Pharmacokinetics, Pharmacodynamics and Drug Transport and Metabolism

Application of a Human Intestinal Epithelial Cell Monolayer to the Prediction of Oral Drug Absorption in Humans as a Superior Alternative to the Caco-2 Cell Monolayer

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A R T I C L E   I N F O

Article history:
Received 3 September 2015
Revised 28 October 2015
Accepted 18 November 2015

Keywords:
intestinal absorption
membrane conductance and resistance
paracellular transport
permeability
tight junction
transcellular transport
ABC transporters
cell culture
intestinal epithelia

A B S T R A C T

A human small intestinal epithelial cell (HIEC) monolayer was recently established in our laboratories as a novel system to evaluate the \( P_{app} \) (apparent permeability coefficient) of compounds during their absorption in humans. An effusion-based analysis using polyethylene glycol oligomers with molecular weights ranging from 194-898 indicated that HIEC and Caco-2 cell monolayers both had paracellular pores with 2 distinct radii (≈5 and 9-14 Å), whereas the porosity of large pores was 11-fold higher in the HIEC monolayer (44 \( \times \) 10\(^{-8} \)) than in the Caco-2 cells (4 \( \times \) 10\(^{-8} \)). A comparison between the fraction-absorbed (F\( a \)) values observed in humans and those predicted from \( P_{app} \) values in both monolayers indicated that the HIEC monolayer had markedly higher precision to predict F\( a \) values with root mean square error of 9.40 than the Caco-2 cells (root mean square error = 16.90) for 10 paracellularly absorbed compounds. Furthermore, the accuracy of the HIEC monolayer to classify the absorption of 23 test drugs with diverse absorption properties, including different pathways in the presence or absence of susceptibility to efflux transporters, was higher than that of the Caco-2 cell monolayer. In conclusion, the HIEC monolayer exhibited advantages over Caco-2 cells in the ranking and prediction of absorption of compounds in humans.

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I n t r o d u c t i o n

The passive diffusion of drugs across the epithelium of the small intestine during absorption involves paracellular permeation through tight junctions, hydrophilic pores between epithelial cells, and/or transcellular permeation through the lipoidal cell membrane.\(^1\) Permeating compounds prefer either or both paracellular and transcellular pathways, depending not only on their properties but also on the morphology of the epithelium, such as pore size and porosity.\(^2\) Hydrophilic compounds, which are primarily absorbed through a paracellular route, generally exhibit low permeability and a low extent of absorption.\(^3\) Because drugs with low permeability (i.e., class 3 + class 4 compounds in the BCS [Biopharmaceutics Classification System] criteria)\(^6\) represent 37% of 263 oral generic drugs approved in the United States from 2000-2011,\(^2\) it is particularly advantageous for the \textit{in vitro} permeability model in a screening setting to accurately distinguish the absorption class and predict the fraction absorbed (F\( a \)) of incompletely absorbed compounds in humans.\(^3\,6\)

Compared with hydrophilic (classes 3 and 4) compounds, the extent of oral bioavailability and rate of absorption of class 2 compounds (i.e., compounds with high permeability and low solubility), which account for 21% of recently approved generic drugs in the United States,\(^5\) are more likely to be affected by active transport by the ABC family of efflux transporters, such as P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), and multidrug resistance protein 2 (MRP2), which are highly abundant at the apical (luminal) membrane of enterocytes. Overlapping substrate specificities between P-gp and BCRP, BCRP and MRP2, and P-gp and MRP2 suggested that a concerted function among efflux transporters may comprise a significant barrier to the intestinal absorption of class 2 drugs and compounds under development.\(^8\) Therefore,
conventional in vitro absorption models need to be carefully used for the screening of development candidates potentially subject to poor absorption in humans because the expression of human efflux transporters in these models are completely lacking or not necessarily representative of that in vivo.\textsuperscript{6,8}

We recently reported the utility of a human small intestinal epithelial cell (HIEC) monolayer differentiated from adult intestinal stem cells as a novel system for evaluating permeability (\(P_{\text{app}}\); apparent permeability coefficient) during the absorption of drugs in humans.\textsuperscript{3} The HIEC monolayer had in vivo–like loose tightness, exhibiting very high permeability for paracellularly absorbed compounds, such as atenolol, terbutaline, and fluorescein isothiocyanate–dextran 4000. Furthermore, the functions of P-gp and BCRP were both confirmed in the HIEC monolayer by the vectorial transport of marker substrates and their disappearance in the presence of specific inhibitors. These findings suggested that the HIEC monolayer has the potential to accurately predict the oral absorption of drugs that are permeated through paracellular and/or transcellular pathways regardless of their susceptibility to efflux transporter-mediated active transport in humans.

