Article

Development of a Perfusing Small Intestine–Liver Microphysiological System Device

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Abstract: There is an increasing need to develop alternatives to animal modeling and testing for pre-clinical studies as researchers face major challenges, such as the study of dynamic systems in laboratory settings. Microphysiological system (MPS) technology has recently shown great potential for addressing such limitations. We developed a perfusing small intestine–liver-connected device that is easy to operate and highly reproducible. In non-clinical pharmacokinetics and safety studies, the use of human-derived materials is necessary. We used human iPS cell-derived small intestinal epithelial cells (HiEs) and cryopreserved human primary hepatocytes. Hepatocytes in 3D culture were co-cultured with swiss-albino 3T3 cells as feeder cells. We evaluated the effects of co-culturing hepatocytes and HiEs using our small intestine–liver device. The mRNA expression levels of CYP1A2 and CYP3A4 in hepatocytes were significantly increased in the 3D culture. The TEER values were increased in HiEs co-cultured with hepatocytes in the 3D culture. We evaluated the differential proliferation and function characteristics of the hepatocytes and HiEs following perfusion and verified the utility of our proposed small intestine–liver device for evaluating multiple cell populations. The perfusion culture system of our small intestine–liver device can be used to investigate distinct effects on co-cultured hepatocytes and HiEs.

Keywords: microphysiological system (MPS); cryopreserved human primary hepatocyte (hepatocytes); human iPS cell-derived small intestinal epithelial cell (HiE); perfusion culture; small intestine–liver connected device (small intestine–liver device)

1. Introduction

The 3Rs—replacement, reduction, and refinement—of scientific experimentation are increasingly being regulated globally, and in Europe, animal testing has been banned for certain safety evaluation studies [1]. Therefore, methods for evaluating safety and toxicity without using animals are essential to the development of novel treatments and drug interventions. The alleviation of animal models can be challenging for certain biomedical research techniques; for example, the study of organ–organ interactions in static culture systems, such as cell cultures in dishes and plates, for predicting pharmacokinetics and safety in humans is complicated.

The microphysiological systems (MPS) model has garnered increasing attention as a new tool for cell assays in drug discovery research by combining microfluidic devices and cell culture technologies [2]. The MPS model functions as a technological system that mimics physiological blood flow, which enhances expression and functions in cells that cannot be suitably tested using technologies such as conventional static culture [3].
the past few years, numerous research grants, including one from the Japan Agency for Medical Research and Development (AMED), have been invested in MPS globally [4]. Such an investment reveals the magnitude of expectations for research related to MPS. The functions of various organs and tissues, such as the lung, liver, kidney, and gut, have been studied using MPS models [5]. Liver MPS models are being developed to accurately predict metabolic abilities and toxicity during the drug discovery process [6]. However, the applicability of liver MPS models is unknown. Researchers have developed a polydimethylsiloxane (PDMS) liver-on-a-chip device to evaluate drug absorption by investigating the drug responsiveness to culture cryopreserved human primary hepatocytes and investigating its responsiveness to determine its drug absorption [7]. Furthermore, MPS devices have been proposed for investigating multi-organ functions for predicting organ interactions [5]. The MPS reported by Imura et al., which comprised a slide glass and PDMS sheets with microchannels fabricated via photolithography, cultured Caco-2 cells in the intestine component and HepG2 cells in the liver component [8]. Based on this report, we employed MPS technology to imitate the small intestine and liver in vivo in our laboratory with the aim of developing a suitable model for evaluating pharmacokinetics and safety in a multi-organ system. Therefore, we developed a perfusing small intestine–liver connected device (hereafter referred to as a small intestine–liver device).

To evaluate our device, we selected human iPS cell-derived small intestinal epithelial cells (HiEs) produced in our laboratory [9] and hepatocytes, which are widely used for pharmacokinetic and safety research by pharmaceutical companies. We then co-cultured hepatocytes and HiEs using the proposed small intestine–liver device and determined its utility for pharmacokinetic analyses.

