Characteristic analysis of intestinal transport in enterocyte-like cells differentiated from human induced pluripotent stem cells

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Running Title: Intestinal Transport in Human iPS Cell-Derived Enterocytes

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Nonstandard abbreviations

A-83-01,

3-(6-methyl-2-pyridinyl)-N-phenyl-4-(4-quinolinyl)-1H-pyrazole-1-carbothioamide;

ABC, ATP-binding cassette; BCRP, breast cancer resistance protein; CYP, cytochrome P450; DAPI, 4',6-diamidino-2-phenylindole; DMEM/F12, Dulbecco’s modified Eagle’s
medium and Ham’s nutrient mixture F-12; EGF, epidermal growth factor; ER, efflux ratio; \( F_a \), fraction absorbed; FBS, fetal bovine serum; FDA, Food and Drug Administration; FGF, fibroblast growth factor; FITC, fluorescein isothiocyanate; HIEC, human small intestinal epithelial cell; iPS, induced pluripotent stem cells; KSR, KnockOut Serum Replacement; Matrigel, Matrigel matrix Growth Factor Reduced; MEF, mouse embryonic fibroblast; MHLW, Ministry of Health, Labour and Welfare; MRP, multidrug resistance-associated protein; NEAA, MEM nonessential amino acid solution; \( P_{\text{app}} \), apparent membrane permeability coefficient; PBS, phosphate-buffered saline; PD98059, 2-(2-amino-3-methoxyphenyl)4H-1-benzopyran-4-one; PEG, polyethylene glycol; PEPT1, peptide transporter 1; P-gp, P-glycoprotein; RPMI, Roswell Park Memorial Institute; SULT, sulfotransferase; TEER, transepithelial electrical resistance; UGT, UDP-glucuronosyltransferase; Y-27632, (+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide dihydrochloride
Abstract

We previously demonstrated that differentiated enterocytes from human induced pluripotent stem (iPS) cells exhibited drug-metabolizing activities and cytochrome P450 (CYP) 3A4 inducibility. The aim of this study was to apply human iPS cell-derived enterocytes in pharmacokinetic studies by investigating the characteristics of drug transport into enterocyte-like cells. Human iPS cells cultured on feeder cells were differentiated into endodermal cells using activin A. These endodermal-like cells were then differentiated into intestinal stem cells by fibroblast growth factor 2. Finally, epidermal growth factor and small-molecule compounds induced the maturation of the intestinal stem cell-like cells. After differentiation, we performed transepithelial electrical resistance (TEER) measurements, immunofluorescence staining, and transport studies. TEER values increased in a time-dependent manner and reached approximately 100 Ω×cm². Efflux transport of Hoechst 33342, a substrate of breast cancer resistance protein (BCRP), was observed and inhibited by the BCRP inhibitor Ko143. The uptake of peptide transporter 1 substrate glycylsarcosine was also confirmed and suppressed when the temperature was lowered to 4°C. Using immunofluorescence staining, villin and Na⁺–K⁺ ATPase were expressed. These results suggest that human iPS cell-derived enterocytes had loose tight junctions, polarity, as well as uptake and efflux transport functions. In addition, the rank order of apparent membrane permeability coefficient ($P_{app}$) values of these test compounds across the enterocyte-like cell membrane corresponded to the fraction absorbance ($F_a$) values. Therefore, differentiated enterocytes from human iPS cells may provide a useful comprehensive evaluation model of drug transport and metabolism in the small intestine.
Introduction

The small intestine is an important organ in the pharmacokinetics of orally administered drugs due to the presence of drug transporters and drug-metabolizing enzymes (Choi et al., 2013; Li et al., 2013; Yoshida et al., 2013; Kostewicz et al., 2014). Thus, Caco-2 cells, a human colon carcinoma cell line, are widely used to evaluate the intestinal transport of orally administered drugs. While immortalized cells offer many advantages, the extrapolation of data generated with these cell lines to in vivo conditions is often difficult. This is because these cells originated from tumors and are therefore not representative of the natural physiological environment (Le Ferrec et al., 2001). However, these cells have different characteristics compared with human enterocytes due to the fact that the expression pattern of drug transporters is different (Sun et al., 2002; Harwood et al., 2015). Additionally, the level of cytochrome P450 (CYP) 3A4, a major drug-metabolizing enzyme in the small intestine, is also very low (Nakamura et al., 2002). Thus, it is difficult to comprehensively estimate drug transport and metabolism in the small intestine appropriately using Caco-2 cells. To precisely predict intestinal pharmacokinetics, it is desirable to use human primary small intestinal epithelial cells. However, it is difficult to obtain such cells, and there is no appropriate model that exists for intestinal pharmacokinetic prediction.