Based on the aforementioned characteristics of loose tightness and high paracellular permeability in the HIEC monolayer,\textsuperscript{3} the tight junctions of the HIEC monolayer were expected to constitute a higher number of and/or larger sized hydrophilic paracellular pores than those in conventional models, such as the Caco-2 cell monolayer. Thus, we first examined paracellular pore radius and porosity (i.e., fraction of the paracellular space on the membrane surface) in HIEC and Caco-2 cell monolayers by an effusion-based analysis using the \(P_{\text{app}}\) values of polyethylene glycol (PEG) oligomers. The relationship between \(F_a\) values observed in humans and those predicted from the \(P_{\text{app}}\) values for 10 test drugs mainly absorbed through paracellular pathways indicated that the HIEC monolayer had markedly higher precision to predict \(F_a\) values than Caco-2 cells. Furthermore, for 23 drugs with diverse absorption properties, the HIEC monolayer exhibited higher accuracy than Caco-2 cells to classify these test drugs into 3 absorption classes (i.e., complete, \(40\%-80\%\); incomplete, \(0\%-40\%\); and poor, \(<40\%\)) and rank them in order of absorbability (as evaluated by the Spearman rank correlation coefficient between \(F_a\) and \(P_{\text{app}}\)). In summary, the HIEC monolayer serves as a superior alternative to the conventional Caco-2 cell monolayer for predicting oral absorption in humans, especially for incompletely absorbed hydrophilic compounds that are of particular interest at the drug discovery and lead optimization stages.

Materials and Methods

HIECs (ACBRI 519) and Caco-2 cells (HTB-37) were purchased from Cell Systems (Kirkland, WA) and the American Type Culture Collection (Rockville, VA), respectively. Dulbecco’s modified Eagle’s medium, Dulbecco’s modified Eagle’s medium mixed 1:1 with Ham’s F-12, 0.25% trypsin–EDTA, Hanks’ balanced salt solution, nonessential amino acids, penicillin–streptomycin, and GlutaMAX were obtained from Life Technologies (Carlsbad, CA). Bovine pituitary extract was purchased from Kojinh Bio (Saitama, Japan). Fetal bovine serum was obtained from SAFC Biosciences (Lenexa, KS). Recombinant human insulin and epidermal growth factor were purchased from Sigma-Aldrich (St. Louis, MO). BioCoat Fibbrilar collagen-coated 24-well inserts and 12-well transwell membrane inserts were obtained from Corning (Corning, NY). PEG200, 400, 600, and 1000 were obtained from Nacalai Tesque (Kyoto, Japan) and Wako Pure Chemicals (Osaka, Japan). All drugs for the transport assay were purchased from Sigma-Aldrich, LKT Laboratories (St. Paul, MN), and Tokyo Kasei Kogyo (Tokyo, Japan). All other chemicals and reagents were of analytical grade.

Cell Culture

HIECs were maintained on type I collagen-coated culture dishes in Dulbecco’s modified Eagle’s medium F-12 supplemented with 10% fetal bovine serum, 1% GlutaMAX, 10 µM dexamethasone, 1 µg/mL insulin, 20 ng/mL epidermal growth factor, 50 µM 2-mercaptoethanol, 50 U/mL penicillin, and 50 µg/mL streptomycin. Caco-2 cells were grown on culture flasks in Dulbecco’s modified Eagle’s medium with 4.5 g/L glucose, supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 1% GlutaMAX, 50 U/mL penicillin, and 50 µg/mL streptomycin. Both cell lines were cultured at 37°C in a humidified atmosphere of 5% CO\textsubscript{2} and 95% air. Before reaching confluence, HIECs and Caco-2 cells were seeded at 10 × 10\textsuperscript{4} and 6.3 × 10\textsuperscript{4} cells/well onto 24-well fibbrilar collagen-coated and 12-well noncoated membrane inserts, respectively. HIECs were fed tridaily with culture medium, supplemented with 50 µg/mL bovine pituitary extract. Culture medium for Caco-2 cells was replaced once in the first week and every other day thereafter. HIECs and Caco-2 cells were grown for 8–9 and 18–20 days, respectively, before assays were performed.

Estimation of the Pore Radius and Porosity of the Membrane

To estimate the pore radius and porosity (\(r\), i.e., the fraction of the paracellular space of the membrane surface) of HIEC and Caco-2 cell monolayers, the following effusion-based method was applied:\textsuperscript{2,10,11}

\[
P_{\text{app}} = \frac{RT\varepsilon}{12\pi N_A \lambda} \cdot \frac{1}{r^2} = \text{slope} \cdot \frac{1}{r^2} \tag{1}
\]

