A Novel Model of P-Glycoprotein Inhibitor Screening Using Human Small Intestinal Organoids

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Abstract: P-glycoprotein (P-gp), an important efflux transporter in intestine, regulates the bioavailability of orally taken drugs. To develop an in vitro model that preferably mimics the physiological microenvironment of human intestine, we employed the three-dimensionally (3D) cultured organoids from human normal small intestinal epithelium. It was observed that the intestinal crypts could efficiently form cystic organoid structure with the extension of culture time. Furthermore, the physiological expression of ABCB1 was detected at both mRNA and protein levels in cultured organoids. Rhodamine 123 (Rh123), a typical substrate of P-gp, was actively transported across 3D organoids and accumulated in the luminal space. This transport process was also inhibited by verapamil and mitotane. In summary, the above-mentioned model based on human small intestinal 3D organoids is suitable to imitate the small intestinal epithelium and could be used as a novel in vitro model especially for P-gp inhibitor screening.

P-glycoprotein (P-gp; ABCB1; MDR1), a member of the ATP-binding cassette (ABC) transporter superfamily, is a 170-kDa transmembrane protein [1]. It is the product of the multi-drug resistance 1 (MDR1) gene and is a single polypeptide with 1280 residues [2]. P-gp has a tandemly duplicated structure, and each part contains a transmembrane domain (TMD) and a nucleotide-binding domain (NBD). The TMD is hydrophobic and considered to form the pathway through which the substrates are secreted out of cells. In contrast, the NBD is relatively hydrophilic and considered as the sites for ATP binding and hydrolysis [3,4]. P-gp is found in a variety of normal tissues and organs, extremely in which involved in drug pharmacokinetics such as the brain–blood barrier, liver, kidney and small intestine [5,6]. It functions as an efflux pump to transport a broad spectrum of substrates including endogenous (e.g. phospholipids, glycolipids, steroid hormones) and exogenous (e.g. anticancer drugs, anticonvulsants, anti-HIV drugs) substances from intra- to extra-cellular under consumption of ATP, protecting the body from toxins and xenobiotics [7–9]. Therefore, P-gp limits drug absorption, facilitates drug elimination and thus decreases the bioavailability of orally taken drugs [10]. Moreover, the overexpression of P-gp in cancer cells could prevent the intracellular accumulation of chemotherapeutics, leading to multi-drug resistance (MDR) phenomenon and consequent chemotherapy failure [11–13]. Based on mentioned above, the P-gp-mediated drug transports are of great clinical importance and require pre-clinical investigations.

Now, there are several in vitro models used for assessing P-gp and drug interactions. In particular, the Caco-2 cell model is the most commonly used method [14–16]. Caco-2 cells are derived from human colon carcinoma and can form a well-polarized monolayer joined by tight junctions when cultured for 21 days on the Transwell membrane, providing a selective barrier that can be used for bidirectional (influx and efflux) transport studies [17,18]. This cell-based bidirectional permeability system is currently accepted as the 'method of choice' for evaluating the P-gp role [19]. However, some factors such as the tighter cellular junctions than normal enterocytes and the long culture period of at least 21 days limit the usefulness of this model [20,21].

Three-dimensional (3D) organoid is the newly developed model which could be used to study P-gp-mediated drug transports and screen P-gp inhibitors [22–24]. In 2009, the culture condition of mouse normal intestinal epithelium was developed [22]. When isolated and cultured three dimensionally, the intestinal crypts could form enclosed crypt-like structures with their apical side facing to the luminal space. Based on this, two novel methods for investigating P-gp-mediated drug transports were emerged [23,24]. Nevertheless, the species difference between human beings and rodents would hinder the organoids from mouse from mimicking the human physiological microenvironment to some extent. Recently, the method for in vitro culture of human normal intestinal epithelium has been reported [25]. In the light of this, we are interested in applying the newly developed culture technology to the P-gp transport studies.
In this study, we aimed to explore whether the three-dimensional (3D) organoids from human normal intestinal epithelium could imitate the native small intestinal epithelium and develop a new in vitro model for P-gp inhibitor screening.

Materials and Methods

**Chemicals and reagents.** Rhodamine 123 (Rh123) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Advanced DMEM/F12 and mouse recombinant EGF were obtained from Invitrogen (Carlsbad, CA, USA). Human recombinant R-spondin 1 and mouse recombinant noggin were purchased from Pepro Tech Inc. (Rocky Hill, NJ, USA). Mouse recombinant Wnt-3a was purchased from Millipore (Billerica, MA, USA). Matrigel (GFR, phenol-free) was obtained from BD Biosciences (Bedford, MA, USA). Trizol reagent was purchased from Zhiyuan (Yong’an, China). Primer Script RT Reagent Kit was supplied by Takara (Dalian, China). Primary antibody for P-gp was from Abcam (Cambridge, UK). The anti-rabbit antibody, DAB kit and streptavidin–peroxidase complex were purchased from Neobioscience Co. (Shenzhen, China). Verapamil was bought from MP Biomedicals, LLC. (Illkirch, France). Mitotane was obtained from J & K Scientific Ltd. (Beijing, China).

