time PCR was performed to investigate prospective alterations in the gene expression of hepatocytes that could be associated with their co-culture with Caco-2-3PU in the gut-liver-on-a-chip. The study focused on a group of genes associated with hepatic metabolism, specifically α 1-antitrypsin, albumin, hepatocyte nuclear factor 4 α (HNF4 α), MRP2, CYP1A2, CYP2C9, CYP2C19, CYP3A4, CYP3A5, and UGT1A1. Besides CYP2C19, no statistically significant difference in gene expression was observed relative to co-culture with Caco-2-3PU (Figure 5). Thus, the magnitude of these changes was insignificant, and no clear pattern emerged.

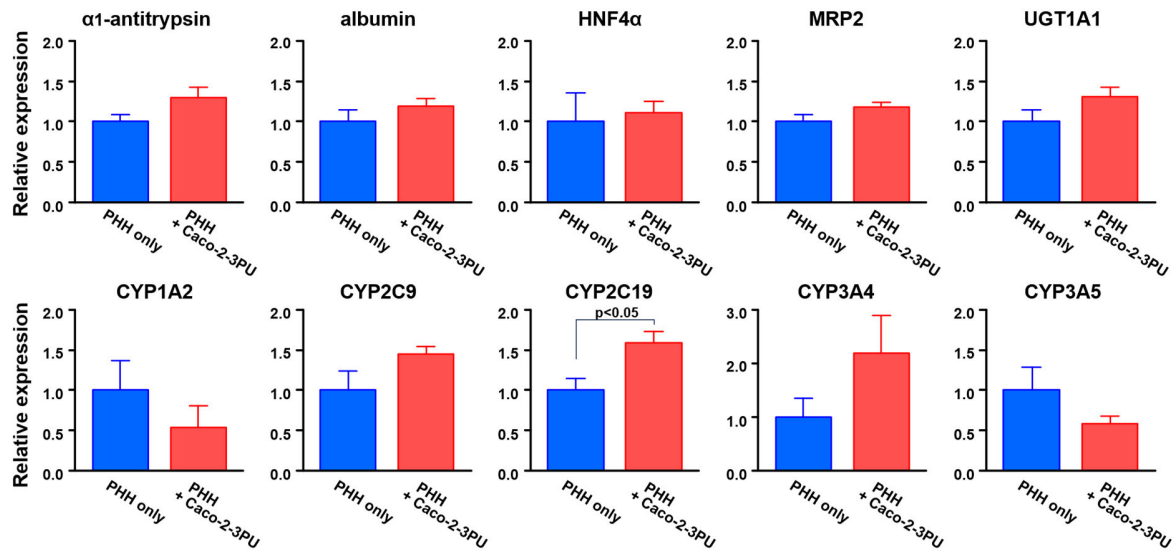


Figure 5. Gene expression of primary human hepatocytes (PHHs) cultured in the gut-liver-on-a-chip. Comparisons were made with and without co-culture with Caco2-3PU. Gene expression of each hepatic marker was corrected based on that of GAPDH, and further normalized by the relative expression in the PHH only group. All data are presented as mean $\pm$ SE (n = 4).

4. Discussion

Compared with previous developed chips³⁸⁻⁴², the gut-liver-on-a-chip fabricated in this study is noteworthy, particularly in its specialized ability to evaluate the first-pass effects of drugs. A key factor in its functionality is the minimization of the dead volume arising from the connection between the gut and liver compartments. Physiologically, approximately 80% of the blood flowing into the liver is portal blood⁴⁸. Therefore, substances secreted or absorbed from the gut into the bloodstream may be channeled directly to the liver with minimal dilution by the hepatic arterial blood. When a substantial amount of a drug is absorbed orally, its hepatic metabolism can reach saturation, leading to increased oral bioavailability⁴⁹⁻⁵¹. Another crucial design feature of our gut-liver-on-a-chip is its open system, which ensures that drugs pass through the liver compartment only once. Oral bioavailability can be directly evaluated by sampling and analyzing the effluent from the liver compartment. Circulating drugs can re-enter the liver in living systems; however, because their concentration is diluted owing to their distribution throughout the body, the impact of this re-entered drug on the hepatic first-pass effect is generally negligible. Most of the gut-liver-on-a-chips developed to date³⁸⁻⁴² employ closed systems in which drug metabolism recurs. Such systems may not always be optimal for evaluating oral bioavailability.