where \(P_{\text{app}}\), \(R\), \(T\), \(\eta\), \(N_A\), \(\lambda\), and \(r\) represent apparent drug permeability, the gas constant, temperature, the viscosity of water (0.6915 mPa · s at 37°C), Avogadro number, the jump length (3.1 Å), and the radius of the drug molecule, respectively. This equation indicated that the measured \(P_{\text{app}}\) was inversely proportional to the radius of the drug molecule. Thus, the porosity of the membrane was evaluated from the slope of the regression line for the relationship between measured \(P_{\text{app}}\) values and the reciprocals of radiiuses for molecules of various sizes. Furthermore, the value of the intercept on the x-axis (at \(P_{\text{app}} = 0\)) obtained from the extrapolation gave estimation for the critical value of the molecular radius still able to permeate through the paracellular pores. Thus, the critical value obtained served as an estimate for the pore radius of the membrane. To calculate the pore radius and porosity of HIEC and Caco-2 cell monolayers based on Equation 1, apical-to-basal \(P_{\text{app}}\) values were determined as described in the section for calculations for the following PEG oligomers with molecular radiiuses (\(r_s\)) in parentheses: PEG\textsubscript{104} (4.13 Å), PEG\textsubscript{238} (4.51 Å), PEG\textsubscript{282} (4.87 Å), PEG\textsubscript{326} (5.15 Å), PEG\textsubscript{370} (5.47 Å), PEG\textsubscript{414} (5.78 Å), PEG\textsubscript{458} (6.03 Å), PEG\textsubscript{502} (6.27 Å), PEG\textsubscript{546} (6.55 Å), PEG\textsubscript{590} (6.77 Å), PEG\textsubscript{634} (7.03 Å), PEG\textsubscript{678} (7.24 Å), PEG\textsubscript{722} (7.45 Å), PEG\textsubscript{766} (7.69 Å), PEG\textsubscript{810} (7.89 Å), PEG\textsubscript{854} (8.09 Å), and PEG\textsubscript{986} (8.27 Å), where \(r_s\) values were obtained from a previous study, except for that of PEG\textsubscript{194}, which was calculated by the following equations using molecular weight (MW):\textsuperscript{10,12}

\[
\eta = 0.02 + 2.4 \times 10^{-4} \times \text{MW}^{0.73} \tag{2}
\]

\[
r_s = 2.268 \times (\eta \times \text{MW})^{1/3} \tag{3}
\]

Before the addition of PEG oligomers, the growth medium was removed and monolayers were rinsed twice with transport medium (TM, Hanks’ balanced salt solution with 4.2 mM NaHCO\textsubscript{3} and 20 mM glucose) adjusted to pH 7.4 by HEPES (10 mM). Monolayers were preincubated in TM (pH 7.4) for 30 minutes at 37°C in 95%
humidity. Transport assays were carried out with apical and basal buffers consisting of TM (pH 6.5) adjusted by 10 mM MES and TM (pH 7.4) with 4.5% wt/vol bovine serum albumin, respectively. The following buffer volumes were used for the apical and basal chambers: 0.4 and 1.2 mL, respectively, for HIECs; and 0.5 and 1.5 mL, respectively, for Caco-2 cells. As donor solutions of PEG oligomers, PEG200, PEG400, PEG600, and PEG1000, were dissolved in TM (pH 6.5). On the basis of the mean MW of PEG oligomers, the final donor concentrations of each PEG were adjusted to be 100 μM. Monolayers were incubated for 240 minutes at 37°C in 95% humidity with 80 rpm reciprocal shaking. Following the initiation of the transport assay, basal compartments were sampled at 60, 120, and 240 minutes. Samples were added to twice their volume of methanol:acetonitrile (2:1, vol/vol) and centrifuged for 15 minutes before being analyzed with the liquid chromatography—tandem mass spectrometry (LC-MS/MS) system, as described in the section for sample assay. Transepithelial electrical resistance (TEER) values before and after experiments using Millicell-ERS (Millipore, Bedford, MA). No significant decreases were observed in TEER values during the transport assays in any of the experiments performed.

**Transport Assay for Test Compounds**

The apical-to-basal $P_{app}$ values of test compounds were evaluated as described earlier with the following slight modifications: briefly, the donor concentration for all test compounds was set at 50 μM. Basal compartments were sampled at 30, 60, and 120 minutes. Samples were added to twice their volume of methanol:acetonitrile (2:1, vol/vol) and centrifuged for 15 minutes at 10,000 × g before being analyzed on the LC-MS/MS system, as described in the section for sample assay. TEER values before transport assays were consistently in the range of 70–120 Ω × cm² and 600–1100 Ω × cm² for HIEC and Caco-2 cell monolayers, respectively. No significant decreases were observed in TEER values during the transport assays in any of the experiments performed.

**Sample Analysis**

An analysis of samples for PEG oligomers was performed using the LC-MS/MS system, consisting of a Waters Quattro Micro Mass Spectrometer and Waters Alliance 2795 HT (Waters,Milford, MA). The ionization source was an electrospray. The multiple-reaction monitoring mode was used to monitor ions as follows: PEG194 (195.3 > 88.8), PEG238 (239.3 > 89.2), PEG282 (283.3 > 89.2), PEG326 (327.3 > 89.2), PEG370 (371.3 > 89.2), PEG414 (415.3 > 89.2), PEG458 (459.3 > 89.2), PEG502 (503.3 > 89.2), PEG546 (547.3 > 89.2), PEG590 (591.3 > 89.2), PEG634 (635.3 > 89.2), PEG678 (679.3 > 89.2), PEG722 (723.3 > 89.2), PEG766 (767.3 > 89.2), PEG810 (811.3 > 89.2), PEG854 (855.3 > 89.2), and PEG898 (899.3 > 89.2). The Xterra MS C18 column (100 mm, 2.1 mm i.d.; Waters) was used for chromatographic separation. The gradient condition for the elution was as follows: at 0, 1.5, 4, 8 minutes, the percentages of acetonitrile were 2, 2, 95, 95, respectively, with 10 mM ammonium acetate as the aqueous mobile phase. The flow rate was 0.2 mL/min, and the injection volume was 5 μL. The column temperature was maintained at 40°C. All data processing was performed with Waters QuanLynx software (Waters).