2. Materials and Methods

2.1. Materials

The human induced pluripotent stem cell (iPS cell) line (Windy) derived from the human MRC-5 embryonic lung fibroblast cell line was provided by Umezawa et al. from the National Center for Child Health and Development. Cryopreserved human primary hepatocytes (lot. HC10-10; female Caucasian donor aged 56 years) were obtained from Sekisui XenoTech (Lenexa, KS, USA). The Swiss-albino 3T3 cells were purchased from the Japan Collection of Research Bioresources Cell Bank (JCRB9019, Osaka, Japan). All reagents used were of the highest quality available.

2.2. Small Intestine–Liver Device

A small intestine–liver device model was developed in our laboratory to serve as a multi-organ MPS (Figure 1) and common evaluation system for pharmacokinetics and safety studies.

The small intestinal cells are seeded on cell culture inserts, which are widely used for absorption evaluation. Hepatocytes require direct seeding on the device. The device material was made of tissue culture PS (TCPS). In addition, the hepatocyte seeding area is designed to allow flat (no patterning; 2D) or spheroid-forming (with patterning; 3D) culture. The 3D plate is aligned regularly at 100 µm intervals on the bottom of the device. The cell culture area is located inside the circle coated with collagen type I. Non-adherent areas of the cells are covered with super-hydrophilic poly (ethyleneglycol) molecules. The 3D plate on the device is similar to the Cell-able® plate system [10].
**Figure 1.** Architecture of the small intestine–liver device. The perfusion culture device consisted of a small intestine–liver-connected device plate, six-strand pump, and holder. The perfusing small intestine–liver-connected device plate contained a six-strand well. Adapters are available for the device as various types of cell culture inserts can be set in the small intestine culture section of the six-strand well plate. To prevent cells from drying, there is a groove in the gap between each well where sterile water can be added. A cassette-type set is inserted in the small intestine culture section of the small intestine–liver device body. The hepatocyte culture section comprised two culture types, with or without patterning. The plate with patterning allows for the combination of co-culture with other cells and 3D culture. The small intestine–liver device was perfused with culture medium at a flow rate of 172 µL/h. The holder was designed to ease mobility and prevent contamination.

### 2.3. Differentiation of Human iPS Cell-Derived Small Intestinal Epithelial Cells and Culture of Cryopreserved Hepatocytes on Small Intestine–Liver Device

The iPS cells were differentiated over 26 days, as previously reported [11]. The small intestine–liver device was produced by Shinko Chemical Co., Ltd. (Kanazawa, Japan). At least 24 h before hepatocytes were thawed and seeded, 2D plates were coated with 0.01% collagen solution (Research Institute for the Functional Peptides, Yamagata, Japan) and washed with PBS solution. 3.0 × 10^5 cells/lane of hepatocytes were seeded on the 2D plate using OptiThaw Hepatocyte Media contained in OptiThaw Hepatocyte Kit K8000 (Sekisui Xenotech). The feeder cells, Swiss-albino 3T3 cells, were cultured in D-MEM (high glucose) with phenol red containing 10% FBS, 1% L-glutamine solution (Biological Industries, Beit-Haemek, Israel), 1% PS, and 1% NEAA. The small intestine–liver device plate was designed in a spheroid (with patterning: 3D) culture in the hepatocyte seeding area by Toyo Gosei Co., Ltd. (Tokyo, Japan). When swiss-albino 3T3 cells were confluent to approximately 80%, cells were detached using 0.5%/v% trypsin-EDTA and seeded at 3.84 × 10^4 cells/lane in the hepatocyte seeding area of the 3D plate. The following day, hepatocytes were seeded at 1.92 × 10^5 cells/lane using OptiThaw Hepatocyte Media. Immediately after seeding, the cells were placed on a clean bench for approximately 10 min to facilitate adherence. The cells were incubated at 37 °C for 4 h and then replaced with OptiPlate Hepatocyte Media K8200 (Sekisui Xenotech). The cells on the 2D plate were incubated at 37 °C for 20 h before co-culturing with HiEs that had been differentiated for 24 d. Swiss-albino 3T3 cells were not seeded in 2D plates. The cells on the 3D plate were incubated at 37 °C for 72 h before co-culturing with HiEs that had been differentiated for 26 d (Figure 2).
Figure 2. Technical explanation and schematic of combined cryopreserved human primary hepatocytes (hepatocytes) and human iPS cell-derived small intestinal epithelial cells (HiEs) by perfusion. To evaluate the small intestine–liver device, swiss-albino 3T3 cells were seeded at $3.84 \times 10^4$ cells/lane in the hepatocyte seeding area of the 3D plate. The following day, hepatocytes were seeded at $1.92 \times 10^5$ cells/lane. Hepatocytes on the 3D plate were incubated at 37 °C for 72 h before co-culturing with HiEs on day 24. Hepatocytes on the 2D plate were seeded at $3.0 \times 10^5$ cells/lane. Swiss-albino 3T3 cells were not seeded in 2D plates. Hepatocytes on the 2D plate were incubated at 37 °C for 24 h before co-culturing with HiEs after differentiation for 24 d. The medium was allowed to flow at 172 µL/h and changed every 24 h for 96 h of perfusion. Transepithelial electrical resistance (TEER) values were measured from 23 to 30 days after iPS cell differentiation. The lid of the device was removed each time when measuring.