In the present study, MDZ was selected as the model drug. By reducing the initial dose concentration of MDZ in the gut-top channel from 200 μM to 10 μM , the ratio of the metabolite to the intact form in the liver compartment effluent significantly increased. This result suggests that at higher concentrations, the absorption rate of MDZ increases proportionally, leading to the saturation of its metabolism in the liver compartment. However, of note, this observation is only relevant when low-sorption PFPE is used as the chip material. The use of PDMS caused intense

sorption of MDZ during the experiment (Table 1), complicating the accurate evaluation of the absorption and metabolism kinetics.

A distinctive feature of the current gut-liver-on-a-chip is its ability to evaluate the hepatic metabolism of drugs by introducing the drug as a bolus injection into the liver compartment inlet. The estimated hepatic availability (F_H) of MDZ using this chip was approximately 0.8, which was higher than the human F_H of 0.56⁵². The F_H value is determined by the balance between flow rate and metabolic clearance. Halving the flow rate to double the passage time within the liver compartment or doubling the number of liver cells to increase metabolic clearance can achieve hepatic availability comparable to that under in vivo conditions. However, a significant concern is the decrease in the metabolic activity of hepatocytes during cultivation, a challenge that persists even with Matrigel overlay sandwich culture⁵³⁻⁵⁵ and fluid shear stress^{56, 57}. This concern has been a long-standing issue, and a solution to this inherent problem in primary hepatocyte culture is still anticipated.

In this study, kinetic analyses were conducted by measuring only the primary metabolite of MDZ, 1'-OH MDZ. However, it is also known that 1'-OH MDZ can undergo further glucuronidation, as well as MDZ undergoing N-glucuronidation^{58,59}. In a preliminary experiment, the effluent samples were thus divided and treated with glucuronidase⁶⁰ to assess the increase in intact and 1'-OH MDZ compared to untreated samples (Supplementary Figure S1). As a result, there was little observed increase in their amount thought to be due to deconjugation, suggesting the contribution of glucuronide conjugation be minimal in this experimental system. Since this experiment evaluates metabolism in a single pass, increases may not be adequately detected due to short contact time with cells. It is presumed that diffusion into the extracellular space surpassed subsequently occurring glucuronidation for 1'-OH MDZ. The use of kinetic data for

only intact and 1'-OH MDZ would be supported by the deconjugation experiments with glucuronidase.

Several studies have evaluated changes in hepatocyte gene expression and metabolic ability due to co-culture with gut cells. In this study, co-culture with Caco-2 cells did not significantly alter the metabolic clearance of MDZ (Figure 4). Moreover, no notable variations in gene expression were observed (Figure 5). Shinohara et al.³⁹ reported increased gene expression of various CYPs and UGTs when hepatocytes isolated from chimeric humanized liver mice (PXB mice) were co-cultured with intestinal cells derived from human iPS; however, the mechanism was not revealed. In contrast, Arakawa et al.³⁷, using the same gut-liver-on-a-chip as Shinohara et al.³⁹, found no effect of co-culture on CYP gene expression. Notably, Arakawa et al.³⁷ selected different cell types, namely Caco-2 and HepaRG cells, and a shorter co-culture duration³⁷. In our study, primary human hepatocytes were utilized, and owing to metabolic activity concerns, the co-culture duration was limited to three days. Owing to the use of Caco-2 cells, our experimental setup aligns more closely with that of Arakawa et al.³⁷ than Shinohara et al.³⁹.

Known *in vivo* interactions in the gut-liver axis include the regulation of CYP by gut bacterial metabolites⁶¹⁻⁶³ and the suppression of gluconeogenesis and bile acid synthesis by gut-derived FGF15/19^{64, 65}. Although the gene expression results did not reveal a strong humoral gut-liver interaction, our chip design, with minimized dead volume and the capability to deliver high concentrations of gut-derived substances to the liver compartment, holds potential for such investigations. We observed the inhibition of the hepatic metabolism of MDZ when co-administered with ketoconazole, a potent CYP3A4 inhibitor. Only few reports have highlighted drug-drug interactions in the gut-liver-on-a-chip, making the findings of this study particularly significant.