**Calculations**

$P_{app}$ values for HIEC and Caco-2 cellular transport were calculated according to the following equation using the linear part of the time versus transported amount:

$$ P_{app} = \frac{dQ}{dt} \times \frac{1}{A \times C_0} $$

(4)

where $dQ/dt$, $A$, and $C_0$ represent the total amount of the test compound transported to the acceptor chamber per unit time, the surface area of the transport membrane (0.33 and 1 cm² for HIECs and Caco-2 cells, respectively), and the initial compound concentration in the donor chamber, respectively. The relationship between apical-to-basal $P_{app}$ and known $Fa$ data was described using the following equation 1:

$$ Fa = 100 \times (1 - \exp(-a \times P_{app})) $$

(5)

where $a$ is the scaling factor. The best-fitting curves were calculated by nonlinear regression using XLfit software (IDBS, Guildford, UK).

**Comparison of Classification Performance for Absorption Between HIEC and Caco-2 Cell Monolayers**

Sensitivity, precision, and accuracy are statistical measures of the performance of a classification test and were analyzed by the confusion matrix of classification for 23 test drugs; the drugs were classified by the boundary values of $P_{app}$ at $Fa = 80\%$ (complete/incomplete absorption) and 40% (incomplete/poor absorption), which were calculated using the best-fitted sigmoidal relationship (Eq. 5) for each monolayer. The correct and false assignments for absorption classes were summarized in the confusion matrix of classification. In the matrix, sensitivity measures the percentage of
drugs belonging to a class that are correctly classified as such for each class of actual absorption properties; precision measures the percentage of drugs classified as a class that are actually as such for each class of predicted absorption properties; and accuracy represents the percentage of drugs correctly classified in total.

**Statistics**

The coefficient of determination ($r^2$) was used to measure the strength of the association between $P_{\text{app}}$ values and $F_a$. In addition, the precision of $P_{\text{app}}$ for the prediction of observed $F_a$ was compared between HIEC and Caco-2 cell monolayers using the root mean square error (RMSE) according to the following equation:

$$\text{RMSE} = \sqrt{\frac{\sum (\text{observed } F_a - \text{predicted } F_a)^2}{n}}$$

where $n$ is the number of test compounds.

Spearman rank correlation coefficient ($\rho$) was calculated for the monotonical association between the rank orders of $P_{\text{app}}$ determined in the HIEC or Caco-2 cell monolayer and observed $F_a$ in humans according to the following equation:

$$\rho = 1 - \frac{6\Sigma d^2}{(n^3 - n)}$$

where $d$ is the difference of ranks for each compound and $n$ is the number of compounds. The value of $\rho$ ranges between $-1$ and $+1$ and a coefficient of $+1$ indicated that there was a perfect positive relationship between the rank orders of determined $P_{\text{app}}$ and observed $F_a$ in humans (i.e. the rank orders of $F_a$ and $P_{\text{app}}$ were the same).

**Results**

**Paracellular Pore Radius and Porosity in HIEC and Caco-2 Cell Monolayers**

The relationships between the determined $P_{\text{app}}$ values of PEG oligomers across HIEC and Caco-2 cell monolayers and their MWs ranging from 194 to 898 are shown in Figure 1a. The $P_{\text{app}}$ values of PEG oligomers in the HIEC monolayer were higher than those in Caco-2 cells for all oligomers tested, indicating that overall paracellular permeability was higher in the HIEC monolayer than in the Caco-2 cells. An effusion-based analysis (Eq. 1) was carried out for the biphasic relationships between the obtained $P_{\text{app}}$ values and reciprocal radius of PEG oligomers in HIEC and Caco-2 cell monolayers (Figs. 1b-1c, respectively). The analysis indicated that each monolayer had pores with 2 distinct radiuses. To separately estimate the radius and porosity of small and large pores, curve stripping was further applied to the $P_{\text{app}}$ data in HIEC and Caco-2 cell monolayers; the contribution of large pores to overall permeability was calculated by extrapolating the relationship between $P_{\text{app}}$ and the radius of PEG oligomers, and $P_{\text{app}}$ obtained was then subtracted from overall $P_{\text{app}}$ for large and small pores to calculate the $P_{\text{app}}$ through small pores. The pore radius and porosity determined in monolayers are listed in Table 1 with those reported for the human jejunum. The radiuses of paracellular pores were similar among the HIEC and Caco-2 cell monolayers and the human jejunum for large (9.3-14.3 Å) and small pores (4.8-6.6 Å). In