2.4. Static and Perfusion Culture Conditions

Inlet septum (3007-16101, GL Sciences, Tokyo, Japan) was attached to the small intestine–liver device (Shinko Chemical Co., Ltd.). We prepared six 7 cm PEEK color tubes (JR-T-6001-M10) (Shimadzu GLC Ltd., Tokyo, Japan), 7 cm general-purpose silicone rubber tubing (SR1554, inner diameter 1 mm, outer diameter 2 mm, Tigers Polymer Corporation, Osaka, Japan), and 27 cm PEEK color tubes; then attached them to the six strand-pump (Takasago Electric, Inc., Nagoya, Japan) part of the small intestine–liver device. The tubes of silicone were used in six-strand pumps only. The PEEK color tubes were bent moderately, being careful not to stop the flow of the medium upon folding the silicon tube. In 2D and 3D plates, 450, 480, and 80 µL of the medium for hepatocytes were added to the lower part of the insert and hepatocyte seeding area and exit area, respectively, and the prepared tubes were filled with the medium. The medium was perfused at 172 µL/h and changed every 24 h for a total perfusion period of 96 h.
2.5. RNA Extraction and Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis

After perfusion culture of HiEs and hepatocytes for 96 h, total RNA was extracted using Buffer RLT (QIAGEN, Venlo, The Netherlands) according to the manufacturer’s instruction manual. RNA concentration was calculated by measuring absorbance at 260 nm using NanoDrop One (Thermo Fisher Scientific, Waltham, MA, USA). ReverTra Ace® qPCR RT Master Mix (Toyobo Co., Ltd., Osaka, Japan) was used to synthesize cDNA from total RNA according to the manufacturer’s instruction manual. KAPA SYBR FAST qPCR Kit Master mix (2×) ABI Prism (Sigma-Aldrich Co., St. Louis, MO, USA) was used to perform real-time RT-PCR. The PCR primer sequences used are shown in Table 1. Samples were reacted using the LightCycler® 96 System (Roche Diagnostics, Basel, Switzerland). mRNA expression levels were standardized using hypoxanthine phosphoribosyl transferase (HPRT).

Table 1. Primers used for real-time RT-PCR.

<table>
<thead>
<tr>
<th>Gene Names</th>
<th>Forward Primer Sequences (5′-3′)</th>
<th>Reverse Primer Sequences (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AhR</td>
<td>GCCACGAGAGGGTACAGGTTATCA</td>
<td>GTGCAATGACTGGACCCACGTTCA</td>
</tr>
<tr>
<td>CAR</td>
<td>TGACGCAAGCTGCAAGAAGGAGA</td>
<td>TGGATGTGCTGGATTTGGTA</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>CTTTGCAGAAGAGAAGGAGA</td>
<td>AGTGCCAGCTCCCTCTCGGAT</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>GACATGAACAACCTACAGACTTT</td>
<td>TGCTTGTCGTCCCTGTCGCA</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>GAAACACCAAGAAATCGATGACA</td>
<td>TCAGCAGGAGAAGAGAGCACT</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>CCTACGGTTCGAAAAAGGCTT</td>
<td>AGAGACAGGCTCGACCACT</td>
</tr>
<tr>
<td>HPRT</td>
<td>CTTTGCAGAAGAAGGAGA</td>
<td>TGGATGACTGACCCACGTTCA</td>
</tr>
<tr>
<td>MDR1</td>
<td>CCCATCATGGCAATAGCAGG</td>
<td>TGCTCAACTGTCGTCGTCGTA</td>
</tr>
<tr>
<td>PXR</td>
<td>AGGATGGCCAGTGCTGGAAC</td>
<td>AGGGAGATGCTGCTGGAT</td>
</tr>
</tbody>
</table>

2.6. TEER Measurement

Transepithelial electrical resistance (TEER) values were measured from days 23 to 30 after iPS cell differentiation. Millicell ERS-2 (Millipore, Bedford, MA, USA) was used for measurements. Plates were left at room temperature (24 ± 4°C) for 10 min before measurement. Each value was subtracted by the value in the insert where no cells were seeded.

