Supplemental Data

Application of Intestinal Epithelial Cells Differentiated from Human Induced Pluripotent Stem Cells for Studies of Prodrug Hydrolysis and Drug Absorption in the Small Intestine

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Cultivation of hiPSCs. Undifferentiated hiPSCs were maintained on a feeder layer of mitomycin C–treated murine embryonic fibroblasts (MEFs) in hiPSC maintenance medium [DMEM/F-12 (Sigma-Aldrich) supplemented with 20% (v/v) Knockout Serum Replacement (KSR) (Thermo Fisher Scientific), 0.8% (v/v) MEM Non-Essential Amino Acids (NEAA) solution (Thermo Fisher Scientific), 1% (v/v) L-Glutamine–Penicillin–Streptomycin solution (Sigma-Aldrich), 100 μM β-mercaptoethanol (Thermo Fisher Scientific), and 5 ng/mL basic fibroblast growth factor (bFGF; ReproCell, Yokohama, Japan)] at 37°C in humidified atmosphere of 5% CO_2 and 95% air. The medium was changed every day.

Differentiation from hiPSCs into Intestinal Organoids. To generate intestinal organoids, hiPSCs were subjected to stepwise differentiation toward DEs, HGs, and eventual intestinal organoids. The differentiation methods were conducted with minor modifications of the previous report (Spence et al., 2011).

After hiPSCs were pre-incubated with 10 μM Y27632 for 1 hr, feeder cells were removed using dissociation solution (ReproCELL) and colonies of hiPSCs were subsequently dissociated into single cells using accutase (Innovative Cell Tech, San Diego, CA). Dissociated hiPSCs were seeded on 24 well plates (Corning Inc.) coated with Corning Matrigel hESC-Qualified matrix (Corning Inc.) at 3.0×10^5 cells/well, and cultured in hiPSC maintenance medium with 10 μM Y27632 for 24 hr. Next, hiPSCs were cultured for 3 days in DE differentiation medium [RPMI 1640 medium (Thermo Fisher Scientific) supplemented with 100 ng/mL Activin A (R&D systems), 2 mM L-glutamine, 100 units/ml penicillin-streptomycin, and varying concentrations of FBS] to differentiate into DEs. In this culture, the concentrations of FBS were set at 0% on

day 1, 0.2% on day 2, and 2% on day 3. DEs were subsequently cultured for 4 days in HG differentiation medium [RPMI 1640 medium supplemented with 500 ng/ml Wnt3a (R&D systems), 500 ng/ml fibroblast growth factor 4 (FGF4; R&D systems), 2 mM L-glutamine, 100 units/ml penicillin-streptomycin, and 2% FBS] with medium change once daily to obtain HGs. After differentiation into HGs, the whole cells in each well were collected using a cell scraper, and embedded in 55 μl of Corning Matrigel Basement Membrane Matrix (Corning Inc.) containing 500 ng/ml R-Spondin 1, 100 ng/ml Noggin, and 50 ng/ml EGF on Nunclon delta surface tissue culture dishes (Thermo Fisher Scientific). After the Matrigel was solidified at 37°C, the cells were cultured with intestinal differentiation medium [Advanced DMEM/F12 supplemented with 500 ng/ml R-Spondin 1, 100 ng/ml Noggin, 50 ng/ml EGF, 2 mM L-glutamine, 100 units/ml penicillin-streptomycin, 15 mM HEPES, and 2% (v/v) B27 supplement] for 51-69 days to further differentiate into intestinal organoids. In this incubation, the medium was changed every 3 days. All cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95 % air.

Isolation of Intestinal Epithelial Cells from Intestinal Organoids. Intestinal epithelial cells were isolated by using MACS technique (Miltenyi Biotec, Bergisch Gladbach, Germany) with a magnetic microbeads antibody of CD326 (epithelial cell adhesion molecule; EpCAM), an epithelial cell marker protein. After intestinal organoids were pre-incubated with 10 μM Y27632 for 1 hr, the organoids were washed once with PBS, collected into a tube, and incubated in PBS with 2 mM EDTA (Thermo Fisher Scientific) and 1 mM dithiothreitol (Sigma-Aldrich) for 30 min at 4°C. The suspension was centrifuged at 260 × g for 4 min at room temperature, and the pellet was

suspended and incubated in DMEM/F12 (Thermo Fisher Scientific) with 3 units/ml Dispase II (Roche, Mannheim, Germany), 1% FBS, and 10 μM Y27632 for 20 min at 37°C. The cells were filtrated through a 70 μm cell strainer (Corning Inc.) and centrifuged at 260 × g for 4 min at room temperature. The pellet was suspended in 500 μL of MACS buffer (PBS with 0.1 mM EDTA and 0.5% BSA) containing 2% (v/v) FcR Blocking Reagent (Miltenyi Biotec) and incubated for 10 min at 4°C to avoid non-specific cell labeling. Subsequently, 125 μL of EpCAM MicroBeads (Miltenyi Biotec) was added and the cells were incubated for 15 min at 4°C. After addition of 10 mL of MACS buffer, the suspension was centrifuged at 260 × g for 4 min at room temperature. The obtained pellet was resuspended with MACS buffer, applied onto a MACS column (Miltenyi Biotec) placed in a MACS magnetic holder (Miltenyi Biotec), and 4 mL of MACS buffer was passed through the column three times. Then, the column was removed from a magnetic holder, 5 mL of MACS buffer was added to this column, and EpCAM positive cells were flushed out by firmly pushing the plunger into a tube. After centrifuge at 260 × g for 4 min at room temperature, the resulting pellet was defined as hiPSC-IECs in the present study and suspended with IEC maintenance medium containing 10 μM Y27632. The cell suspension of hiPSC-IECs was subsequently seeded on 24 well Transwell inserts for transcellular transport studies as described in Materials and Methods.