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**Figure 1.** Effusion-based analysis for pore size and porosity in HIEC and Caco-2 cell monolayers. (a) The relationship between the permeability and MW of PEG oligomers in monolayers. Open and closed circles represent the mean values with SD of 3 independent experiments in HIEC and Caco-2 cell monolayers, respectively. (b and c) The relationship between permeability and the reciprocal radius of PEG oligomers in HIEC and Caco-2 cell monolayers. (b) and Caco-2 cell (c) monolayers. Open squares represent the observed permeability through small and large pores, whereas open circles only represent permeability through large pores. Closed squares represent the calculated permeability through small pores. Porosity was calculated from the slope of the regression line (solid line: large pores; dashed line: small pores) according to the effusion-based theory (Eq. 1).
contrast, a large difference was noted in porosity among the membranes: the porosities of large and small pores were higher in the HIEC monolayer than in the Caco-2 cell monolayer, and the porosity of large pores was 11-fold higher in the HIEC monolayer (44 ± 2) than in the Caco-2 cell monolayer (4 ± 8). The porosities of both pores in the HIEC monolayer (large, 44 ± 2; small, 52 ± 8) were closer to those in the human jejunum (large, 89 ± 10; small, 442 ± 10) than those in the Caco-2 cell monolayer (large, 4 ± 8; small, 52 ± 8).

Prediction of the Fa by HIEC and Caco-2 Cell Monolayers

Twenty-three test drugs were chosen to cover a wide range of Fa values after their oral administration in humans (13%-100%) (Table 2). The test drugs were also diverse in their pathways (transcellular vs. paracellular passive diffusion) and susceptibilities to efflux transporters during absorption. The P_{app} values of the test drugs in the present study ranged from 0.40 × 10^{-6} to 62 × 10^{-6} cm/s and from 0.029 × 10^{-6} to 110 × 10^{-6} cm/s in HIEC and Caco-2 cell monolayers, respectively. The drugs with almost complete absorption (Fa = 80%-100%) showed similar P_{app} values between the HIEC and Caco-2 cell monolayers, whereas the drugs absorbed paracellularly with low Fa values (Fa < 80%) had markedly higher P_{app} values in the HIEC monolayer than in the Caco-2 cell monolayer.

Table 1

| Pore Radius and Porosity in HIEC and Caco-2 Cell Monolayers and the Human Jejunum |
|-----------------|-----------------|-----------------|
|                  | HIEC Monolayer  | Caco-2 Cell Monolayer  | Human Jejunum*  |
| Radius (Å)       | Large pores     | Small pores       | Large pores     | Small pores |
|                  |                 |                  |                 |            |
|                  | 14.3            | 4.8              | 10.1            | 5.4         |
| Porosity (× 10^{-9}) | Large pores     | Small pores      | Large pores     | Small pores |
|                  | 4               | 4                | 89              | 88          |

* Values in the human jejunum were obtained from published data.2

Table 2

<table>
<thead>
<tr>
<th>Fa (%)</th>
<th>P_{app} (× 10^{-6} cm/s)*</th>
<th>Pathway for Absorption/Transporter Susceptibility</th>
<th>MW</th>
<th>Charge Reference for Fa Valueb</th>
<th>Reference for Permeation Pathway and Transporters</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>HIEC Monolayer</td>
<td>Caco-2 Cell Monolayer</td>
<td>Transcellular/BCRP, MRP2</td>
<td>398.4 ± 15</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Paracellular</td>
<td>225.2 ± 14,15</td>
<td>16</td>
</tr>
<tr>
<td>Sulfasalazine</td>
<td>13</td>
<td>0.40 ± 0.04</td>
<td>0.029 ± 0.000</td>
<td>26 22</td>
<td>13</td>
</tr>
<tr>
<td>Acyclovir</td>
<td>18</td>
<td>0.77 ± 0.05</td>
<td>0.11 ± 0.03</td>
<td>26 22</td>
<td>13</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>20</td>
<td>0.51 ± 0.06</td>
<td>0.094 ± 0.019</td>
<td>26 22</td>
<td>13</td>
</tr>
<tr>
<td>Nadolol</td>
<td>33</td>
<td>0.82 ± 0.26</td>
<td>0.30 ± 0.04</td>
<td>26 22</td>
<td>13</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>34</td>
<td>0.98 ± 0.08</td>
<td>0.11 ± 0.01</td>
<td>26 22</td>
<td>13</td>
</tr>
<tr>
<td>Sulpiride</td>
<td>36</td>
<td>0.89 ± 0.07</td>
<td>0.17 ± 0.02</td>
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<td>13</td>
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<tr>
<td>Famotidine</td>
<td>38</td>
<td>0.98 ± 0.03</td>
<td>0.31 ± 0.03</td>
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<td>13</td>
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<tr>
<td>Etoposide</td>
<td>50</td>
<td>1.9 ± 0.2</td>
<td>0.41 ± 0.04</td>
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<td>13</td>
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<tr>
<td>Ranitidine</td>
<td>50</td>
<td>0.89 ± 0.13</td>
<td>0.31 ± 0.02</td>
<td>26 22</td>
<td>13</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>56</td>
<td>2.5 ± 0.1</td>
<td>0.17 ± 0.03</td>
<td>26 22</td>
<td>13</td>
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<tr>
<td>Cimetidine</td>
<td>62</td>
<td>1.4 ± 0.9</td>
<td>0.29 ± 0.03</td>
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<td>13</td>
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<tr>
<td>Indinavir</td>
<td>63</td>
<td>5.6 ± 0.5</td>
<td>4.3 ± 0.3</td>
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<td>13</td>
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<tr>
<td>Hydrochlorothiazide</td>
<td>67</td>
<td>3.3 ± 0.7</td>
<td>0.17 ± 0.04</td>
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<td>13</td>
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<tr>
<td>Metformin</td>
<td>71</td>
<td>3.0 ± 0.7</td>
<td>0.24 ± 0.02</td>
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<td>13</td>
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<tr>
<td>Diclofenac</td>
<td>75</td>
<td>3.5 ± 0.2</td>
<td>0.96 ± 0.03</td>
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<td>Procainamide</td>
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<td>5.9 ± 0.3</td>
<td>3.0 ± 0.3</td>
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<td>13</td>
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<td>8.5 ± 0.4</td>
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<td>41 ± 3</td>
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<td>44 ± 0</td>
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<td>13</td>
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<tr>
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<td>98</td>
<td>20 ± 1</td>
<td>16 ± 1</td>
<td>26 22</td>
<td>13</td>
</tr>
<tr>
<td>Carbamazepine</td>
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<td>62 ± 2</td>
<td>110 ± 0</td>
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<td>13</td>
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<tr>
<td>Quinidine</td>
<td>100</td>
<td>26 ± 1</td>
<td>48 ± 1</td>
<td>26 22</td>
<td>13</td>
</tr>
</tbody>
</table>