2.7. Statistical Analysis

The level of statistical significance was assessed using a Student’s t-test for comparison between two groups and Dunnett’s test for multiple comparisons (SPSS Student Version 13.0 J). Data are presented as means ± standard deviations of at least three wells in three separate experiments. Statistical significance was set at p < 0.05.

3. Results and Discussion

3.1. Configuration of Small Intestine–Liver Device

The body is composed of multi-organ systems that maintain integrated functions. When conducting safety and pharmacokinetics analyses, it is particularly important to consider the interaction between each organ. We developed an MPS small intestine–liver device that may be useful in evaluating drug bioavailability in this organ–organ system (Figure 1). The small intestine–liver device can be used to evaluate the interaction of tissues (particularly gut and liver tissues) via the perfusion system comprising six-strand pumps. This device model is an open system, which facilitates cell seeding and collection as well as substrate addition and sampling. In addition, the small intestine portion can be easily detached as a cassette.

PDMS and polystyrene (PS) are commonly used materials for cell plates. PDMS is useful for biological applications because it is impermeable to water and gases while being non-toxic to cells. The major advantage of PDMS over glass and silicon is the ease
with which it can be fabricated and bonded to other surfaces. However, there is a risk of potential adsorption of medium components on the device, particularly those made of PDMS. Therefore, we selected tissue culture PS (TCP5) as the plate material.

We selected two different tube materials for the model. The silicone tube was used for the driving part of six-strand pumps; owing to its excellent heat resistance, it can be autoclaved, allowing for sterilization. However, silicone may cause drug sorption. To avoid this limitation, we also used PEEK for the remainder of the tubing, as it is suitable for collecting biological samples and exhibits almost no drug adsorption or sorption. In our small intestine–liver device, mediums can be simultaneously placed in six wells to flow one way or circulate from inlet to outlet. In our experiments, we allowed the medium to circulate to evaluate the effects of co-culture with gut and liver cells.

Furthermore, we designed planar (without patterning: 2D) and spheroid (with patterning: 3D) cultures in the hepatocyte seeding area. This would also reflect the effect of different hepatocyte culture methods while perfusing. Notably, 3D cultures of hepatocytes with Cell-able® plates enable high functional activity and long-term culture compared to 2D culture [10]. Moreover, the 3D culture of hepatocytes co-cultured with feeder cells, swiss-albino 3T3 cells, yields more robust hepatocyte spheroid formation and higher metabolic activity [12]. We believe that the 3D culturing of hepatocytes using this device would yield sufficiently high functional activity for the evaluation of pharmacokinetics.

3.2. Hepatocytes Morphology at 0, 2, and 4 Days after Seeding on Small Intestine–Liver Device (2D and 3D Cultures)

Figure 3 shows the morphological changes in hepatocytes on days 0, 2, and 4 after 2D or 3D culture. In terms of morphology, Hepatocytes on day 4 after 2D culture were large and flat. Unattached hepatocytes tended to be flowed by perfusion for 2 days. Hepatocyte morphology on days 2 and 4 was unchanged. Hepatocytes (HC10-10) had a poor adhesion rate and could not be 3D cultured without Swiss-albino 3T3 cells. Hepatocytes with swiss-albino 3T3 cells exhibited immediate gathering in cell adhesion areas after seeding on day 0 after 3D culture. In addition, hepatocytes after 3D culture were distinguishable in a spheroid of 100 µm on day 4. Therefore, we illustrated that hepatocytes (HC10-10) with Swiss-albino 3T3 cells could yield more robust spheroid formation than hepatocytes alone (HC10-10) (Figure 3). The expression of metabolic enzymes and the adhesion rate of hepatocytes vary significantly [13]; therefore, the lot selection of hepatocytes is particularly important. Additionally, co-culturing hepatocytes exhibiting weak adhesion with swiss-albino 3T3 cells when using our device is critical.