Human induced pluripotent stem (iPS) cells (Takahashi et al., 2007) have the potential to form almost any type of cell and are expected to be a useful tool in regenerative medicine and drug discovery research. The hepatic differentiation of human iPS cells has been frequently reported (Kondo et al., 2014; Takayama et al., 2014; Faulkner-Jones et al., 2015; Ishikawa et al., 2015); however, intestinal differentiation remains relatively unexplored in the literature. Spence et al. (2011)
reported the generation of three-dimensional gut-like organoids from human iPS cells and demonstrated that the organoids had the morphological characteristics of intestinal-tract tissues. Ogaki et al. showed that all four intestinal differentiated cell types (absorptive enterocytes, goblet cells, enteroendocrine cells, and Paneth cells) could be efficiently differentiated from human pluripotent stem cells (Ogaki et al., 2013; Ogaki et al., 2015). However, the pharmacokinetic functions of organoids and intestinal cells were only briefly explored in these studies. Kauffman et al. (2013) and Ozawa et al. (2015) have reported the pharmacokinetic characteristics of human iPS-derived enterocyte-like cells, but the characteristics of intestinal transport have been insufficiently investigated.

We have previously established a method for differentiating human iPS cells into enterocytes (Iwao et al., 2014; Iwao et al., 2015) and found several small-molecule compounds that were effective in promoting the differentiation of human iPS cells. In addition, these enterocytes were associated with a gain of pharmacokinetic function. Moreover, we also demonstrated that the enterocyte-like cells had various pharmacokinetic functions such as drug-metabolizing activities by CYPs, UDP-glucuronosyltransferase (UGT), and sulfotransferase (SULT), as well as CYP3A4 induction by 1α,25-dihydroxyvitamin D₃ and peptide uptake through peptide transporters.

In the present study, we investigated the characteristics of drug transport in enterocyte-like cells. The enterocyte-like cells had tight junctions and exhibited activities of efflux transporter breast cancer resistance protein (BCRP). Moreover, our findings indicated that the enterocyte-like cell membrane may be able to predict fraction absorbance ($F_a$) in humans. These results suggest that the human iPS cell-derived
enterocyte-like cells would be useful for a human intestinal pharmacokinetic prediction, including transport and metabolism.
Materials and Methods

Materials. Fibroblast growth factor (FGF) 2, activin A, and epidermal growth factor (EGF) were purchased from PeproTech Inc. (Rocky Hill, NJ, USA). BD Matrigel matrix Growth Factor Reduced (Matrigel) was purchased from BD Biosciences (Bedford, MA, USA). KnockOut Serum Replacement (KSR) was purchased from Invitrogen Life Technologies Co. (Carlsbad, CA, USA). (+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide dihydrochloride (Y-27632), 2-(2-amino-3-methoxyphenyl)4H-1-benzopyran-4-one (PD98059), 5-aza-2′-deoxycytidine, 3-(6-methyl-2-pyridinyl)-N-phenyl-4-(4-quinolinyl)-1H-pyrazole-1-carbothioamide (A-83-01), ibuprofen, paraformaldehyde, and Hoechst33342 were purchased from Wako Pure Chemical Industries (Osaka, Japan). Ko143 was purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA). Anti-villin and anti-BCRP/ABCG2 antibodies were purchased from Abcam (Cambridge, UK). Anti-Na⁺–K⁺ ATPase antibody purchased from GeneTex. (Woburn, MA). Anti-PEPT1 antibody was purchased from Santa Cruz Biotechnology, Inc (Dallas, TX). [N-methyl-¹⁴C]antipyrine was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). [Ring-³H]atenolol, [³H]metoprolol, and [³H]glycylsarcosine were purchased from Moravek Biochemicals, Inc. (Brea, CA). (−)-[Methoxy-³H]sulpiride and [¹⁴C]polyethylene glycol (PEG) 4000 were purchased from PerkinElmer, Inc. (Boston, MA). All other reagents were of the highest quality available. Triton X-100 was purchased from AMRESCO (Cleveland, OH).