Caco-2 cells, which are often used as a model for drug absorption, are associated with challenges due to their low metabolic activity. Recently, Negoro et al.⁶⁶ introduced Caco-2-3PU, in which the CYP3A4 and UGT1A1 genes were incorporated using genome editing. Although not as high as that in the human small intestine, Caco-2-3PU cells show significant metabolic activity, which is entirely absent in Caco-2-WT⁶⁶. Using Caco-2-3PU cells in our gut-liver-on-a-chip resulted in an increase in 1'-OH MDZ in the liver compartment effluent compared to that observed with Caco-2-WT cells. Further, we detected a significant amount of the metabolite in the gut-top channel. However, the calculated intestinal availability (F_g) was close to unity, suggesting that MDZ was primarily absorbed in its intact form. With oral bioavailability and hepatic availability (F_H) values of 0.30 and 0.56, respectively, for MDZ in humans⁵², assuming a fraction absorbed (F_a) of one yields a human F_g value of 0.54. The metabolic activity of Caco-2-3PU cells remained relatively low, indicating the need for further improvements of the small intestine model.

However, developing organs-on-a-chip that faithfully reproduce clinical pharmacokinetics appears challenging. Realistically, extrapolating results from organs-on-a-chip using scaling factors seems more feasible. Empirical methods, such as modeling the correlation between in vitro and in vivo data, could be considered for this purpose, yet human clinical data are far from sufficient. Utilizing mechanistic approaches, akin to physiologically based pharmacokinetic models, is imperative³⁷. Hence, it is crucial to dissect individual processes even in evaluations conducted with organs-on-a-chip. We propose a method to separately evaluate intestinal absorption and hepatic metabolism by integrating the administration method into the gut-liver chip system. Such an approach to devising dosing modes undoubtedly proves beneficial for extracting information from organs-on-a-chip.

5. Conclusion

Although the metabolic activity within the gut and liver compartments can be improved, our gut-liver-on-a-chip can not only be used to evaluate oral bioavailability, but through combination with different administration methods, can be used to carry out individual assessment of both intestinal and hepatic availability. Moreover, this chip confirmed the inhibition of hepatic metabolism when co-administered with metabolic inhibitors, suggesting its potential for evaluating drug-drug interactions.

SUPPORTING INFORMATION.

Method S1. Evaluation of glucuronide conjugation in the gut-liver-on-a-chip

Table S1. HPLC conditions in LC-MS/MS

Table S2. Ionization mode and multiple-reaction monitoring (MRM) transition in LC-MS/MS

Table S3. Primers used for qPCR

Figure S1. Contribution of glucuronidation to the absorption and metabolism of midazolam in the gut-liver-on-a-chip.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Parrott, N.; Lave, T. Applications of physiologically based absorption models in drug discovery and development. *Molecular Pharmaceutics* **2008**, *5* (5), 760-775. DOI: 10.1021/mp8000155.

(2) Jamei, M.; Turner, D.; Yang, J. S.; Neuhoff, S.; Polak, S.; Rostami-Hodjegan, A.; Tucker, G. Population-Based Mechanistic Prediction of Oral Drug Absorption. *Aaps Journal* **2009**, *11* (2), 225-237. DOI: 10.1208/s12248-009-9099-y.

(3) Darwich, A. S.; Neuhoff, S.; Jamei, M.; Rostami-Hodjegan, A. Interplay of Metabolism and Transport in Determining Oral Drug Absorption and Gut Wall Metabolism: A Simulation Assessment Using the "Advanced Dissolution, Absorption, Metabolism (ADAM)" Model. *Current Drug Metabolism* **2010**, *11* (9), 716-729.

(4) Wang, J. M.; Hou, T. J. Advances in computationally modeling human oral bioavailability. *Advanced Drug Delivery Reviews* **2015**, *86*, 11-16. DOI: 10.1016/j.addr.2015.01.001.

(5) Dahlgren, D.; Lennernas, H. Intestinal Permeability and Drug Absorption: Predictive Experimental, Computational and In Vivo Approaches. *Pharmaceutics* **2019**, *11* (8). DOI: 10.3390/pharmaceutics11080411.

(6) Cheng, K. C.; Li, C.; Uss, A. S. Prediction of oral drug absorption in humans - from cultured cell lines and experimental animals. *Expert Opinion on Drug Metabolism & Toxicology* **2008**, *4* (5), 581-590. DOI: 10.1517/17425255.4.5.581.

(7) Fedi, A.; Vitale, C.; Ponschin, G.; Ayehunie, S.; Fato, M.; Scaglione, S. In vitro models replicating the human intestinal epithelium for absorption and metabolism studies: A systematic review. *Journal of Controlled Release* **2021**, *335*, 247-268. DOI: 10.1016/j.jconrel.2021.05.028.