Quantification of Gene Expression of Markers of Undifferentiated Cells, DEs, HGs, and Small Intestine Using qPCR Analysis. Unlike the qPCR for transporters and metabolic enzymes as described in Materials and Methods, qPCR for 15 genes [OCT3/4, SOX2, SOX17, FOXA2, GATA4, CDX2, LGR5, ISX, VIL, SI,

LYZ, CHGA, MUC2, TFF3, and GAPDH (primer 2)] was performed by using an Applied Biosystems 7500 Real Time PCR System using an One Step SYBR® PrimeScript PLUS RT-PCR Kit (Takara Bio Inc., Otsu, Japan), where cDNA synthesis and PCR amplification steps are performed in a single reaction. The reaction solution consisted of 2 μL of RNA, 0.8 μL of 10 μM forward primer (0.4 μM in reaction solution), 0.8 μL of 10 μM reverse primer (0.4 μM in reaction solution), 10 μL of 2 × One Step SYBR® RT-PCR Buffer 4, 0.4 μL of PrimeScript PLUS RTase Mix, 1.2 μL of TaKaRa Ex Taq HS Mix, 0.4 μL of 50 × ROX Reference Dye II, and 4.4 μL of distilled water. The reverse transcription reactions were conducted under the following conditions: 42°C for 5 min, 95°C for 10 sec, followed by 50 cycles of 95°C for 5 sec, 60°C for 34 sec. At the end of each run, a melt curve was generated under the following conditions: 95°C for 15 sec, 60°C for 1 min, and then increasing temperature up to 95°C at 0.5°C increments. The primer information is shown in Table S3. The expression level of each target gene was normalized by that of GAPDH (primer 2). Relative expression of the target gene to GAPDH was calculated using the comparative ΔCt method where ΔCt is obtained by subtracting Ct of GAPDH from the target gene. The relative expression levels of target gene to GAPDH were expressed as $2^{-\Delta Ct}$.

Immunofluorescence Staining. HiPSCs, differentiated DEs and HGs were washed with PBS, and fixed with 4% formaldehyde for 20 min at room temperature. The cells were then washed with PBS and blocked with PBS containing 0.1% Triton-X-100 and 10% normal donkey serum (Jackson Immuno Research Labs., West Grove, PA) for 45 min at room temperature. After washing with PBS, the cells incubated with primary antibodies, 10 μg/mL goat anti-human SOX17 antibody (R&D

Systems, Cat. No. AF1924) or prediluted mouse anti-human CDX2 antibody (BioGenex, San Ramon, CA, Cat. No. AM392), overnight at 4°C. Primary antibodies were removed by washing with PBS, the cells incubated with secondary antibodies, a 1:500 dilution of Alexa Fluor 455-labeled donkey anti-goat antibody (Invitrogen, Cat. No. A-11055) or a 1:500 dilution of Alexa Fluor 455-labeled donkey anti-mouse antibody (Invitrogen, Cat. No. A-31570), for 1 hr at room temperature. Secondary antibodies were removed by washing with PBS, and the cells were incubated with PBS containing Hoechst 33342 (1.62 μM) for 15 min at room temperature. After washing with PBS, PBS was finally added to the cells, and then the fluorescence was measured using a BZ-X710 All-in-One fluorescence microscope (Keyence, Osaka, Japan).

Tight Junction formation in hiPSC-IEC monolayers. An intercellular tight junction barrier restricts permeation through the paracellular pathway in the small intestine. A previous study demonstrated that the P_{app} value of lucifer yellow, a marker for paracellular transport, in Caco-2 cells conspicuously increased when the TEER values were below 200 $\Omega \times cm^2$, but was almost independent of TEER values when they were over 200 $\Omega \times cm^2$ (Hashimoto et al., 1994). This suggested that TEER values exceeding 200 $\Omega \times cm^2$ are needed to study paracellular transport in epithelial cells. The present study showed that the TEER values of hiPSC-IECs increased gradually over time, reaching 299 $\Omega \times cm^2$ at 21 days after seeding on Transwell inserts (Fig. 2A). Also, the P_{app} value of [^{14}C]mannitol, a marker for paracellular transport, in hiPSC-IECs was statistically similar to that of Caco-2 cells showing high TEER values (891 $\Omega \times cm^2$) (Fig. 2B). These results suggested that hiPSC-IECs formed tight junctions strong enough to allow study of paracellular transport.

Membrane localization of the P-gp, BCRP, and PEPT1 in hiPSC-IECs from the Result of Functional Analysis in the Transcellular Transport Study. It is important to examine the membrane localization of the P-gp, BCRP, and PEPT1 for discussing whether hiPSC-IECs can reflect small intestine. In the present study, the membrane localization of P-gp, BCRP, and PEPT1 in hiPSC-IECs was not measured by immunofluorescence confocal microscopy. However, the membrane localization of these transporters can be discussed from the result of functional analysis in the transcellular transport study. P-gp and BCRP play a role for the efflux of substrates from the intracellular-to-extracellular space by driving force of ATP hydrolysis at the intracellular ATP-binding pockets (Higgins and Linton, 2004). PEPT1 transports the substrates from the extracellular-to-intracellular space by the proton gradient (Rubio-Aliaga and Daniel, 2008). As described in Fig. 3, the P_{app} values of [^{3}H]digoxin (a P-gp substrate) and sulfasalazine (a BCRP substrate) in the basolateral-to-apical direction were significantly higher than that in apical-to-basolateral direction, and the transport of [^{14}C]glycylsarcosine was observed in apical-to-basolateral direction (Fig. 3). Therefore, P-gp and BCRP in hiPSC-IECs is consider to mediate the efflux of intracellular-to-apical side, and PEPT1 in hiPSC-IECs is considered to mediate influx of apical-to-intracellular side, indicating that P-gp, BCRP, and PEPT1 could be expressed in the apical membrane of hiPSC-IECs, likewise human small intestine.

Pharmacokinetic Theory for Comparison between CL_{int} Determined Using hiPSC-IECs and Intestinal Absorption Process of Six Ester Prodrugs. The CL_{int} values of six prodrugs in hiPSC-IECs were determined by using (Eq. 4) as described in

Materials and Methods. According the pharmacokinetic model of Fig. S4, CL_{int} could also be expressed by Eq. S1.

$$CL_{int} = PS_{a,\,inf\,(PD)} \times \frac{CL_{hydro\,(PD)}}{PS_{a,\,eff\,(PD)} + PS_{b,\,eff\,(PD)} + CL_{hydro\,(PD)}} \quad \text{(Eq. S1)}$$

where $PS_{a,\,inf\,(PD)}$ (μL/min), $PS_{a,\,eff\,(PD)}$ (μL/min), and $PS_{b,\,eff\,(PD)}$ (μL/min) represent the PS product, the product of the permeability coefficient and the surface area of plasma membrane of per intestinal epithelial cell, for the apical influx, apical efflux, and basolateral efflux of a prodrug, respectively. $CL_{hydro\,(PD)}$ (μL/min) represents the hydrolysis-mediated clearance of a prodrug into its active form in the intestinal epithelial cell.