**Human tissue material.** All the experimental procedures involving human beings have been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) guidelines. Surgically resected small intestine tissue was obtained from a patient from the Shanghai Fengxian District Central Hospital, and a distance of more than 10 cm to the tumours was kept. The patient was a 65-year-old man with colon cancer. This study was approved by the ethical committee of Shanghai Fengxian District Central Hospital, and all samples were obtained with informed consent.

**3D organoid culture.** The isolation of human small intestinal crypts was performed according to the method reported before [25]. After purified and counted, the crypts were embedded in Matrigel at a density of 10,000 crypts/mL. Then, 5 μL of Matrigel was seeded in 96-well plates. After the Matrigel became solid at 37°C for 10 min., 100 μL of Advanced DMEM/F12 medium containing several growth factors (50 ng/mL mouse recombinant EGF, 100 ng/mL mouse recombinant noggin, 500 ng/mL human recombinant R-spondin 1 and 100 ng/mL mouse recombinant Wnt-3a) was added. The crypts were cultured in a humidified atmosphere with 5% CO2 at 37°C, and the culture medium was replaced every other day.

Morphological structure observations. On the different cultured days, the morphological structure of organoids was observed on a microscope IX71 (Olympus, Tokyo, Japan). All images were acquired with the equipped Olympus DP 71 photographic system.

Semi-quantitative reverse transcription–polymerase chain reaction (RT-PCR). On the second cultured day, the organoids were isolated from the Matrigel. Total cellular RNA in small intestine tissue, crypts and organoids was extracted with Trizol reagent. The RNA concentration was detected by spectrophotometric analysis at 260 nm, and a portion of 1000 ng RNA was reverse-transcribed into cDNA in a 20-μL reaction mixture performed with Takara RR036A RT-kit according to supplier’s instructions. To detect the transcription levels of genes, selective primers were designed and synthesized. The primer sequences were as follows: forward (F): 5'-GAGGCCCAACATACATGCTTC-3' and reverse (R): 5'-GTCAAGAAGCCAGACGATCT-3' for ABCB1; F: 5'-GTACGCC AACACAGTCTG-3' and R: 5'-CGTCACTCTCCGTCGTCG-3' for β-actin (internal reference). PCR was performed, and the lengths of the products were 127 bp for ABCB1 and 212 bp for β-actin, respectively. The amplified products were subjected to 2% agarose gel electrophoresis, and images were acquired with the Bio-Imaging System 910 (Jerusalem, Israel).

Immunohistochemistry. On the second cultured day, the organoids were isolated from the Matrigel. The small intestine tissue and organoids were fixed with 4% paraformaldehyde overnight, and thereafter, they were dehydrated with gradient ethanol. For immunohistochemical staining, paraformin-embedded sections were taken at approximately 4-μm intervals. The sections were subsequently deparaffinized, endogenous peroxidases inhibited and subjected to antigen retrieval. Sections were incubated with the primary antibody at 4°C overnight, rinsed and incubated with the secondary antibody at room temperature for 30 min. Sections were further incubated with the streptavidin–peroxidase complex at room temperature for 30 min., followed by incubation with diaminobenzidine (DAB) for 3 min. Sections were counter-stained with haematoxylin, dehydrated and finally mounted for long-term preservation. Images were achieved from the microscope (LEICA DM4000 B LED, Wetzlar, Germany).

Rhodamine 123 transport study in 3D organoids. On the second cultured day, the organoids were taken from the incubator and counted. The transport experiments were divided into control group (Rh123 alone) and treated groups (Rh123 together with verapamil or mitotane). The concentration of Rh123 was 5 μM [26]. The concentration of P-gp inhibitors was 20 μM as in our previous study [24]. Incubated with drugs for 20, 40, 60, 80 and 100 min., respectively, the culture medium was taken entirely. After washing five times with 150 μL of pre-warmed PBS, the organoids were exposed to blank PBS at 37°C for 4 hr to allow the Rh123 to release thoroughly. To determine the fluorescence intensity of Rh123, 80 μL of supernatant was taken and detected by the automatic microplate reader (FLUOSTar OPTIMA) (BMG LABTECH, Ortenberg, Germany) under the condition of λex/λem = 485 nm/520 nm.

The calibration curve was obtained by plotting the fluorescence intensity of Rh123 versus the Rh123 concentration in blank PBS, covering a range from 5 to 500 nM. The concentration of Rh123 in organoids was calculated from the standard curve.

Statistical analysis. All data were expressed as the mean ± S.E.M. One-way analysis of variance was used to estimate the significance of differences. There was statistical significance between control and test groups if \( p < 0.05 \).

Results

Morphological structure observations.

As shown in fig. 1, the isolated intestinal crypts efficiently formed cystic organoid structure with their upper openings closed. The size of organoids increased gradually in the initial 7 days with budding in the second cultured day.

Semi-quantitative reverse transcription–polymerase chain reaction (RT-PCR).

The expression of ABCB1 in small intestine tissue, crypts and organoids was analysed at the mRNA level. The mRNA amount of P-gp in organoids was no less than that in small intestine tissue and crypts (fig. 2).

Immunohistochemistry.