Human iPS cell culture. The human iPS cell line Windy, which was derived from
the human embryonic lung fibroblast cell line MRC-5, was provided by Dr. Akihiro Umezawa of the National Center for Child Health and Development (Tokyo, Japan). Human iPS cells were maintained in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s nutrient mixture F-12 (DMEM/F12) containing 20% KSR, 2 mM L-glutamine, 1% MEM nonessential amino acid solution (NEAA), 0.1 mM 2-mercaptoethanol, and 5 ng/mL FGF2 at 37°C in humidified air with 5% CO₂. The human iPS cells were cultured on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts (MEFs), and the medium was changed daily.

**Differentiation into enterocyte-like cells.** The human iPS cells were differentiated into enterocytes based on our previous report (Iwao et al., 2015). Briefly, human iPS cells were differentiated into endodermal cells by incubating the cells in the presence of 100 ng/mL activin A for 72 h, and these endodermal-like cells were then differentiated into intestinal stem cells via 250 ng/mL FGF2 for 96 h. Finally, the cells were passaged on Matrigel-coated 24-well plates or cell culture inserts and cultured in medium containing 20 ng/mL EGF. Subsequently, 20 μM PD98059, 5 μM 5-aza-2′-deoxycytidine, and 0.5 μM A-83-01 were also added to the medium on day 14 after differentiation. The medium was subsequently changed every 3 days. Transepithelial electrical resistance (TEER) values were measured to check the integrity of the membrane before the transport assay.

**Uptake assay.** The culture medium was removed, and the differentiated cells were preincubated with the transport buffer (Hank’s balanced salt solution containing 10 mM MES, pH 6.0) at 37°C for 15 min. Uptake assays were initiated by the replacement of a
transport buffer containing 135 nM \[^{3}H\]glycylsarcosine at 37°C in the presence or absence of 3 mM ibuprofen, a known PEPT1 inhibitor (Omkvist et al., 2010) or at 4°C. Assays were stopped by the addition of ice-cold transport buffer, and the cells were washed twice with the same buffer. The cells were solubilized with 0.2 M NaOH solution (0.5 mL) containing 0.5% sodium dodecyl sulfate. Radioactivity was measured by liquid scintillation counting using 3 mL of Clear-sol I (Nakarai Tesque, Kyoto, Japan) as a scintillation fluid.

To correct for the uptake of glycylsarcosine, the total protein of the differentiated cells was measured using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA), according to the manufacturer's instructions.

**Immunofluorescence staining.** Differentiated cells were washed three times with phosphate-buffered saline (PBS) with 1 mM CaCl\(_2\) and 1 mM MgCl\(_2\), following which they were fixed and permeabilized in methanol (−20°C) for 5 min at 4°C for staining villin, Na\(^{+}\)–K\(^{+}\) ATPase, and PEPT1. Differentiated cells were washed three times with PBS, following which they were fixed in a 4% (w/v) paraformaldehyde solution for 30 min at room temperature, and permeabilized in a Triton X-100 solution for 5 min at room temperature for staining BCRP. After washing three times with PBS, the cells were blocked in PBS containing 2% skim milk for 20 min at room temperature. Following the blocking step, the cells were incubated for 60 min at room temperature, with anti-villin 1 and Na\(^{+}\)–K\(^{+}\) ATPase antibody diluted at 1:100. The cells were incubated overnight at 4°C, with the Na\(^{+}\)–K\(^{+}\) ATPase antibody diluted at 1:100, or BCRP and PEPT1 antibodies diluted at 1:50. The cells were washed three times with PBS and incubated with a 1:500 dilution of Alexa Fluor 488- and 568-labeled secondary
antibody for 60 min at room temperature. After washing three times with PBS, the cells were incubated with 1 µg/mL 4',6-diamidino-2-phenylindole (DAPI) for 5 min at room temperature and washed with PBS. The cells were mounted on a glass slide using a 9:1 mixture of glycerol and PBS (without magnesium and calcium) and were viewed using an LSM 510Meta confocal microscope (Carl Zeiss Inc., Oberkochen, Germany).

**TEER value measurements.** The TEER values of the human iPS cell-derived enterocyte-like cell membrane on cell culture inserts were measured by Millicell ERS-2 (Millipore, Bedford, MA).