As shown in Fig. S4, the equation of CL_{int} reflects two processes: permeation of the prodrug into the cells and hydrolysis of the prodrug to its active form in the cells. Whereas, besides these two processes, intestinal absorption of prodrugs includes the process of the basolateral efflux of the active form produced by hydrolysis of prodrugs in the cells. In the present study, we define $CL_{a\,to\,b\,(PD\,to\,AF)}$ (μL/min) as the conversion clearance from a prodrug in the apical side to its active form in the basolateral side via permeation and hydrolysis in intestinal epithelial cells. The equation of $CL_{a\,to\,b\,(PD\,to\,AF)}$ reflects these three processes: the apical influx of a prodrug, hydrolysis of a prodrug into the active form in the cells, and the basolateral efflux of the active form as follows:

$$CL_{a\,to\,b\,(PD\,to\,AF)} = PS_{a,\,inf\,(PD)} \times \frac{CL_{hydro\,(PD)}}{PS_{a,\,eff\,(PD)} + PS_{b,\,eff\,(PD)} + CL_{hydro\,(PD)}} \times \frac{PS_{b,\,eff\,(AF)}}{PS_{a,\,eff\,(AF)} + PS_{b,\,eff\,(AF)}} \quad \text{(Eq. S2)}$$

where $PS_{a,\ eff\ (AF)}$ (μL/min), and $PS_{b,\ eff\ (AF)}$ (μL/min) represent the PS product for the apical efflux and basolateral efflux of the active form, respectively.

Hence, Eq. S2 can be converted to Eq. S3 using Eq. S1.

$$CL_{a\ to\ b\ (PD\ to\ AF)} = CL_{int} \times \frac{PS_{b,\ eff\ (AF)}}{PS_{a,\ eff\ (AF)} + PS_{b,\ eff\ (AF)}} = CL_{int} \times \alpha \quad \text{(Eq. S3)}$$

The value of α reflects the process of the basolateral efflux of the hydrolyzed active form. Since the six prodrugs we investigated were all hydrolyzed into a common active form in the present study, the α value in Eq. S3 is the same for the six prodrugs (Fig. S1).

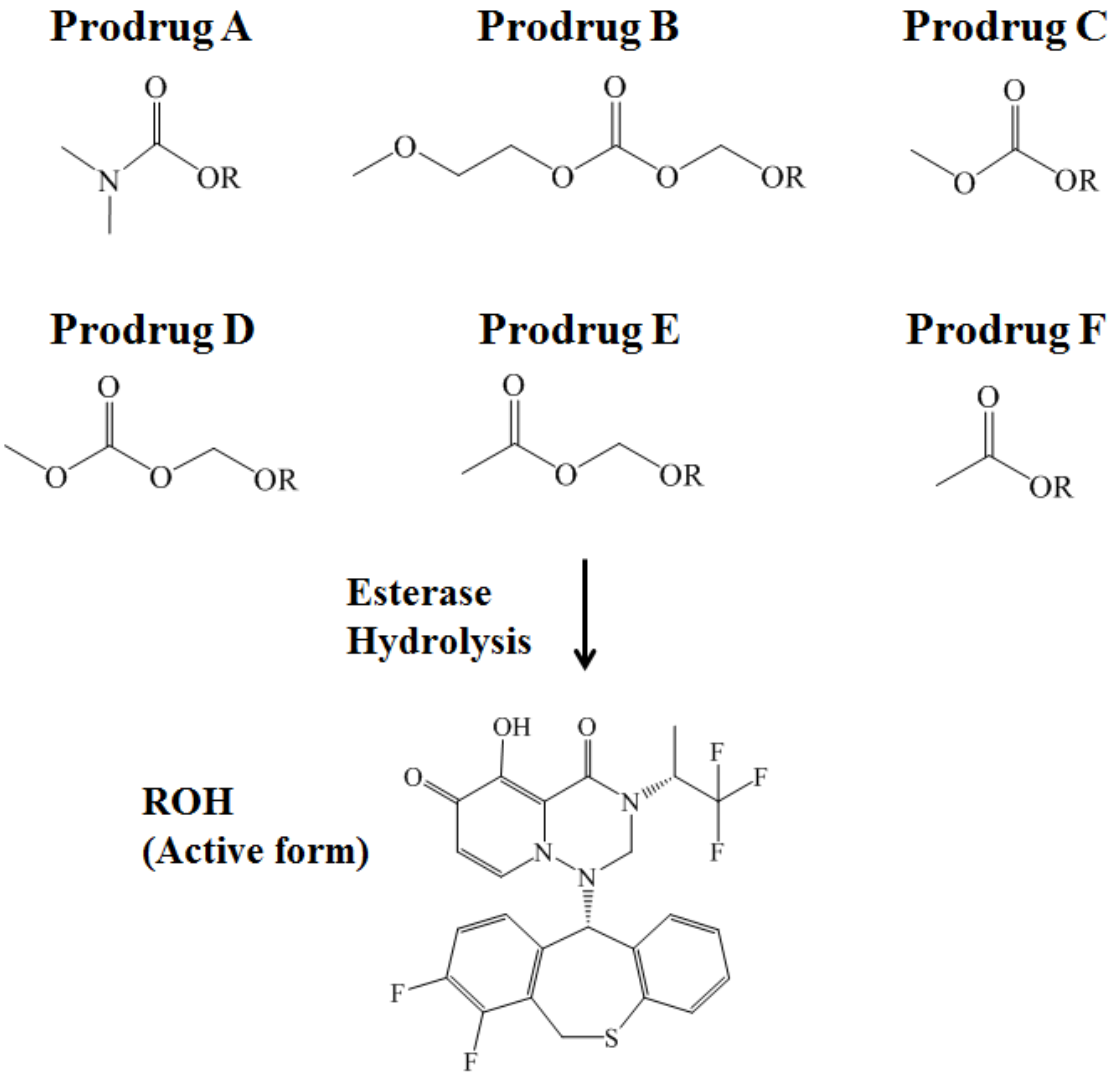

Fig. S1. Structures of six ester prodrugs and their hydrolysis into the active form by esterase. Six ester prodrugs (prodrugs A, B, C, D, E, and F) and their active form (ROH), the candidates of anti-influenza virus agents, are in-house compounds synthesized by Shionogi & Co., Ltd. These six prodrugs are all hydrolyzed into the same active form (ROH). The study of structure-activity relationships in the development of anti-influenza virus agents containing these compounds will be summited for publication.