- (8) Galetin, A.; Gertz, M.; Houston, J. B. Potential role of intestinal first-pass metabolism in the prediction of drug-drug interactions. *Expert Opinion on Drug Metabolism & Toxicology* **2008**, *4* (7), 909-922. DOI: 10.1517/17425255.4.7.909.
- (9) Lenneras, H. Animal data: The contributions of the Ussing Chamber and perfusion systems to predicting human oral drug delivery in vivo. *Advanced Drug Delivery Reviews* **2007**, *59* (11), 1103-1120. DOI: 10.1016/j.addr.2007.06.016.
- (10) Harloff-Helleberg, S.; Nielsen, L. H.; Nielsen, H. M. Animal models for evaluation of oral delivery of biopharmaceuticals. *J Control Release* **2017**, *268*, 57-71. DOI: 10.1016/j.jconrel.2017.09.025 From NLM.
- (11) Heimbach, T.; Lakshminarayana, S. B.; Hu, W. Y.; He, H. D. Practical Anticipation of Human Efficacious Doses and Pharmacokinetics Using In Vitro and Preclinical In Vivo Data. *Aaps Journal* **2009**, *11* (3), 602-614. DOI: 10.1208/s12248-009-9136-x.
- (12) Heikkinen, A. T.; Baneyx, G.; Caruso, A.; Parrott, N. Application of PBPK modeling to predict human intestinal metabolism of CYP3A substrates - An evaluation and case study using GastroPlus (TM). *European Journal of Pharmaceutical Sciences* **2012**, *47* (2), 375-386. DOI: 10.1016/j.ejps.2012.06.013.
- (13) Malmborg, J.; Ploeger, B. A. Predicting human exposure of active drug after oral prodrug administration, using a joined in vitro/in silico-in vivo extrapolation and physiologically-based pharmacokinetic modeling approach. *Journal of Pharmacological and Toxicological Methods* **2013**, *67* (3), 203-213. DOI: 10.1016/j.vascn.2012.12.002.

(14) Olivares-Morales, A.; Kamiyama, Y.; Darwich, A. S.; Aarons, L.; Rostami-Hodjegan, A. Analysis of the impact of controlled release formulations on oral drug absorption, gut wall metabolism and relative bioavailability of CYP3A substrates using a physiologically-based pharmacokinetic model. *European Journal of Pharmaceutical Sciences* **2015**, *67*, 32-44. DOI: 10.1016/j.ejps.2014.10.018.

(15) Abaci, H. E.; Shuler, M. L. Human-on-a-chip design strategies and principles for physiologically based pharmacokinetics/pharmacodynamics modeling. *Integrative Biology* **2015**, *7* (4), 383-391. DOI: 10.1039/c4ib00292j.

(16) Fowler, S.; Chen, W. L. K.; Duignan, D. B.; Gupta, A.; Hariparsad, N.; Kenny, J. R.; Lai, W. G.; Liras, J.; Phillips, J. A.; Gan, J. P. Microphysiological systems for ADME-related applications: current status and recommendations for system development and characterization. *Lab on a Chip* **2020**, *20* (3), 446-467. DOI: 10.1039/c9lc00857h.

(17) Ishida, S. Organs-on-a-chip: Current applications and consideration points for in vitro ADME-Tox studies. *Drug Metabolism and Pharmacokinetics* **2018**, *33* (1), 49-54. DOI: 10.1016/j.dmpk.2018.01.003.

(18) Lee, S. H.; Choi, N.; Sung, J. H. Pharmacokinetic and pharmacodynamic insights from microfluidic intestine-on-a-chip models. *Expert Opinion on Drug Metabolism & Toxicology* **2019**, *15* (12), 1005-1019. DOI: 10.1080/17425255.2019.1700950.

(19) Prantil-Baun, R.; Novak, R.; Das, D.; Somayaji, M. R.; Przekwas, A.; Ingber, D. E. Physiologically Based Pharmacokinetic and Pharmacodynamic Analysis Enabled by Microfluidically Linked Organs-on-Chips. In *Annual Review of Pharmacology and Toxicology*,

Vol 58, Insel, P. A. Ed.; Annual Review of Pharmacology and Toxicology, Vol. 58; 2018; pp 37-64.

(20) Sung, J. H. Multi-organ-on-a-chip for pharmacokinetics and toxicokinetic study of drugs. *Expert Opinion on Drug Metabolism & Toxicology* **2021**, *17* (8), 969-986. DOI: 10.1080/17425255.2021.1908996.