* P_{app} values represent the mean ± SD (n = 3).

** When Fa values were obtained from >1 reference, the mean values were used.

The Fa values for 10 test drugs that mainly undergo paracellular absorption (acyclovir, nadolol, pravastatin, sulpiride, famotidine, ranitidine, norfloxacin, cimetidine, hydrochlorothiazide, and metformin in Table 2) were predicted by Equation 5 using the P_{app} values determined in both monolayers. The results of curve fitting indicated that the P_{app} values in the HIEC monolayer correlated better with the Fa values observed in humans than those in the Caco-2 cells (Fig. 2a): the coefficients of determination (r^2) for the HIEC and Caco-2 cell monolayers were 0.73 and 0.11, respectively. The relationships between the Fa values observed in humans and those predicted by Equation 5 best fitted for each cell line (shown in Fig. 2a) were further compared between the HIEC and Caco-2 cell monolayers (Fig. 2b). The value of RMSE, a measure for the deviation of prediction, was smaller in the HIEC monolayer (RMSE = 9.40) than in the Caco-2 cells (RMSE = 16.90). These results suggested that P_{app} values in the HIEC monolayer had markedly higher precision than those in the Caco-2 cells for predicting Fa in humans for paracellularly absorbed compounds. These results were also consistent with those of the effusion-based analysis in which the porosities of large and small pores in the HIEC monolayer were closer to those in the human jejunum than those in the Caco-2 cells (Table 1).

All 23 test drugs not only including those absorbed paracellularly but also those transcellularly absorbed with or without susceptibility to efflux transporters (P-gp, BCRP, and MRP2) listed in Table 2 were used to investigate the relationship between the Fa values observed in humans and P_{app} values determined in the HIEC and Caco-2 cell monolayers. Good sigmoidal relationships between these parameters were obtained in both the HIEC and Caco-2 cell monolayers (Fig. 3), and the calculated r^2 value was higher in the HIEC monolayer (0.92) than in the Caco-2 cells (0.78).

Absorption Classification by HIEC and Caco-2 Cell Monolayers

The classification of absorbability for the development candidates facilitates lead optimization at the discovery stage. Therefore, the accuracy to classify 23 test drugs into 3 absorption classes (i.e.
complete, \( Fa = 80\%-100\% \); incomplete, \( Fa = 40\%-80\% \); and poor, \( Fa < 40\% \) was compared between HIEC and Caco-2 cell monolayers. The boundary values of \( P_{\text{app}} \) at \( Fa = 80\% \) (complete/incomplete absorption) and 40\% (incomplete/poor absorption) were calculated using the best-fitted sigmoidal relationship in Figure 3 for each monolayer and then used for the absorption classification. Table 3 shows the confusion matrices for the classification of test drugs into the earlier described 3 classes based on \( P_{\text{app}} \) values determined in the HIEC and Caco-2 cell monolayers. Among all absorption classes, the HIEC monolayer showed higher sensitivity and precision than Caco-2 cells in the classes of incomplete and poor absorptions: sensitivity for incompletely absorbed drugs, 75\% and 50\% in HIECs and Caco-2 cells, respectively; precision for incompletely absorbed drugs, 100\% and 67\% in HIECs and Caco-2 cells, respectively; sensitivity for poorly absorbed drugs, 100\% and 71\% in HIECs and Caco-2 cells, respectively; and precision for poorly absorbed drugs, 88\% and 71\% in HIECs and Caco-2 cells, respectively. On the other hand, HIEC and Caco-2 cell monolayers had equally high sensitivity and precision for completely absorbed drugs; sensitivity, 100\% and 100\% in HIECs and Caco-2 cells, respectively; and precision, 89\% and 80\% in HIECs and Caco-2 cells, respectively. As a result, overall accuracy was higher in the HIEC monolayer (91\%) than in Caco-2 cells (74\%).