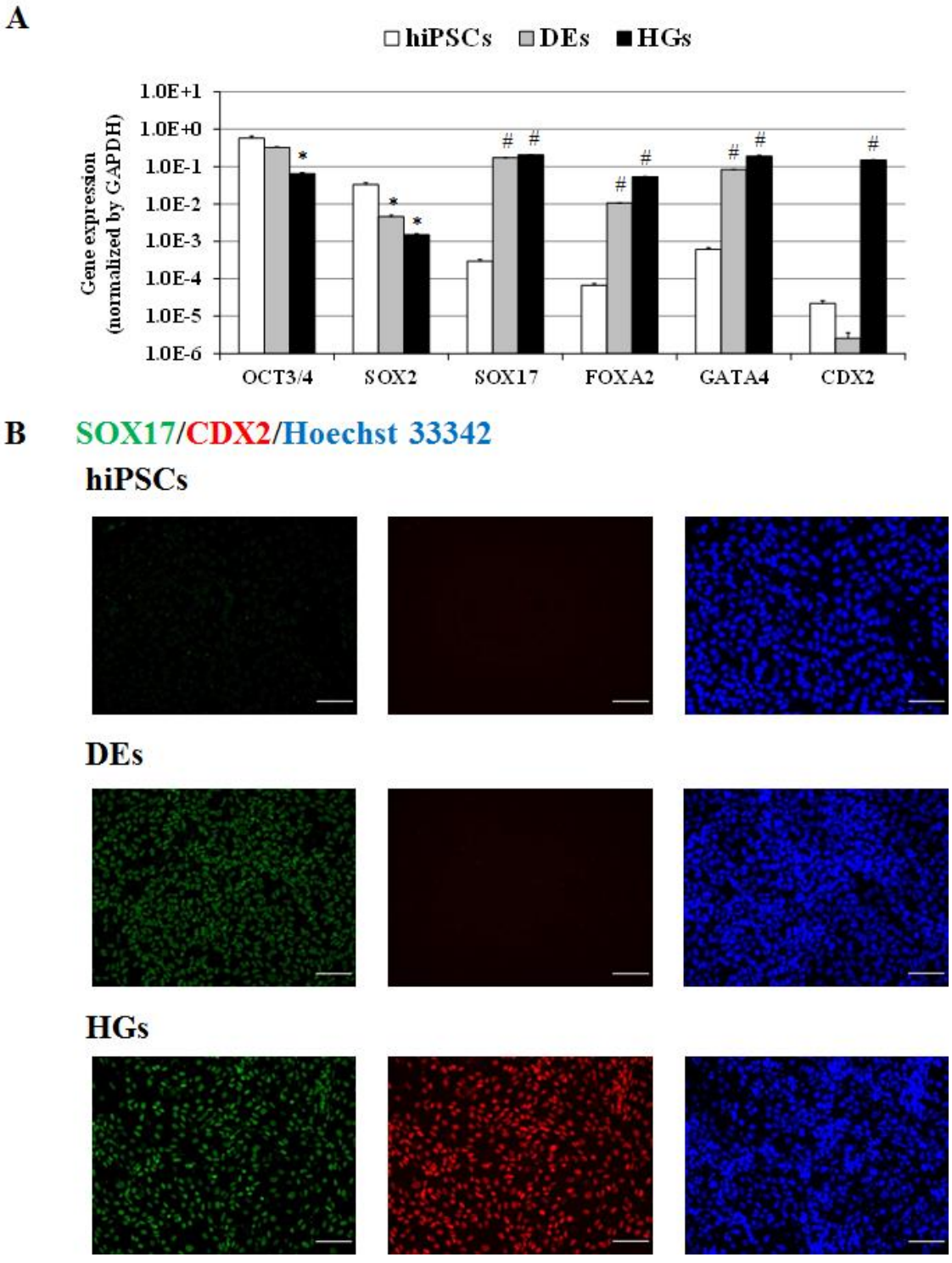

Fig. S2. Marker expressions in hiPSCs, differentiated DEs, and HGs.

HiPSCs were cultured in the presence of Activin A for 3 days to differentiate DEs, and

then the DEs was cultured in the presence of Wnt3a and FGF4 for 4 days to differentiate HGs. (A) Gene expression levels of undifferentiated markers (OCT3/4 and SOX2), DE markers (SOX17, FOXA2, and GATA4), a HG marker (CDX2) in hiPSCs, DEs, and HGs. Total RNA of each cell was extracted at each differentiated stage. Gene expression levels of markers were determined by qPCR analysis. Gene expression level of each marker was normalized by that of GAPDH and the relative expression ($2^{-\Delta Ct}$) calculated as described in Materials and Methods. Each data item represents the means (± S.E.M.) of triplicate analyses in a single sample. * and $^{\#}$ indicate significant difference of the quantitative value compared with hiPSCs (*$p < 0.05$; $^{\#}p < 0.01$). (B) Immunofluorescence analysis of SOX17 and CDX2 in hiPSCs, DEs, and HGs. Left (green), middle (red), and right (blue) panel showed staining of SOX17, CDX2 and Hoechst 33342, respectively. Scale bar represents 100 μm.