(21) Ramadan, Q.; Fardous, R. S.; Hazaymeh, R.; Alshmmari, S.; Zourob, M. Pharmacokinetics-On-a-Chip: In Vitro Microphysiological Models for Emulating of Drugs ADME. *Adv Biol (Weinh)* **2021**, *5* (9), e2100775. DOI: 10.1002/adbi.202100775 From NLM.

(22) Wang, M.; Tsuda, M.; Deguchi, S.; Higuchi, Y.; So, K.; Torisawa, Y. S.; Takayama, K.; Yamashita, F. Application of perfluoropolyether elastomers in microfluidic drug metabolism assays. *Int J Pharm* **2022**, *627*, 122253. DOI: 10.1016/j.ijpharm.2022.122253 From NLM.

(23) Deguchi, S.; Tsuda, M.; Kosugi, K.; Sakamoto, A.; Mimura, N.; Negoro, R.; Sano, E.; Nobe, T.; Maeda, K.; Kusuvara, H.; et al. Usability of Polydimethylsiloxane-Based Microfluidic Devices in Pharmaceutical Research Using Human Hepatocytes. *Acs Biomaterials Science & Engineering* **2021**, *7* (8), 3648-3657. DOI: 10.1021/acsbmaterials.1c00642.

(24) Sano, E.; Deguchi, S.; Matsuoka, N.; Tsuda, M.; Wang, M. Y.; Kosugi, K.; Mori, C.; Yagi, K.; Wada, A.; Yamasaki, S.; et al. Generation of Tetrafluoroethylene-Propylene Elastomer-Based Microfluidic Devices for Drug Toxicity and Metabolism Studies. *Acs Omega* **2021**, *6* (38), 24859-24865. DOI: 10.1021/acsomega.1c03719.

(25) Ueno, R.; Kuninori, M.; Sumi, T.; Sadeghian, R. B.; Takata, Y.; Iguchi, A.; Tsuda, M.; Yamashita, F.; Ichikawa, K.; Yokokawa, R. Relationship between Adsorption and Toxicity of

Nephrotoxic Drugs in Microphysiological Systems (MPS). *Micromachines* **2023**, *14* (4). DOI: 10.3390/mi14040761.

(26) Banan Sadeghian, R.; Ueno, R.; Takata, Y.; Kawakami, A.; Ma, C.; Araoka, T.; Takasato, M.; Yokokawa, R. Cells sorted off hiPSC-derived kidney organoids coupled with immortalized cells reliably model the proximal tubule. *Commun Biol* **2023**, *6* (1), 483. DOI: 10.1038/s42003-023-04862-7.

(27) Sasaki, Y.; Tatsuoka, H.; Tsuda, M.; Sumi, T.; Eguchi, Y.; So, K. A. K.; Higuchi, Y.; Takayama, K.; Torisawa, Y.; Yamashita, F. Intestinal Permeability of Drugs in Caco-2 Cells Cultured in Microfluidic Devices. *Biological & Pharmaceutical Bulletin* **2022**, *45* (9), 1246-1253.

(28) Chantarasrivong, C.; Okada, R.; Yamane, Y.; Yang, X.; Higuchi, Y.; Konishi, M.; Komura, N.; Ando, H.; Yokokawa, R.; Yamashita, F. Disposition of E-selectin-targeting liposomes in tumor spheroids with a perfusable vascular network. *Drug Metabolism and Pharmacokinetics* **2022**, *47*. DOI: 10.1016/j.dmpk.2022.100469.

(29) Herland, A.; Maoz, B. M.; Das, D.; Somayaji, M. R.; Prantil-Baun, R.; Novak, R.; Cronic, M.; Huffstater, T.; Jeanty, S. S. F.; Ingram, M.; et al. Quantitative prediction of human pharmacokinetic responses to drugs via fluidically coupled vascularized organ chips. *Nat Biomed Eng* **2020**, *4* (4), 421-436. DOI: 10.1038/s41551-019-0498-9.

(30) Wikswo, J. P.; Curtis, E. L.; Eagleton, Z. E.; Evans, B. C.; Kole, A.; Hofmeister, L. H.; Matloff, W. J. Scaling and systems biology for integrating multiple organs-on-a-chip. *Lab Chip* **2013**, *13* (18), 3496-3511. DOI: 10.1039/c3lc50243k From NLM.