The expression of ABCB1 in small intestine tissue and organoids was assessed at the protein level by immunohistochemistry with...
an anti-P-gp antibody. The results are shown in fig. 3. As the arrowheads indicate, P-gp protein is expressed on the apical surface of small intestinal epithelium (fig. 3A), while it is located on the inner surface of cultured organoids (fig. 3B).

Rhodamine 123 transport study in 3D organoids.
Rh123, a typical substrate of P-gp with autofluorescence under the condition of $\lambda_{em}/\lambda_{ex} = 485$ nm/520 nm, was chosen as the probe in this study. Good linearity ($r^2 = 0.99$) was obtained covering a range from 5 to 500 nM for Rh123 in PBS (fig. 4A). Based on this method, the Rh123 concentration in organoids was calculated. Furthermore, we assessed the influence of P-gp inhibitors, verapamil and mitotane, on the transport of Rh123 across organoids. As shown in fig. 4B, the inner concentration of Rh123 increased with the extension of incubation time in both control and treated organoids. At the same time, both verapamil and mitotane exhibited significant inhibitory effects on the transport of Rh123.

Discussion
In the present study, we developed a human small intestinal 3D organoid model and further applied it to study P-gp-mediated Rh123 transport. To determine whether the 3D organoid model could represent the small intestinal epithelium, several methods were adopted. Results showed that the isolated crypts could efficiently form cystic organoid structure and grow gradually with the extension of culture time (fig. 1). Except for morphological structure observation, the expression of $ABCB1$ was detected at both mRNA and protein levels. The P-gp mRNA level in cultured organoids is as similar as that in human normal small intestine tissue and isolated crypts (fig. 2). The assay of immunohistochemistry also indicated that P-gp protein kept physiological expression and remained at correct location in cultured organoids compared with small intestine tissue (fig. 3). All above-mentioned data proved that the 3D organoids could imitate the human normal small intestinal epithelium perfectly.

Rh123, a typical substrate of P-gp with autofluorescence, was selected as the probe in the P-gp-mediated transport study [23,24]. Our previous study has successfully measured Rh123 in mouse 3D organoids [24]. In this study, the concentration of Rh123 was also detected via automatic microplate reader. Good linearity ($r^2 = 0.99$) was obtained covering a range from 5 to 500 nM for Rh123 (fig. 4A). Verapamil and mitotane, as the first-generation and third-generation P-gp inhibitor, respectively, were used to make a validation about this developed approach. The concentrations of them were both 20 µM like in our previous study [24]. After
incubation, the organoids were washed five times to clean the remaining Rh123 in Matrigel. To ensure the liveness of organoids, total cleaning time was within 15 min. The present new cleaning condition was more effective compared with previous method [24]. The organoids were disrupted, and the inner Rh123 was released thoroughly after exposure to PBS for 4 hr. Then, the supernatant was taken to detect the fluorescence intensity and the Rh123 concentration in organoids was calculated. In fact, the structure of organoid was no longer enclosed after incubation with PBS for 4 hr and the inner Rh123 was subsequently released. Therefore, although the duration of P-gp inhibitory effect of different P-gp inhibitor varied significantly after removing the inhibitor solution due to different inhibitory mechanisms [27], the experimental results were not significantly influenced. Moreover, mitotane exhibited more potent inhibition on P-gp than verapamil, which also agreed with the previous reports [24,28].

Human-derived Caco-2 cell line as the most common in vitro model is widely used to study P-gp-mediated drug transports [14–16]. However, some limitations may exist in Caco-2 cell model such as the tighter cellular junctions than normal enterocytes and the long culture period. Recently, the mouse 3D organoid model has been employed to investigate P-gp-mediated drug transports [23,24]. Compared with Caco-2 cells, the organoids are more similar to the native small intestinal epithelium because they are built from the ‘normal’ stem cells. Meanwhile, the shorter culture period of organoids also shortened the time cost of studies. Our previous study firstly exploited ultrasonic cell disruptor to smash the
organoids and then developed a rapid and efficient method to measure Rh123 in 3D organoids [24]. Until now, however, all previous studies have been based on the mouse intestinal organoids. In fact, P-gp expressed in different species possesses different substrate affinities, and thus, it is very difficult to extrapolate the results from one specific animal species to human beings. Fortunately, the method for in vitro culture of human normal intestinal epithelium has been reported in 2011 [25]. In the light of this, we employed this newly developed culture technology to the P-gp transport studies. To our knowledge, this study is the first to investigate P-gp-mediated Rh123 transport via the 3D organoid model from human small intestinal epithelium. Compared with the previous models [23,24], this newly developed organoid model will eliminate the species difference and therefore preferably mimic the human physiological microenvironment. In addition, the new method in this study to collect Rh123 by incubation with PBS for 4 hr is more convenient and labour-saving, compared with the reported methods such as real-time analysis and ultrasonication [23,24].

In conclusion, human small intestinal 3D organoid model was developed to successfully imitate the native small intestinal epithelium and study P-gp-mediated drug transport. In particular, human small intestinal 3D organoids plus Rh123 could serve as a novel model for P-glycoprotein inhibitor screening.

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