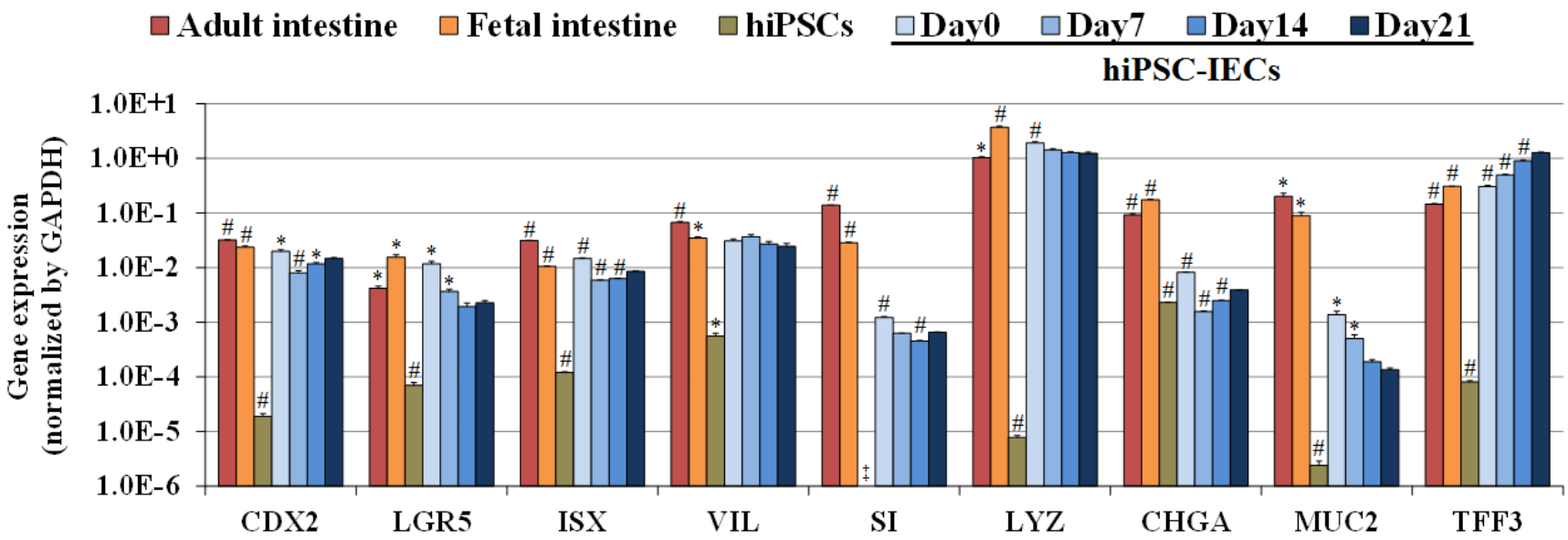

Fig. S3. Gene expression levels of intestinal markers in adult small intestine, fetal small intestine, hiPSCs, and hiPSC-IECs.

The gene expression levels of intestinal markers in adult small intestine (adult intestine), fetal small intestine (fetal intestine), hiPSCs, and hiPSC-IECs were examined by qPCR analysis as described in Materials and Methods section. The total RNAs of adult intestine and fetal intestine were purchased from Clonetech and BioChain Institute Inc., respectively. The total RNAs of hiPSCs and hiPSC-IECs were isolated using PureLink® RNA Mini Kit. hiPSC-IECs were cultured on Transwell inserts for defined periods; day 0 represents the cells before seeding on the Transwell insert, and day 7, day 14, and day 21 represent the cells cultured on the Transwell insert for 7, 14, and 21 days. The expression level of each gene was normalized by that of GAPDH, and the relative expression ($2^{-\Delta Ct}$) calculated as

described in Materials and Methods. Each data represents the means (± S.E.M.) of triplicate analyses in a single sample. * and # indicate significant difference of the quantitative value compared with day 21 (*$p < 0.05$; #$p < 0.01$). ‡The gene was not detected.

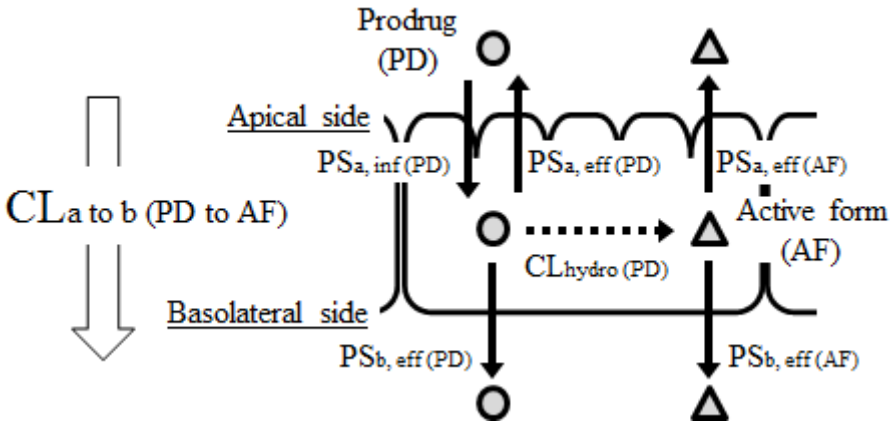

Fig. S4. Schematic diagram illustrating behavior of a prodrug around intestinal epithelial cells. $CL_{a\ to\ b\ (PD\ to\ AF)}$ is defined as the conversion clearance from a prodrug in the apical side to its active form in the basolateral side through the permeation and hydrolysis in intestinal epithelial cells. PS product is also defined as the product of the permeability coefficient and the surface area of plasma membrane of per intestinal epithelial cell in the present study. $PS_{a,\ inf\ (PD)}$, $PS_{a,\ eff\ (PD)}$, and $PS_{b,\ eff\ (PD)}$ represent the PS product for the apical influx, apical efflux, and basolateral efflux of a prodrug, respectively. $PS_{a,\ eff\ (AF)}$ and $PS_{b,\ eff\ (AF)}$ represent the PS product for the apical efflux and basolateral efflux of the active form, respectively. $CL_{hydro\ (PD)}$ represents the hydrolysis-mediated clearance of a prodrug into its active form in intestinal epithelial cell.