(31) Zhao, Y.; Kankala, R. K.; Wang, S. B.; Chen, A. Z. Multi-Organs-on-Chips: Towards Long-Term Biomedical Investigations. *Molecules* **2019**, *24* (4). DOI: 10.3390/molecules24040675.

(32) Maschmeyer, I.; Lorenz, A. K.; Schimek, K.; Hasenberg, T.; Ramme, A. P.; Hübner, J.; Lindner, M.; Drewell, C.; Bauer, S.; Thomas, A.; et al. A four-organ-chip for interconnected long-term co-culture of human intestine, liver, skin and kidney equivalents. *Lab Chip* **2015**, *15* (12), 2688-2699. DOI: 10.1039/c5lc00392j From NLM.

(33) Maass, C.; Stokes, C. L.; Griffith, L. G.; Cirit, M. Multi-functional scaling methodology for translational pharmacokinetic and pharmacodynamic applications using integrated microphysiological systems (MPS). *Integr Biol (Camb)* **2017**, *9* (4), 290-302. DOI: 10.1039/c6ib00243a.

(34) Picollet-D'hahan, N.; Zuchowska, A.; Lemeunier, I.; Le Gac, S. Multiorgan-on-a-Chip: A Systemic Approach To Model and Decipher Inter-Organ Communication. *Trends Biotechnol* **2021**, *39* (8), 788-810. DOI: 10.1016/j.tibtech.2020.11.014.

(35) Choe, A.; Ha, S. K.; Choi, I.; Choi, N.; Sung, J. H. Microfluidic Gut-liver chip for reproducing the first pass metabolism. *Biomed Microdevices* **2017**, *19* (1), 4. DOI: 10.1007/s10544-016-0143-2 From NLM.

(36) Tsamandouras, N.; Chen, W. L. K.; Edington, C. D.; Stokes, C. L.; Griffith, L. G.; Cirit, M. Integrated Gut and Liver Microphysiological Systems for Quantitative In Vitro Pharmacokinetic Studies. *AAPS J* **2017**, *19* (5), 1499-1512. DOI: 10.1208/s12248-017-0122-4.

(37) Arakawa, H.; Sugiura, S.; Kawanishi, T.; Shin, K.; Toyoda, H.; Satoh, T.; Sakai, Y.; Kanamori, T.; Kato, Y. Kinetic analysis of sequential metabolism of triazolam and its extrapolation to humans using an entero-hepatic two-organ microphysiological system. *Lab Chip* **2020**, *20* (3), 537-547. DOI: 10.1039/c9lc00884e From NLM.

(38) Marin, T. M.; Indolfo, N. C.; Rocco, S. A.; de Carvalho, M.; Dias, M. M.; Vasconcelos Bento, G. I.; Bortot, L. O.; Schuck, D. C.; Lorencini, M.; Pagani, E. An Intestine/Liver Microphysiological System for Drug Pharmacokinetic and Toxicological Assessment. *J Vis Exp* **2020**, (166). DOI: 10.3791/60184.

(39) Shinohara, M.; Arakawa, H.; Oda, Y.; Shiraki, N.; Sugiura, S.; Nishiuchi, T.; Satoh, T.; Iino, K.; Leo, S.; Kato, Y.; et al. Coculture with hiPS-derived intestinal cells enhanced human hepatocyte functions in a pneumatic-pressure-driven two-organ microphysiological system. *Sci Rep* **2021**, *11* (1), 5437. DOI: 10.1038/s41598-021-84861-y From NLM.

(40) Milani, N.; Parrott, N.; Ortiz Franyuti, D.; Godoy, P.; Galetin, A.; Gertz, M.; Fowler, S. Application of a gut-liver-on-a-chip device and mechanistic modelling to the quantitative in vitro pharmacokinetic study of mycophenolate mofetil. *Lab Chip* **2022**, *22* (15), 2853-2868. DOI: 10.1039/d2lc00276k.

(41) Chen, W. L. K.; Edington, C.; Suter, E.; Yu, J.; Velazquez, J. J.; Velazquez, J. G.; Shockley, M.; Large, E. M.; Venkataramanan, R.; Hughes, D. J.; et al. Integrated gut/liver microphysiological systems elucidates inflammatory inter-tissue crosstalk. *Biotechnol Bioeng* **2017**, *114* (11), 2648-2659. DOI: 10.1002/bit.26370 From NLM.