**Rank Correlation Analysis**

In the late stage of drug discovery, multiple compounds must often be ranked in order of oral absorbability in humans to facilitate the prioritization of development candidates from a development feasibility point of view. A Spearman rank-order correlation analysis between the \( Fa \) values observed for the 23 tested drugs and their \( P_{\text{app}} \) values determined in each monolayer indicated that a
better monotonic correlation was found in the HIEC monolayer \( (p = 0.97) \) than in the Caco-2 cell monolayer \( (p = 0.89) \) (Fig. 4). In the Caco-2 cell monolayer, most drugs that disturbed the monotone relationship belonged to paracellularly absorbed drugs (open symbols in Fig. 4).

**Discussion**

We recently demonstrated the utility of a HIEC monolayer differentiated from adult intestinal stem cells as a novel in vitro system for evaluating permeability during absorption. The TEER across the morphologically matured HIEC monolayer was markedly lower \( (98.9 \ \Omega \ \times \ cm^2) \) than that in the Caco-2 cells \( (900 \ \Omega \ \times \ cm^2) \), indicating that the looseness of the tight junction in the HIEC monolayer was similar to that in the human small intestine \( (ca. 40 \ \Omega \ \times \ cm^2) \). The present study revealed that the porosity of the paracellular pores was higher in the HIEC monolayer than in the Caco-2 cells. Considering the enlargement of surface area \( (4.5 \text{ times}^{27}) \) by the villi structure in the human intestine, the values of porosity of large and small pores in HIEC and Caco-2 cell monolayers were recalculated by multiplying by 4.5, and the resultant values in HIECs \( (198 \times 10^{-8} \text{ and } 491 \times 10^{-8} \text{ in large and small pores, respectively}) \) became more closer to those in the human jejunum \( (89 \times 10^{-8} \text{ and } 442 \times 10^{-8}, \text{ respectively}) \) than those in the Caco-2 cells \( (18 \times 10^{-8} \text{ and } 234 \times 10^{-8}, \text{ respectively}) \). The \( P_{app} \) values determined in the HIEC monolayer consistently had markedly higher precision than Caco-2 cells for the prediction of \( F_a \) in humans for paracellularly absorbed compounds. Therefore, the ability of the HIEC monolayer to classify and rank the absorption of the 23 test drugs with diverse absorption properties, including different pathways regardless of susceptibility to efflux transporters during absorption, was superior to that of the Caco-2 cell monolayer.

The Caco-2 cell monolayer has been recognized as an inappropriate model to predict \( F_a \) in humans for paracellularly absorbed drugs because it forms an excessively tight monolayer. The present study demonstrated that \( P_{app} \) values in the HIEC monolayer had markedly higher precision than those in Caco-2 cells for predicting \( F_a \) in humans, especially for the paracellularly absorbed 10 test drugs (Fig. 2). For small hydrophilic drugs, paracellular permeation plays a more dominant role in absorption than transcellular passive diffusion. The paracellular permeation pathway is quantitatively characterized by the paracellular porosity \( (\text{the fraction of the paracellular space of the membrane surface}) \) of the membrane and pore size \( (\text{radius}) \) of tight junctions. An effusion-based analysis previously revealed that all conventional cell models (Caco-2 cells, Madin–Darby canine kidney [MDCK] II cells, and 2/4/A1 cells [a conditionally immortalized rat intestinal cell line]) deviated from the human small intestine either with respect to porosity \( (\text{Caco-2 cell and MDCKII cell monolayers}) \) or pore size distribution \( (2/4/A1 \text{ cell monolayer}) \); the paracellular porosities of the human small intestine and 2/4/A1 monolayer were larger than those of MDCKII and Caco-2 cells; and 2 different pore sizes \( (5-6 \text{ and } >10\AA) \) were detected in the human intestinal epithelium, Caco-2 cells, and MDCKII cells, whereas only 1 \( (<15 \AA) \) was found in the 2/4/A1 cell monolayer. Therefore, these findings together with the present results suggested that porosity and

![Graph](image1.png)

**Table 3**

Confusion Matrix of Classification into Completely (\( F_a > 80\% \)), Incompletely (\( F_a: 40\%-80\% \)), and Poorly (\( F_a: 0\%-40\% \)) Absorbed Drugs Based on \( P_{app} \) Values Determined in HIEC and Caco-2 Cell Monolayers

<table>
<thead>
<tr>
<th>Monolayer</th>
<th>Actual</th>
<th>Predicted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Complete</td>
<td>Incomplete</td>
</tr>
<tr>
<td>HIEC Monolayer</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Caco-2 Cell</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Monolayer</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>Precision</td>
<td>80%</td>
<td>67%</td>
</tr>
</tbody>
</table>
pore size distribution in the HIEC monolayer (Table 1) were the most similar to those in the human small intestine among the differentiated epithelial cell models that have been conventionally used to predict absorption in humans.