Table S1. Generic name of prodrugs A, B, C, D, E, and F and the active form

Compound	Generic name
Prodrug A	1-((S)-7,8-difluoro-6,11-dihydrodibenzo[b,e]thiepin-11-yl)-4,6-dioxo-3-((R)-1,1,1-trifluoropropan-2-yl)-2,3,4,6-tetrahydro-1H-pyrido[2,1-f][1,2,4]triazin-5-yl dimethylcarbamate
Prodrug B	((1-((S)-7,8-difluoro-6,11-dihydrodibenzo[b,e]thiepin-11-yl)-4,6-dioxo-3-((R)-1,1,1-trifluoropropan-2-yl)-2,3,4,6-tetrahydro-1H-pyrido[2,1-f][1,2,4]triazin-5-yl)oxy)methyl (2-methoxyethyl) carbonate
Prodrug C	1-((S)-7,8-difluoro-6,11-dihydrodibenzo[b,e]thiepin-11-yl)-4,6-dioxo-3-((R)-1,1,1-trifluoropropan-2-yl)-2,3,4,6-tetrahydro-1H-pyrido[2,1-f][1,2,4]triazin-5-yl methyl carbonate
Prodrug D	((1-((S)-7,8-difluoro-6,11-dihydrodibenzo[b,e]thiepin-11-yl)-4,6-dioxo-3-((R)-1,1,1-trifluoropropan-2-yl)-2,3,4,6-tetrahydro-1H-pyrido[2,1-f][1,2,4]triazin-5-yl)oxy)methyl methyl carbonate
Prodrug E	((1-((S)-7,8-difluoro-6,11-dihydrodibenzo[b,e]thiepin-11-yl)-4,6-dioxo-3-((R)-1,1,1-trifluoropropan-2-yl)-2,3,4,6-tetrahydro-1H-pyrido[2,1-f][1,2,4]triazin-5-yl)oxy)methyl acetate
Prodrug F	1-((S)-7,8-difluoro-6,11-dihydrodibenzo[b,e]thiepin-11-yl)-4,6-dioxo-3-((R)-1,1,1-trifluoropropan-2-yl)-2,3,4,6-tetrahydro-1H-pyrido[2,1-f][1,2,4]triazin-5-yl acetate
Active form	1-((S)-7,8-difluoro-6,11-dihydrodibenzo[b,e]thiepin-11-yl)-5-hydroxy-3-((R)-1,1,1-trifluoropropan-2-yl)-2,3-dihydro-1H-pyrido[2,1-f][1,2,4]triazine-4,6-dione

Table S2. Primer information in real-time RT-PCR using a TaqMan® Universal PCR Master Mix

Gege name	GenBank accession number	TaqMan Assay number
CYP3A4	NM_017460	Hs00430021_m1
CYP3A7	NM_000765	Hs00426361_m1
CES1	NM_001025195	Hs00275607_m1
CES2	NM_003869	Hs01077945_m1
UGT1A1	NM_000463	Hs02511055_s1
P-gp	NM_000927	Hs00184500_m1
BCRP	NM_004827	Hs01053790_m1
PEPT1	NM_005073	Hs00953898_m1
GAPDH (primer 1)	NM_002046	Hs02758991_g1

Table S3. Primer information in real-time RT-PCR using a One Step SYBR® PrimeScript™ PLUS RT-PCR Kit

Gege name	GenBank accession number	Primer sequence	
		Sense (5'→3')	Antisense (5'→3')
OCT3/4	NM_002701	TGAAGCTGGAGAAGGAGAAGCTG	GCAGATGGTCGTTTGGCTGA
SOX2	NM_003106	TGAGCGCCCTGCAGTACAA	GCTGCGAGTAGGACATGCTGTAG
SOX17	NM_022454	CTGCAGGCCAGAAGCAGTGTTA	CCCAAACTGTTCAAGTGGCAGA
FOXA2	NM_021784	GGTGTACTCCCGGCCCATTA	CAGAGTTAGCCGGGCCTGAA
GATA4	NM_001308093	TTACACGCTGATGGGACTGGAG	TCGGTGCTAGAAACACAATGCAA
CDX2	NM_001265	TTCACTACAGTCGCTACATCACC	CTGCGGTTCTGAAACCAGATT
LGR5	NM_003667	CAGCCATCAAGCAGGTGTTCA	ATGCTGGAATGTTTCAGGCTCA
ISX	NM_001303508	TCTCTAGCACCGCTGGATGAA	CTGCTACCCAAGTGATGAGCTACTG
VIL	NM_007127	GCTTGGCAACTCTAGGGACTGG	TGAGGTTGCTGTTAGCATTGAACAC
SI	NM_001041	AGACAACTATGCACGATGGGACAA	CATCCAGCGGGTACAGAGATGA
LYZ	NM_000239	GAGTTACACTCCACAACCTTGAACA	CCGTGATCCACAAGGCATTA
CHGA	HM_001275	TTGGAGAGCGAGGTCTTGGAG	TGCCTGTCAGCCAGGAATGT

MUC2	NM_002457	GATGCAAATGCTGGCATCAAAG	CAACCAGCACGTCATCCTGAA
TFF3	NM_003226	CTGCTGCTTTGACTCCAGGAT	CAGCTGGAGGTGCCTCAGAA
GAPDH (primer 2)	NM_002046	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA

Table S4. Conditions for LC-MS/MS analysis.

Compound	LC conditions					MS conditions	
	Instrument, column	Mobile phase A	Mobile phase B	Gradient condition Time (min); B concentration %	Flow rate (mL/min)	Instrument, Ionization Mode	SRM transition (m/z)
Transcellular transport studies across hiPSC-IECs							
Sulfasalazine	1, 1	0.1 % FA in water	MeCN	0-0.20 min; 20% → 0.50-0.90 min; 60% → 0.91-1.10 min; 95% → 1.11-1.40 min; 20%	0.75	1, Positive	399.1 /223.1
Midazolam	1, 1	0.1 % FA in water	MeCN	0-0.20 min; 20% → 0.50-0.90 min; 60% → 0.91-1.10 min; 95% → 1.11-1.40 min; 20%	0.75	1, Positive	326.1 /291.1
1-OH Midazolam	1, 1	0.1 % FA in water	MeCN	0-0.20 min; 20% → 0.50-0.90 min; 60% → 0.91-1.10 min; 95% → 1.11-1.40 min; 20%	0.75	1, Positive	342.1 /203.1
Irinotecan	1, 1	0.1 % FA in water	MeCN	0-0.20 min; 20% → 0.50-0.90 min; 60% → 0.91-1.10 min; 95% → 1.11-1.40 min; 20%	0.75	1, Positive	587.2 /543.1
SN38	1, 1	0.1 % FA	MeCN	0-0.20 min; 5% → 0.30-0.90 min; 40% →	0.75	1, Positive	393.2