(42) Jeon, J. W.; Choi, N.; Lee, S. H.; Sung, J. H. Three-tissue microphysiological system for studying inflammatory responses in gut-liver Axis. *Biomedical Microdevices* **2020**, 22 (4). DOI: 10.1007/s10544-020-00519-y.

(43) Jeon, J. W.; Lee, S. H.; Kim, D.; Sung, J. H. In vitro hepatic steatosis model based on gut-liver-on-a-chip. *Biotechnol Prog* **2021**, 37 (3), e3121. DOI: 10.1002/btpr.3121.

(44) Toepke, M. W.; Beebe, D. J. PDMS absorption of small molecules and consequences in microfluidic applications. *Lab Chip* **2006**, 6 (12), 1484-1486. DOI: 10.1039/b612140c From NLM.

(45) van Meer, B. J.; de Vries, H.; Firth, K. S. A.; van Weerd, J.; Tertoolen, L. G. J.; Karperien, H. B. J.; Jonkheijm, P.; Denning, C.; Ijzerman, A. P.; Mummery, C. L. Small molecule absorption by PDMS in the context of drug response bioassays. *Biochemical and Biophysical Research Communications* **2017**, 482 (2), 323-328. DOI: 10.1016/j.bbrc.2016.11.062.

(46) Campbell, S. B.; Wu, Q. H.; Yazbeck, J.; Liu, C. A.; Okhovatian, S.; Radisic, M. Beyond Polydimethylsiloxane: Alternative Materials for Fabrication of Organ-on-a-Chip Devices and Microphysiological Systems. *Acs Biomaterials Science & Engineering* **2021**, 7 (7), 2880-2899. DOI: 10.1021/acsbmaterials.0c00640.

(47) Negoro, R.; Yamada, N.; Watanabe, K.; Kono, Y.; Fujita, T. Generation of Caco-2 cells stably expressing CYP3A4·POR·UGT1A1 and CYP3A4·POR·UGT1A1*6 using a PITCH system. *Arch Toxicol* **2022**, 96 (2), 499-510. DOI: 10.1007/s00204-021-03175-0 From NLM.

(48) Markowitz, J.; Rappaport, A. M. THE HEPATIC ARTERY. *Physiological Reviews* **1951**, 31 (2), 188-204. DOI: 10.1152/physrev.1951.31.2.188.

(49) Ludden, T. M. Nonlinear pharmacokinetics: clinical Implications. *Clin Pharmacokinet* **1991**, 20 (6), 429-446. DOI: 10.2165/00003088-199120060-00001.

(50) Yeh, K. C.; Stone, J. A.; Carides, A. D.; Rolan, P.; Woolf, E.; Ju, W. D. Simultaneous investigation of indinavir nonlinear pharmacokinetics and bioavailability in healthy volunteers using stable isotope labeling technique: Study design and model-independent data analysis. *Journal of Pharmaceutical Sciences* **1999**, 88 (5), 568-573. DOI: 10.1021/js9802392.

(51) Maeda, K.; Takano, J.; Ikeda, Y.; Fujita, T.; Oyama, Y.; Nozawa, K.; Kumagai, Y.; Sugiyama, Y. Nonlinear Pharmacokinetics of Oral Quinidine and Verapamil in Healthy Subjects: A Clinical Microdosing Study. *Clinical Pharmacology & Therapeutics* **2011**, 90 (2), 263-270. DOI: 10.1038/clpt.2011.108.

(52) Thummel, K. E.; O'Shea, D.; Paine, M. F.; Shen, D. D.; Kunze, K. L.; Perkins, J. D.; Wilkinson, G. R. Oral first-pass elimination of midazolam involves both gastrointestinal and hepatic CYP3A-mediated metabolism. *Clin Pharmacol Ther* **1996**, 59 (5), 491-502. DOI: 10.1016/s0009-9236(96)90177-0 From NLM.

(53) Moghe, P. V.; Berthiaume, F.; Ezzell, R. M.; Toner, M.; Tompkins, R. G.; Yarmush, M. L. Culture matrix configuration and composition in the maintenance of hepatocyte polarity and function. *Biomaterials* **1996**, 17 (3), 373-385. DOI: 10.1016/0142-9612(96)85576-1.

(54) Ferrini, J. B.; Pichard, L.; Domergue, J.; Maurel, P. Long-term primary cultures of adult human hepatocytes. *Chemico-Biological Interactions* **1997**, 107 (1-2), 31-45. DOI: 10.1016/s0009-2797(97)00072-0.