Figure 3. Hepatocyte morphology 0, 2, and 4 days after seeding on the small intestine–liver device. Morphological images of 2D or 3D hepatocyte cultures. Images were taken on days 0, 2, and 4 after perfusion. White arrows represent large and flat hepatocytes. Scale bar: 200 µm.
3.3. mRNA Expression of Drug Metabolizing Enzymes and Nuclear Receptors in HiEs or Hepatocytes on Day 4 after Culturing on the Small Intestine–Liver Device (2D and 3D Cultures)

To examine the effect of perfusion and HiE–hepatocytes interactions on gene expression levels, we evaluated the mRNA expression levels of each cell type. The mRNA expression levels of CYP1A2 and CYP3A4 in hepatocytes were significantly increased in the 3D culture compared with those in the 2D culture (Figure 4A). Conversely, the mRNA expression levels in the HiEs of 2D or 3D cultured hepatocytes were almost equivalent. The gene expression levels of HiEs were higher than or almost equivalent to the levels of human adult small intestine cells, excluding CYP3A4 (Figure 4B).

Figure 4. Expression levels of drug-metabolizing enzymes, transporter in HiEs or hepatocytes on day 4 after perfusion. (A) Samples were obtained from hepatocytes in HiEs–hepatocytes interactions cultured under perfusion and hepatocytes at 0 h. HPRT was used as housekeeping gene. Bars indicate means ± SD (n = 3). The graph shows mRNA expression relative to that of hepatocytes at 0 h at considered as 1. * p < 0.05, ** p < 0.01 for comparison between 2D and 3D. (B) Samples were obtained from HiEs in HiEs–hepatocytes interactions cultured under per fusion condition and small intestine (SI). HPRT was used as a housekeeping gene. Bars indicate means ± SD (n = 3). The graph shows mRNA expression relative to that of SI considered as 1.
CYP enzyme induction is regulated by aromatic hydrocarbon receptor (AHR), pregnane X receptor (PXR), and constitutive androstane receptor (CAR) that activate gene transcription [14,15]. Nuclear receptors should be studied to elucidate the regulation of CYP enzyme induction. The mRNA expression levels of the AhR and PXR in hepatocytes were similar to those in hepatocytes (0 h) (Figure 5A). The mRNA expression levels of CAR in hepatocytes were approximately one-tenth the levels of that in hepatocytes (0 h). The mRNA expression levels of PXR and CAR, excluding AhR, did not significantly differ between 2D or 3D cultures. Conversely, the mRNA expression of CAR in HiEs exceeded the levels in the small intestine (Figure 5B). The mRNA expressions of CAR and PXR in HiEs for 2D or 3D culture did not change.

Figure 5. Expression levels of nuclear receptors in HiEs or hepatocytes on day 4 after perfusion. (A) Samples were obtained from hepatocytes in HiEs–hepatocytes interactions cultured under perfusion and hepatocytes at 0 h. HPRT was used as a housekeeping gene. Bars indicate means ± SD (n = 3). The graph shows mRNA expression relative to that of hepatocytes at 0 h considered as 1. * p < 0.05 vs. between 2D and 3D cultures. (B) Samples were obtained from HiEs in HiEs–hepatocytes interactions cultured under perfusion conditions and SI. HPRT was a reference gene. Data are presented as means ± SD (n = 3). The graph shows mRNA expression relative to that of SI considered as 1.

Pleiotrophin, released from Swiss-albino 3T3 cells, is a secreted growth factor with mitogenic properties for fibroblasts, epithelial cells, and endothelial cells [12]. In addition, swiss-albino 3T3 cells produce collagen type I and are involved in the maturation of hepatocytes [13]. Therefore, the function of hepatocytes co-cultured with Swiss-albino 3T3
cells may have been enhanced by pleiotrophin and collagen type I, along with perfusion. Conversely, this dynamic may not have affected the gene expression of HiEs (Figure 4B).