		in water		0.91-1.10 min; 95% → 1.11-1.40 min; 5%			/263.0
Temocapril	1, 1	0.1 % FA in water	MeCN	0-0.10 min; 40% → 0.40-0.70 min; 80% → 0.71-0.90 min; 95% → 0.91-1.20 min; 40%	0.75	1, Positive	477.1 /270.0
Temocaprilat	1, 1	0.1 % FA in water	MeCN	0-0.10 min; 5% → 0.30-0.70 min; 70% → 0.71-0.90 min; 95% → 0.91-1.20 min; 5%	0.75	1, Positive	449.2 /190.1
Ganciclovir	1, 1	0.1 % FA in water	MeCN	0-0.10 min; 5% → 0.50-0.70 min; 20% → 0.71-0.90 min; 95% → 0.91-1.20 min; 5%	0.75	1, Positive	256.1 /152.1
Doxorubicin	1, 1	0.1 % FA in water	MeCN	0-0.10 min; 5% → 0.50-0.70 min; 90% → 0.71-0.90 min; 95% → 0.91-1.20 min; 5%	0.75	1, Positive	544.2 /397.0
Famotidine	1, 1	0.1 % FA in water	MeCN	0-0.20 min; 5% → 0.90-1.10 min; 95% → 1.11-1.40 min; 5%	0.75	1, Positive	338.0 /189.1
Sulpiride	1, 1	0.1 % FA in water	MeCN	0-0.10 min; 5% → 0.50-0.70 min; 60% → 0.71-0.90 min; 95% → 0.91-1.20 min; 5%	0.75	1, Positive	342.1 /112.1
Atenolol	1, 2	0.1 % FA in water	MeCN	0-0.20 min; 5% → 0.60-0.70 min; 80% → 0.71-1.00 min; 95% → 1.01-1.30 min; 5%	0.75	1, Positive	267.2 /190.1

Furosemide	1, 1	0.1 % FA in water	MeCN	0-0.10 min; 20% → 0.60-0.90 min; 60% → 0.91-1.10 min; 95% → 1.11-1.40 min; 20%	0.75	1, Negative	329.0 /284.9
Ranitidine	1, 1	0.1 % FA in water	MeCN	0-0.10 min; 5% → 0.50-0.70 min; 80% → 0.71-0.90 min; 95% → 0.91-1.20 min; 5%	0.75	1, Positive	315.1 /130.1
Hydrochlorothiazide	1, 1	0.1 % FA in water	MeCN	0-0.10 min; 5% → 0.50-0.70 min; 80% → 0.71-0.90 min; 95% → 0.91-1.20 min; 5%	0.75	1, Negative	295.9 /268.9
Acetaminophen	1, 1	0.1 % FA in water	MeCN	0-0.10 min; 5% → 0.50-0.70 min; 80% → 0.71-0.90 min; 95% → 0.91-1.20 min; 5%	0.75	1, Positive	152.1 /110.1
Propranolol	1, 1	0.1 % FA in water	MeCN	0-0.10 min; 25% → 0.60-0.70 min; 60% → 0.71-0.90 min; 95% → 0.91-1.20 min; 25%	0.75	1, Positive	260.2 /183.1
Antipyrine	1, 1	0.1 % FA in water	MeCN	0-0.20 min; 5% → 0.50-0.80 min; 80% → 0.81-1.00 min; 95% → 1.01-1.30 min; 5%	0.75	1, Positive	189.1 /56.1
Prodrug A	1, 3	0.1 % FA in water	MeCN	0-1.30 min; 50% → 0.31-1.50 min; 95% → 1.51-1.80 min; 50%	0.55	1, Positive	595.1 /247.0
Prodrug B	1, 3	0.1 % FA	MeCN	0-0.80 min; 53% → 0.81-1.30 min; 95% →	0.75	1, Positive	656.2

		in water		1.31-1.60 min; 53%			/247.0
Prodrug C	1, 3	0.1 % FA in water	MeCN	0-0.80 min; 53% → 0.81-1.30 min; 95% → 1.31-1.60 min; 53%	0.75	1, Positive	582.1 /247.1
Prodrug D	1, 3	0.1 % FA in water	MeCN	0-0.80 min; 53% → 0.81-1.30 min; 95% → 1.31-1.60 min; 53%	0.75	1, Positive	612.1 /247.1
Prodrug E	1, 3	0.1 % FA in water	MeCN	0-0.80 min; 53% → 0.81-1.30 min; 95% → 1.31-1.60 min; 53%	0.75	1, Positive	596.1 /247.0
Prodrug F	1, 3	0.1 % FA in water	MeCN	0-0.80 min; 53% → 0.81-1.30 min; 95% → 1.31-1.60 min; 53%	0.75	1, Positive	566.4 /247.1
Active form (condition 1)	1, 3	0.1 % FA in water	MeCN	0-1.30 min; 50% → 0.31-1.50 min; 95% → 1.51-1.80 min; 50%	0.55	1, Positive	524.1 /247.1
Active form (condition 2)	1, 3	0.1 % FA in water	MeCN	0-0.80 min; 53% → 0.81-1.30 min; 95% → 1.31-1.60 min; 53%	0.75	1, Positive	524.1 /247.1
Pharmacokinetics studies in rat							
Active form	2, 4	0.1 % FA	MeCN	0-0.90 min; 50% → 0.90-1.10 min; 95% →	0.75	2, Positive	524.0

(condition 3)		/ 0.05 % AA in water		1.11-1.40 min; 50%			/247.0
Active form (condition 4)	2, 4	0.1 % FA / 0.05 % AA in water	MeCN	0-0.20 min; 35% → 0.50-0.90 min; 60% → 0.90-1.10 min; 95% → 1.11-1.40 min; 35%	0.75	3, Positive	524.0 /247.0
Active form (condition 5)	2, 4	0.1 % FA / 0.05 % AA in water	MeCN	0 min; 45% → 0.60-0.80 min; 75% → 0.81-1.00 min; 45%	0.75	3, Positive	524.0 /247.0