**Membrane transport assay.** The culture medium was removed, and the differentiated cells were preincubated with transport buffer (Hank’s balanced salt solution containing 10 mM HEPES, pH 7.4) at 37°C for 15 min. Transport assays were performed by replacing with transport buffer containing substrates such as 1.15 µM [14C]antipyrine, 87.1 nM [3H]atenolol, 9.39 nM [3H]metoprolol, 7.70 nM [3H]sulpiride, or 1.31 nM [14C]PEG4000 on the apical chambers. The solution was collected from the basal chambers at 30, 60, and 120 min. Radioactivity was measured by liquid scintillation counting using 3 mL Clear-sol I (Nakarai Tesque, Kyoto, Japan) as a scintillation fluid. In a bidirectional transport assay using Hoechst33342, after preincubation, the transport buffer containing 20 µM Hoechst33342 was added to the apical or basal chambers, and the cells were incubated at 37°C for 120 min in the presence or absence of 10 µM Ko143. Samples were collected from the receiver chambers. The intensity of the Hoechst33342 fluorescence was measured using a fluorescence plate reader (ARVO MX 1420 Multilabel Counter, Perkin Elmer Inc.,
Waltham, MA) using the wavelengths of 355 nm for excitation and 460 nm for emission.

**Analysis of apparent membrane permeability.** The apparent membrane permeability coefficient \( P_{\text{app}} \) in transport assay was calculated as follows:

\[
P_{\text{app}} = \frac{dQ}{dt} \cdot \frac{1}{A \times C_0}
\]

where \( dQ/dt \) is the amount of the compound permeated per unit of time; \( A \) is the surface area of transwell membrane (0.3 cm\(^2\)); and \( C_0 \) is the initial compound concentration in the donor chamber. Efflux ratio (ER) of Hoechst33342 was calculated by dividing \( P_{\text{app}} \) of the basal-to-apical transport by that of the apical-to-basal transport.

**Statistical Analysis.** The level of statistical significance was assessed using Student's \( t \)-test. The correlation between the \( P_{\text{app}} \) value and \( F_a \) of the five compounds was estimated by a \( P \) and \( R \) value. The best-fitting curves were calculated by nonlinear regression using PASW Statistics 18 system software (IBM, Armonk, NY, USA).
Results

Uptake of glycylsarcosine in the differentiated enterocyte-like cells. The oligopeptide transporter SLC15A1/PEPT1 is expressed in the small intestine and plays an important role in peptide transport from the lumen (Liang et al., 1995; Giacomini et al., 2010). In previous study, we confirmed mRNA expression, but not protein expression, of PEPT1 in enterocyte-like cells. Therefore, we conducted immunofluorescence staining of PEPT1, indicating that the protein was also expressed (Fig. 1). Moreover, we performed uptake analyses of glycylsarcosine, which is a substrate of PEPT1 (Nakanishi et al., 1997). The uptake of glycylsarcosine in the enterocyte-like cells was increased in a time-dependent manner at 37°C (Fig. 2). When the uptake temperature was lowered to 4°C, the uptake was significantly suppressed and reached a plateau after 30 min. Moreover, at 37°C, in the presence of ibuprofen, the uptake was significantly suppressed to a similar extent as that at 4°C. Therefore, these findings indicate that PEPT1-mediated active transport was quantitatively evaluated in the enterocyte-like cells.