- (55) Hamilton, G. A.; Jolley, S. L.; Gilbert, D.; Coon, D. J.; Barros, S.; LeCluyse, E. L. Regulation of cell morphology and cytochrome P450 expression in human hepatocytes by extracellular matrix and cell-cell interactions. *Cell and Tissue Research* **2001**, *306* (1), 85-99.
- (56) Roy, P.; Washizu, J.; Tilles, A. W.; Yarmush, M. L.; Toner, M. Effect of flow on the detoxification function of rat hepatocytes in a bioartificial liver reactor. *Cell Transplantation* **2001**, *10* (7), 609-614.
- (57) Tilles, A. W.; Baskaran, H.; Roy, P.; Yarmush, M. L.; Toner, M. Effects of oxygenation and flow on the viability and function of rat hepatocytes cocultured in a microchannel flat-plate bioreactor. *Biotechnology and Bioengineering* **2001**, *73* (5), 379-389. DOI: 10.1002/bit.1071.
- (58) Wagner, B. K.; O'Hara, D. A. Pharmacokinetics and pharmacodynamics of sedatives and analgesics in the treatment of agitated critically ill patients. *Clin. Pharmacokinet.* **1997**, *33*, 426–453. DOI: 10.2165/00003088-199733060-00003.
- (59) Jeong, W.; Sunwoo, J.; You, Y.; Park, J. S.; Min, J. H.; In, Y. N.; Ahn, H. J.; Jeon, S. Y.; Hong, J. H.; Song, J. H.; Kang H.; Nguyen, M. T. T.; Kim, J.; Kang, C. Distribution and elimination kinetics of midazolam and metabolites after post-resuscitation care: a prospective observational study. *Sci Rep.* **2024**, *14* (1), 4574. DOI: 10.1038/s41598-024-54968-z.
- (60) Moorthy, G. S.; Jogiraju, H.; Vedar, C.; Zuppa, A. F. Development and validation of a sensitive assay for analysis of midazolam, free and conjugated 1-hydroxymidazolam and 4-hydroxymidazolam in pediatric plasma: Application to Pediatric Pharmacokinetic Study. *J Chromatogr B Analyt Technol Biomed Life Sci.* **2017**, *1067*, 1-9. DOI: 10.1016/j.jchromb.2017.09.030.

- (61) Selwyn, F. P.; Cheng, S. L.; Bammler, T. K.; Prasad, B.; Vrana, M.; Klaassen, C.; Cui, J. Y. Developmental Regulation of Drug-Processing Genes in Livers of Germ-Free Mice. *Toxicological Sciences* **2015**, *147* (1), 84-103. DOI: 10.1093/toxsci/kfv110.
- (62) Mosa, A.; Gerber, A.; Neunzig, J.; Bernhardt, R. Products of gut-microbial tryptophan metabolism inhibit the steroid hormone-synthesizing cytochrome P450 11A1. *Endocrine* **2016**, *53* (2), 610-614. DOI: 10.1007/s12020-016-0874-0.
- (63) Haduch, A.; Bromek, E.; Kuban, W.; Daniel, W. A. The Engagement of Cytochrome P450 Enzymes in Tryptophan Metabolism. *Metabolites* **2023**, *13* (5). DOI: 10.3390/metabo13050629.
- (64) Potthoff, M. J.; Boney-Montoya, J.; Choi, M.; He, T. T.; Sunny, N. E.; Satapati, S.; Suino-Powel, K.; Xu, H. E.; Gerard, R. D.; Finck, B. N.; et al. FGF15/19 Regulates Hepatic Glucose Metabolism by Inhibiting the CREB-PGC-1 alpha Pathway. *Cell Metabolism* **2011**, *13* (6), 729-738. DOI: 10.1016/j.cmet.2011.03.019.
- (65) Kliewer, S. A.; Mangelsdorf, D. J. Bile Acids as Hormones: The FXR-FGF15/19 Pathway. *Digestive Diseases* **2015**, *33* (3), 327-331. DOI: 10.1159/000371670.
- (66) Negoro, R.; Yamada, N.; Watanabe, K.; Kono, Y.; Fujita, T. Generation of Caco-2 cells stably expressing CYP3A4·POR · UGT1A1 and CYP3A4 · POR · UGT1A1*6 using a PITCH system. *Arch Toxicol* **2022**, *96* (2), 499-510. DOI: 10.1007/s00204-021-03175-0 From NLM.