Compounds were quantified by coupling a triple quadrupole mass spectrometer to a high-performance liquid chromatography (HPLC) or ultra-performance liquid chromatography (UPLC) system. Compounds were separated and eluted from the column under gradient conditions of mobile phase A and B; mobile phase A consisted of either 0.1 % of formic acid (FA) in water or 0.1% of FA and 0.05% acetylacetone (AA) in water, and mobile phase B consisted of acetonitrile (MeCN). The eluted compounds were detected by electrospray ionization using selected reaction monitoring (SRM) modes. The LC instruments used were either a Nexera X2 UHPLC system (Shimadzu, Kyoto, Japan; LC instrument 1) or an Acquity UPLC system (Waters; LC instrument 2). The LC columns used were YMC-Triart C18 column (2.1 mm × 50 mm, i.d., 3 μm; YMC, Ltd., Kyoto, Japan; column 1), CAPCELL PAK ADME column (2.1 mm

× 50 mm, i.d., 3 μm; Shiseido, Tokyo, Japan; column 2), L-column ODS (2.1 mm × 50 mm, i.d., 3 μm; Chemicals Inspection and Institute, Tokyo, Japan; column 3), or Acquity UPLC BEH C18 column (2.0 mm or 2.1 mm × 50 mm, i.d., 1.7 μm; Waters; column 4), and the all column temperatures were set at 40 C. The MS instruments used were a Triple Quad 6500 triple quadruple linear ion trap mass spectrometer (AB SCIEX, Framingham, MA; MS instrument 1), Quattro Ultima Pt (Waters; MS instrument 2), and API-5000 (AB SCIEX; MS instrument 3). The active form was measured under the following conditions. For the hydrolysis studies of prodrugs in hiPSC-IECs, condition 1 was employed in the study of prodrug A and condition 2 was employed in the studies of prodrugs B, C, D, E, and F. For the oral administration studies of prodrugs in rat, condition 3 was employed in the studies of prodrugs A and C, condition 4 was employed in the studies of prodrugs B, E, and F, and condition 5 was employed in the study of prodrug D. For the intravenous administration study of the active form in rat, condition 5 was employed.

Table S5. Hydrolysis activity in hiPSC-IEC monolayers and pharmacokinetic parameters in rats of six ester prodrugs.

Compound	Transcellular transport study across hiPSC-IEC monolayers	PK study in rat		
	CL_{int} (μL/min)	C_{max} (ng/mL)	AUC (ng×hr/mL)	BA (%)
Prodrug A	0.0239 ± 0.0056	13.5	134.7	4.1
Prodrug B	0.632 ± 0.040	141	1777	54.3
Prodrug C	0.655 ± 0.037	59.4	730	22.3
Prodrug D	0.690 ± 0.055	72.9	929	28.4
Prodrug E	1.11 ± 0.06	104	1242	38.0
Prodrug F	1.66 ± 0.13	172	2045	62.5

CL_{int}, a parameter of hydrolysis activity of ester prodrugs into the active form, was determined by means of the apical-to-basolateral transcellular transport study in hiPSC-IECs as described in Materials and Methods. The pharmacokinetic parameters (C_{max}, AUC, and BA) of six prodrugs in rats were calculated from the plasma concentrations of the active form after oral administrations of six prodrugs and intravenous administration of the active form. Six ester prodrugs and the active form are in-house compounds synthesized by Shionogi & Co., Ltd., and these six prodrugs are all hydrolyzed into the same active form. Each value of CL_{int} in hiPSC-IECs represents

the mean ± S.E.M. of three determinations. Each value of C_{max}, AUC, and BA in rat represents the mean of two or three rats.

Table S6. Prediction of human Fa of values 14 drugs from their P_{app} values across hiPSC-IEC monolayers.

Compound	P_{app} in hiPSC-IECs ($\times 10^{-6}$ cm/sec)	Predicted Fa	Observed Fa			
			Median	Zhao et al. (2001)	Sugano et al. (2002)	Verma et al (2010)
Ganciclovir	0.0175 ± 0.0025	0.05	0.06	0.03	0.03	0.09
Doxorubicin	0.0758	0.20	0.12	0.007-0.23	not examined	0.12
[^{14}C]Mannitol	0.222 ± 0.020	0.48	0.16	0.16	0.16	not examined
Famotidine	0.160 ± 0.030	0.38	0.38	0.38	0.38	not examined
Sulpiride	0.0795 ± 0.0010	0.21	0.40	0.44	0.35	0.44
Atenolol	0.0972 ± 0.0016	0.25	0.50	0.50	0.50	0.50
Sulfasalazine	0.221 ± 0.027	0.48	0.59	0.56-0.61	not examined	0.59
Furosemide	0.467 ± 0.098	0.75	0.61	0.61	0.61	0.61
Ranitidine	0.0637 ± 0.0026	0.17	0.64	0.39-0.88	0.50	0.65
Hydrochlorothiazide	0.474 ± 0.119	0.75	0.69	0.65-0.72	not examined	not examined
[^{3}H]Digoxin	1.81 ± 0.04	1.00	0.81	0.81	not examined	0.81
Acetaminophen	17.1 ± 0.4	1.00	0.90	0.80	0.80	1.00

Propranolol	39.3 ± 0.3	1.00	0.95	0.99	0.90	0.99
Antipyrine	23.9 ± 2.5	1.00	0.97	0.97	0.97	0.97

The P_{app} values of 14 drugs were determined by means of the apical-to-basolateral transcellular transport study across hiPSC-IECs. The data of doxorubicin represent the mean of duplicate experiments, and the data of 13 other drugs represent the mean ± S.E.M. of triplicate experiments. Human Fa values were taken from the literature (Zhao et al., 2001; Sugano et al., 2002; Varma et al., 2010). If multiple Fa values are reported in these literatures, the median value was calculated and used. The predicted Fa values were calculated by using Eq. 9 as described in Results.

Reference for Supplemental Data

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