Characteristics of enterocyte-like cells. To investigate whether the enterocyte-like cell membrane was available for drug permeability studies, we characterized the enterocyte-like cells. After seeding on cell culture inserts, the TEER values were increased in a time-dependent manner and finally reached a plateau at approximately 100 Ω× cm² (Fig. 3). Therefore, these results suggest that the enterocyte-like cells formed a membrane with a loose tight junction. Using immunofluorescence staining, we found that almost all the cells expressed the intestinal epithelial marker villin, whereas Na⁺–K⁺ ATPase was located only on the basal side (Fig. 4).
Bidirectional transport across the enterocyte-like cell membrane. It has been found that BCRP is highly expressed in the apical membrane of the small intestinal epithelium and plays an important role in intestinal absorption of drug substrates (Giacomini et al., 2010). Thus, we utilized a bidirectional transport assay to examine the enterocyte-like cell membrane on the cell culture inserts. Apical-to-basal of $P_{\text{app}}$ values were $3.32 \pm 1.09$ and $11.53 \pm 0.82 \times 10^{-6}$ cm/sec in the absence or presence of Ko143, respectively ($n = 4$). The basal-to-apical values were $49.96 \pm 7.98$ and $33.72 \pm 9.55 \times 10^{-6}$ cm/sec in the absence or presence of Ko143, respectively ($n = 4$). As shown in Fig. 5, $P_{\text{app}}$ values of Hoechst 33342, a substrate of BCRP (Doyle and Ross, 2003), in the basal-to-apical direction was significantly higher than for the apical to basal direction. The ER, calculated from the ratio of $P_{\text{app}}$ in the basal-to-apical direction to that in the apical-to-basal direction, was 15. From the addition of Ko143, a BCRP inhibitor (Allen et al., 2002), the basal-to-apical $P_{\text{app}}$ values were decreased and the apical-to-basal $P_{\text{app}}$ values were increased. Additionally, the ER value was reduced to 3. Moreover, in immunofluorescence staining, it was indicated that BCRP was located on the apical side (Fig. 6). Therefore, these results indicate that the enterocyte-like cell membrane had also efflux transporter BCRP activity.

Permeability of test compounds across an enterocyte-like cell membrane. We performed a membrane transport study using five test compounds with various $F_a$ (1–97%) values in humans. The $P_{\text{app}}$ values of the five test compounds ranged from 2.04 to $9.99 \times 10^{-6}$ cm/sec; Table 1). The rank order of $P_{\text{app}}$ values of these test compounds corresponded to those of the $F_a$ values. Moreover, the sigmoidal relationship between
the $P_{\text{app}}$ values and the $F_a$ values of the five compounds were observed in differentiated cells (Fig. 7). These results suggest that drug permeability in the enterocyte-like cell membrane may be able to predict $F_a$ in humans.
Discussion

We previously reported that human iPS cell-derived enterocyte-like cells had metabolic functions, such as drug-metabolizing enzyme activities and CYP3A4 inducibility (Iwao et al., 2015). Moreover, in this study, we demonstrated the drug transport characteristics in the enterocyte-like cells.

Human peptide transporter PEPT1 is primarily responsible for the transport of dietary di- and tripeptides from the lumen of the small intestine. PEPT1 has been exploited with prodrugs designed to introduce peptide and peptide bond-like moieties onto the parent molecule. This method was demonstrated to significantly increase the absorption of drugs with poor oral bioavailability (Gomez-Orellana, 2005; Hamman et al., 2005; Leonard et al., 2006; Majumdar and Mitra, 2006). In the enterocyte-like cells, we confirmed the expression of the PEPT1 protein (Fig. 1) and also quantitatively evaluated the PEPT1-mediated uptake activity of glycylsarcosine (Fig. 2). However, PEPT1-mediated uptake using fluorescence-labeled di- or tripeptides was also qualitatively evaluated in previous reports (Iwao et al., 2014; Ozawa et al., 2015). Therefore, it was considered that the enterocyte-like cells may be used as a quantitative evaluation model of uptake transporters such as PEPT1.

To our knowledge, there are few reports involving drug membrane permeability using human iPS-derived enterocytes (Kauffman et al., 2013; Ozawa et al., 2015). However, this evaluation used only TEER measurements and the permeability of a non-absorbable marker (fluorescein isothiocyanate (FITC)-dextran, molecular weight 4 or 150 kDa) in these reports. Thus, we performed a bidirectional transport assay and drug membrane permeability assay to characterize the enterocyte-like cells. TEER values of the enterocyte-like cell membrane on the cell culture inserts were gradually increased and
finally reached a plateau at approximately 100 Ω×cm² (Fig. 3). It was reported that TEER values in the human small intestine were approximately 40 Ω×cm² (Sjoberg et al., 2013). In addition, we previously reported that the values in human small intestinal epithelial cell (HIEC) monolayer were 98.9 Ω×cm² and were even lower in the Caco-2 cell monolayer (900 Ω×cm²) (Takenaka et al., 2014). It was indicated that the TEER value of the enterocyte-like cell membrane was comparable to that of the HIEC monolayer and the human small intestine. We were also able to use immunofluorescence staining to visualize the presence of the intestinal epithelial marker villin and Na⁺–K⁺ ATPase located in the cytoplasm and on the basal side of the enterocyte-like cell membrane, respectively (Fig. 4). Taken together, these results suggest that the enterocyte-like cell membrane formed a loose tight junction similar to the small intestine and also exhibited a polarity.