Paracellular permeability depends on the charge of compound molecule because the rumen of the tight junctional pores is negatively charged; cationic compounds can easily permeate tight junction than neutral or anionic compounds.15 However, there was no tendency between the total permeabilities of paracellularly absorbed drugs tested in the present study and their charges (Table 2). This may be due to the differences in other factors, such as the size and diffusion coefficient among compounds, because these physicochemical parameters also affect the permeability of compound through paracellular route.15 In addition, any contributions of transcellular pathway and transporters may also influence the total permeability. The permeabilities for all the paracellularly absorbed drugs in the present study showed higher values in HIEC than in the Caco-2 cell monolayers (Table 2). Besides, HIECs had better correlation between Fa values and permeabilities for paracellularly absorbed drugs than the Caco-2 cells (HIECs, \( r^2 = 0.73 \) vs. Caco-2 cells, \( r^2 = 0.11 \) in Fig. 2a). These data suggest that the paracellular permeability in HIEC monolayers would reflect more closely to that in the human small intestine than that in the Caco-2 cells.

Previous studies demonstrated that 2/4/A1 cells formed loose monolayers close to the human intestinal epithelium, similar to the HIEC monolayer, and predicted the Fa values of paracellularly absorbed drugs better than Caco-2 cells.6,14 However, 2/4/A1 cells completely lack the functional activities of efflux transporters, such as P-gp, BCRP, and MRP 2, as indicated by no unidirectional transport for their respective substrates;25 unlike the HIEC monolayer. Matsson et al. (2005) reported that the rank-order correlation between the Fa of P-gp substrates and their \( P_{app} \) values determined in the Caco-2 cell monolayer was better than that in the 2/4/A1 cell monolayer. Furthermore, the Fa value of sulfasalazine, the intestinal absorption of which was limited by BCRP and MRP2 in Caco-2 cells,29 in rats,31,32 and in clinical studies,33-35 was overpredicted in 2/4/A1 cells by the best-fitted regression line for the relationship between the \( P_{app} \) value in 2/4/A1 cells and observed Fa value.6 However, the Fa value for the same drug was predicted well by \( P_{app} \) values in HIECs (observed Fa = 13% vs. predicted Fa = 15% obtained from the best-fitted sigmoidal relationship in Fig. 3 for HIECs) in the present study. Furthermore, the Fa values of efflux-transporter substrates (sulfasalazine, methotrexate, famotidine, etoposide, ranitidine, norfloxacain, cimetidine, indinavir, digoxin, and imatinib) were predicted well by the \( P_{app} \) values determined in the HIEC monolayer (Table 2 and triangles in Fig. 3). Thus, the expression and activity of efflux transporters in the HIEC monolayer further conferred an advantage on the HIEC monolayer over 2/4/A1 cells in predicting the oral absorption of the substrates of efflux transporters.

Some transcellularly absorbed drugs that are the substrates of efflux transporters (sulfasalazine, methotrexate, etoposide, and digoxin) had relatively higher \( P_{app} \) values in HIECs than in Caco-2 cells (Table 2). The reason for this may be partly explained by differences in the protein expression levels of efflux transporters between HIECs and Caco-2 cells. Recent advances in LC-MS/MS-based proteomic approaches have enabled the quantification of absolute protein expression levels.36 To verify the aforementioned possibility, further studies to compare the protein expression levels of transporters in HIECs and Caco-2 cells are needed. These data may also be informative to extrapolate the in vitro contribution of transporters from in vitro permeability by comparing with the reported absolute protein levels in the human small intestinal epithelium.37-39

In the present study, 2 distinct pore sizes were observed in the HIEC and Caco-2 cell monolayers (Table 1). Similar findings have been already reported in the human intestine,40 pig ileum,41 and epithelial cell lines, such as Caco-2 cells, T84 cells (human colon adenocarcinoma cells), and MDCK cells.2,24,42 Previous studies suggested that large and small pores were localized to the intestinal crypts and villi, respectively.40,43,44 If this hypothesis is correct, the close porosity of large pores between HIECs and human jejunum (Table 1) is attributable to the coexistence of intestinal stem cells in HIECs, even after differentiation into enterocytes,5 which are localized to the crypts of the in vivo epithelium. On the other hand, the markedly lower porosity of
large pores in the Caco-2 cell monolayer than in the HIEC monolayer and human intestine (Table 1) may be at least partly explained by differentiated Caco-2 cells entirely consisting of matured enterocytes, as indicated by a gradual decrease in the crypt cell marker, Sox9, in a 21-day culture.45 In contrast to the 2 different pore sizes observed in the epithelial cell lines (such as HIECs, Caco-2 cells, and T84 cells) and human intestine, the total porosity of 2/4/A1 cells only comprised large pores.2 The insufficient morphologic maturation of 2/4/A1 cells, as indicated by their cuboidal cell shape and sparse microvilli, and their origin (i.e. fetal rat intestine at 18 days of gestation)46 may be responsible for the discrepancy in pore distribution between 2/4/A1 cells and the human small intestine.

Conclusions

The HIEC monolayer had similar pore sizes and distribution of large and small pores and porosities to the human small intestine, which likely resulted in higher precision for predicting the absorption fraction in humans than that of Caco-2 cells, especially for paracellularly absorbed hydrophilic compounds. The superior prediction and functional activity of human efflux transporters in the HIEC monolayer afford it an advantage over conventional cell models in the ranking and prediction of the absorption of development candidates in humans, especially at the drug discovery stage when a number of incompletely absorbed compounds are expected for screening and prioritization.

References


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