3.4. Changes in TEER Values

We examined whether the TEER values of HiEs were increased in HiEs–hepatocytes interactions cultured under perfusion conditions. The TEER values of HiEs were increased in a time-dependent manner during the 26-day differentiation induction period and reached 200–250 Ω·cm² on day 26 (Figure 6). This is consistent with the findings of previous studies [11]. In addition, we continued to incubate HiEs and measure TEER values until day 29. TEER values decreased in a time-dependent manner. The values for HiEs co-cultured with hepatocytes in the 2D culture decreased slightly. Conversely, the TEER values increased considerably in HiEs co-cultured with hepatocytes in the 3D culture. The TEER values remained consistent by day 30.

![Figure 6. TEER values of HiEs in HiEs–hepatocytes interactions cultured under perfusion conditions. The TEER values of HiEs seeded on cup-type cell culture inserts were measured during culturing on a perfusing small intestine–liver-connected device. In the control group, cell culture inserts seeding HiEs were set on plates. Data are presented as means ± SD (n = 12).](image)

We used a medium of hepatocytes under the inserts. We also suggest that HiEs can be cultured using a suitable medium to increase TEER values and mRNA expression levels. Our findings verified that our proposed small intestine–liver device was suitable for both planar (2D) or spheroid (3D) hepatocyte cultures to evaluate effects in two cell populations.

4. Conclusions

We evaluated organ–organ interactions in co-cultured hepatocytes and HiEs using our proposed small intestine–liver device. We found that 3D spheroid hepatocytes co-cultured with Swiss-albino 3T3 cells exhibited more optimized gene expressions than flat-cultured hepatocytes. The proposed small intestine–liver device allows for perfusion and facilitates the investigation of organ interactions in co-cultured hepatocytes and HiEs. We expect to mimic in vivo mechanisms with this assay in future studies.

Author Contributions: Y.S. contributed to data curation, formal analysis, investigation, methodology, writing—review and editing, writing—original draft, validation, visualization; M.M. contributed to data curation, formal analysis, investigation, methodology, visualization; H.Y., A.D. and I.S. contributed to data curation, formal analysis, investigation, visualization; T.I. contributed to investigation, methodology, supervision, writing—review and editing, writing—original draft; T.M. contributed to conceptualization, project administration, investigation, methodology, funding acquisition, supervision, writing—review and editing, writing—original draft.
sion, writing—review and editing, writing—original draft. All authors have read and agreed to the published version of the manuscript.

**Funding:** This study was partly supported by AMED under Grant Number JP21be0304203. Furthermore, support was also provided by a Grant-in-Aid for Research at Nagoya City University in 2020.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

**Acknowledgments:** We thank Hidenori Akutsu, Yoshitaka Miyagawa, Hajime Okita, Nobutaka Kiyokawa, Masashi Toyoda, and Akihiro Umezawa for providing human iPSC cells (Windy #51). We wish to thank Atsushi Doi (Shinko Chemical Co., Ltd.) for the small intestine–liver device design.

**Conflicts of Interest:** The authors declare no conflict of interest. This study was partly supported by AMED under Grant Number JP21be0304203. Furthermore, support was also provided by a Grant-in-Aid for Research at Nagoya City University in 2020. Isao Saito, Hideki Yama-da, and Atsushi Doi are employees of Shinko Chemical Co., Ltd.

**Abbreviations**

AhR, aryl hydrocarbon receptor; A-83-01, 3-(6-Methyl-2-pyridinyl)-N-phenyl-4-(4-quinoliny1)-1H-pyrazole-1-carbothioamide; 5-aza-2′-dC, 5-aza-2′-deoxycytidine; CAR, constitutive androstane receptor; CES, carboxylesterase; EGF, epidermal growth factor; FBS, fetal bovine serum; FGF2, basic fibroblast growth factor2; HiEs, human iPSC cell-derived small intestinal epithelial cells; hepatocytes, cryopreserved human primary hepatocytes; HPRT, hypoxanthine phosphoribosyltransferase; iPSC cells, induced pluripotent stem cells; NEAA, non-essential amino acids solution; MDR1, multidrug resistance protein 1; MPS, microphysiological system; PD98059, 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one; PBS, phosphate-buffered saline; PDMS, polydimethylsiloxane; PXR, pregnane X receptor; PS, penicillin–streptomycin mixed solution; SI, small intestine; TCPS, tissue-culture polystyrene; TEER, transepithelial electrical resistance.

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