In the intestine, transporters are localized on the brush border membrane and the basal side of intestinal cells. Four major ATP-binding cassette (ABC) efflux transporters have been shown to localize at the apical/luminal membrane of enterocytes. These are thought to form a barrier to intestinal absorption of the substrate drugs: P-glycoprotein (P-gp), BCRP, multidrug resistance-associated protein (MRP) 2, and MRP4 (Englund et al., 2006; Takano et al., 2006; Maubon et al., 2007). The expression levels of these substrate drugs differed between the segments of the intestine. In general, BCRP, MRP2, and P-gp are expressed at high levels in the small intestine and are considered to be a limiting barrier to oral drug absorption (Shirasaka et al., 2008; Giacomini et al., 2010). In the Food and Drug Administration (FDA) and the Ministry of Health Labour and Welfare (MHLW) guidelines involving in drug interactions, it is indicated that drug candidates should be evaluated to determine whether they are
substrates of efflux transporters such as P-gp and BCRP. Thus, we examined the BCRP-mediated transport activity in the enterocyte-like cells. The basal-to-apical $P_{\text{app}}$ values of Hoechst33342, a substrate of BCRP, were higher than the apical-to-basal $P_{\text{app}}$ values (Fig. 5). By addition of Ko143, a BCRP inhibitor, basal-to-apical $P_{\text{app}}$ values were decreased and apical-to-basal $P_{\text{app}}$ values were increased. ER values were also decreased by the presence Ko143. In addition, BCRP was located on the apical side (Fig. 6). These results demonstrated that the enterocyte-like cells had BCRP-mediated transport activity and that the cell membrane was available for the evaluation of efflux transport.

In our membrane permeability study, the rank order of $P_{\text{app}}$ values of these test compounds corresponded to those of the $F_a$ values (Fig. 7). Takenaka et al. (2014) reported that the HIEC monolayer with loose tight junctions was able to accurately predict the oral absorption of paracellularly absorbed compounds (Takenaka et al., 2014). The enterocyte-like cell membrane could also be useful for the prediction of the $F_a$ values of drugs, including such compounds.

In conclusion, we demonstrated that the enterocyte-like cells exhibited: 1) functions of uptake and efflux transporters; 2) loose tight junctions similar to the human small intestine; and 3) apical/basal polarity. Moreover, it was indicated that the $F_a$ of drugs in humans can be estimated from permeability data of the enterocyte-like cell membrane. In our previous study, we found that the differentiated cells performed drug-metabolizing enzyme activities and CYP3A4 inducibility (Iwao et al., 2015). Taken together, the enterocyte-like cells may be useful as an appropriate model to comprehensively predict drug transport and metabolism in the intestine.
Acknowledgments

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Wrote or contributed to the writing of the manuscript: Nao kodama, Takahiro Iwao, Kinya Ohta, Hiroaki Yuasa, Tamihide Matsunaga
Conflict of Interest

The authors have declared no conflicts of interest.
References


Omkvist DH, Brodin B, and Nielsen CU (2010) Ibuprofen is a non-competitive


Footnotes

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**Figure Legends**

**Fig. 1. Immunofluorescence staining analysis of PEPT1 in the differentiated enterocyte-like cells**

After differentiation, the cells were stained with PEPT1 (red), and DAPI (blue). Scale bar: 50 μm.

**Fig. 2. Uptake of glycylsarcosine in the differentiated enterocyte-like cells**

Following differentiation, the enterocyte-like cells were incubated with a transport buffer (pH 6.0) containing glycylsarcosine at 37°C, with or without 3 mM ibuprofen, or at 4°C. Data are represented as the mean ± S.D. (A: n = 3, B: n = 4). Open and closed symbols bars show the uptake at 37°C and 4°C, respectively, and the gray bar shows the uptake with 3 mM ibuprofen at 37°C. Levels of statistical significance compared with the uptake at 37°C: A: **P < 0.01; B: *P < 0.05.

**Fig. 3. Time-dependent changes of TEER values in the enterocyte-like cell membrane**

The enterocyte-like cells were seeded on Matrigel-coated cell culture inserts. TEER values were measured every three days from day 4 after seeding. Data were represented as the mean ± S.D. (n = 22).

**Fig. 4. Immunofluorescence staining analysis of villin and Na⁺–K⁺ ATPase in the differentiated enterocyte-like cells**

The enterocyte-like cells were seeded on Matrigel-coated cell culture inserts. After differentiation, the cells were stained with villin (green), Na⁺–K⁺ ATPase (red), and
DAPI (blue). Scale bar, 50 μm. I and II are cross-sectional views along the red and green lines, respectively. A: apical side; B: basal side.

**Fig. 5. Bidirectional permeability of Hoechst33342 across the enterocyte-like cell membrane**

The enterocyte-like cells were seeded on Matrigel-coated cell culture inserts. After differentiation, the cells were incubated with the transport buffer (pH 7.4) containing Hoechst33342 (20 μM) for 120 min at 37°C in the presence or absence of Ko143 (10 μM). Data are represented as the mean ± S.D. (n = 4). White or black bars show apical-to-basal or basal-to-apical $P_{\text{app}}$ values, respectively. ER of Hoechst33342 was calculated by dividing $P_{\text{app}}$ of the basal-to-apical transport by that of the apical-to-basal transport. Levels of statistical significance compared with each $P_{\text{app}}$ value in the absence of Ko143: *$P < 0.05$; **$P < 0.01$; and compared with each $P_{\text{app}}$ values for apical-to-basal transport: †$P < 0.01$.

**Fig. 6. Immunofluorescence staining analysis of BCRP in the differentiated enterocyte-like cells**

The enterocyte-like cells were seeded on Matrigel-coated cell culture inserts. After differentiation, the cells were stained with BCRP (green) and DAPI (blue). Scale bar, 50 μm. I and II are cross-sectional views along the red and green lines, respectively. A: apical side; B: basal side.

**Fig. 7. Relationship between $F_a$ values and $P_{\text{app}}$ of test compounds across the enterocyte-like cell membrane**
The enterocyte-like cells were seeded on Matrigel-coated cell culture inserts. Following differentiation, the cells were incubated with the transport buffer (pH 7.4) containing antipyrine, atenolol, metoprolol, sulpiride, or PEG4000 for 120 min at 37°C. The correlation curve was fitted by using the following formula: $F_a = \frac{1}{(0.01 + 5.28 * 0.36^{P_{app}})}$. Data were represented as the mean ± S.D. ($n = 4$). $P < 0.01$; $R = 0.99$. 
Table 1. *P*<sub>app</sub> values of test compounds in the enterocyte-like cell membrane

<table>
<thead>
<tr>
<th>Compounds</th>
<th><em>P</em>&lt;sub&gt;app&lt;/sub&gt; (×10&lt;sup&gt;-6&lt;/sup&gt; cm/sec)</th>
<th>Fa (%)&lt;sup&gt;a&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± S.D.</td>
<td></td>
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<tr>
<td>Antipyrine</td>
<td>9.99 ± 2.59</td>
<td>97</td>
</tr>
<tr>
<td>Metoprolol</td>
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<td>Atenolol</td>
<td>6.10 ± 1.19</td>
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<td>Sulpiride</td>
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<tr>
<td>PEG4000</td>
<td>2.04 ± 0.43</td>
<td>&gt;1</td>
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*P*<sub>app</sub> values were represented as the mean ± S.D. (*n* = 4).

<sup>a</sup>*Fa* values were obtained from published data (Rozehnal et al., 2012).
Fig. 1. Immunofluorescence staining of PEPT1
Fig. 2. Uptake of glycylysarcosine

**A**

![Graph showing uptake of glycylysarcosine over time at 37°C and 4°C. The graph shows a significant increase in uptake at 37°C compared to 4°C.]

**B**

![Bar graph showing % of control for control, 3 mM ibuprofen, and 4°C. The graph indicates a significant decrease in % of control for 3 mM ibuprofen and 4°C compared to control.]
Fig. 3. Time-dependent changes of TEER values
Fig. 4. Immunofluorescence staining of villin and Na⁺-K⁺ ATPase
Fig. 5. $P_{\text{app}}$ of Hoechst33342

- Apical-to-basal
- Basal-to-apical

ER = 15.1

ER = 2.9

Ko143

(-)  (+)
Fig. 6. Immunofluorescence staining of BCRP
Fig. 7. $P_{\text{app}}$ values of antipyrine, atenolol, metoprolol, sulpiride, and PEG4000 across differentiated cell membrane