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## Significance of basal membrane permeability of epithelial cells in predicting intestinal drug

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## Running title page

Running title: Role of basal membrane in intestinal drug absorption

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## Abbreviations

ACAT, advanced compartmental absorption and transit; ADAM, advanced dissolution, absorption and metabolism; ATOM, advanced translocation model; BSA, bovine serum albumin; CPR, NADPHcytocrome P 450 reductase; $\mathrm{F}_{\mathrm{A}}$, intestinal absorption; $\mathrm{F}_{\mathrm{A}} \mathrm{F}_{\mathrm{G}}$, product of $\mathrm{F}_{\mathrm{A}}$ and $\mathrm{F}_{\mathrm{G}} ; \mathrm{f}_{\text {ent }}$, unbound fraction in enterocytes; $\mathrm{F}_{\mathrm{G}}$, intestinal availability; $\mathrm{F}_{\mathrm{H}}$, hepatic availability; $\mathrm{f}_{\mathrm{u}}$, unbound fractions in the incubation buffer; HAC, human artificial chromosome; HBSS, Hank's balanced salt solution; $\mathrm{K}_{\mathrm{m}}$, Michaelis constant; LC-MS/MS, liquid chromatography-tandem mass spectrometry; Napp, Numeric Analysis Program for

Pharmacokinetics; OATP, organic anion transporting polypeptide; OST, organic solute transporter; P-gp,
P-glycoprotein; PS, permeability surface area products; $\mathrm{V}_{\text {max }}$, maximum reaction velocity


#### Abstract

Drug absorption from the gastro-intestinal tract is often restricted by efflux transport by P-glycoprotein (Pgp) and metabolism by cytochrome P450 (CYP) 3A4. Both localize in the epithelial cells and thus their activities are directly affected by the intracellular drug concentration which should be regulated by the ratio of permeability between apical (A) and basal (B) membranes. In this study, using Caco-2 cells with forced expression of CYP3A4, we assessed the transcellular permeation of A-to-B and B-to-A directions and the efflux from the preloaded cells to the both sides of 12 representative P-gp or CYP3A4 substrate drugs, and obtained the parameters for permeabilities, transport, metabolism, and unbound fraction in the enterocytes ( $\mathrm{f}_{\text {ent }}$ ) using simultaneous and dynamic model analysis. The membrane permeability ratios for B to $\mathrm{A}\left(\mathrm{R}_{B A}\right)$ and $\mathrm{f}_{\text {ent }}$ varied by 8.8 -fold and by more than 3,000 -fold, respectively, among the drugs. The $\mathrm{R}_{\mathrm{BA}}$ values for digoxin, repaglinide, fexofenadine, and atorvastatin were greater than 1.0 (3.44, 2.39, 2.27, and 1.90, respectively) in the presence of a P-gp inhibitor, thus suggesting the potential involvement of transporters in the B membrane. The $\mathrm{K}_{\mathrm{m}}$ for quinidine for P -gp transport was $0.077 \mu \mathrm{M}$ for the intracellular unbound concentration. These parameters were used to predict overall intestinal availability $\left(\mathrm{F}_{\mathrm{A}} \mathrm{F}_{\mathrm{G}}\right)$ by applying an intestinal pharmacokinetic model, ATOM, in which permeability of A and B membranes accounted separately. The model predicted changes in the absorption location for P-gp substrates according to its inhibition, and $\mathrm{F}_{\mathrm{A}} \mathrm{F}_{\mathrm{G}}$ values of $10 / 12$ drugs, including quinidine at varying doses, were explained appropriately.


## Significance Statement

Pharmacokinetics has improved predictability by identifying the molecular entities of metabolism and transport, and by using mathematical models to appropriately describe drug concentrations at the locations where they act. However, analyses of intestinal absorption so far have not been able to accurately consider the concentrations in the epithelial cells where P-glycoprotein and CYP3A4 exert effects. In this study, the limitation was removed by measuring the apical and basal membrane permeability separately and then analyzing these values using new appropriate models.

## Introduction

The prediction or precise assessment of the bioavailability of orally administered drugs is one of the most critical issues in selecting new drug candidates and is essential for better use of drugs in clinical practice (Varma et al., 2010). The bioavailability of a drug is, theoretically, the product of intestinal absorption $\left(\mathrm{F}_{\mathrm{A}}\right)$, intestinal availability $\left(\mathrm{F}_{\mathrm{G}}\right)$, and hepatic availability $\left(\mathrm{F}_{\mathrm{H}}\right)$. Even when $\mathrm{F}_{\mathrm{A}}$ is high and $\mathrm{F}_{\mathrm{H}}$ is moderate, if a drug is actively transported in the intestine by efflux transporters, such as P-gp or extensively metabolized by CYP3A4, $\mathrm{F}_{\mathrm{G}}$ will become small (Benet et al., 2004). Substrate recognition of CYP3A4, which accounts for $80 \%$ of CYPs expressed in the intestine (Paine et al., 2006), is known to overlap with that of P-gp (Wacher et al., 1995). For this reason, understanding the mechanism of low $F_{G}$ is often difficult, and various studies have been performed using advanced pharmacokinetic models.

Physiologically based pharmacokinetic (PBPK) models that consider site-specific differences in absorption include the advanced compartmental absorption and transit (ACAT) (Agoram et al., 2001; Huang et al., 2009) and the advanced dissolution, absorption and metabolism (ADAM) model (Jamei et al., 2009). These models divide the gastrointestinal tract into multiple compartments and assume that drugs move to neighboring compartments sequentially with first-order rate constants and excellently explain the absorption profiles of various pharmaceuticals (Bolger et al., 2009; Takano et al., 2016). However, the evaluation of P-gp activity is unreproducible when analyzed in terms of drug concentration in the incubation media, and for this reason, several analyses that consider intracellular concentrations have been reported (Shirasaka, Masaoka, et al., 2008; Shirasaka, Sakane, et al., 2008; Tachibana et al., 2010), but it should be noted that these models do not explicitly analyze the drug concentration in the epithelial cells. A reason for this ambiguity is the lack of an appropriate method for measuring the permeability of the A and B membranes separately. In previous studies in which intracellular drug concentrations were considered, the permeability of the A and B membranes was assumed to be the same for this reason (Jamei et al., 2009). However, this hypothesis seems unreasonable because microvilli exist only on the A membrane, and the surface area is remarkably expanded (DeSesso and Jacobson, 2001; Helander and Fändriks, 2014). Furthermore, it has been reported that the expression of transporters is very different between A and B membranes (Giacomini et al., 2010; Giacomini and Huang, 2013).

We have reported the translocation model and advanced translocation model (ATOM) to solve these issues for physiologically based PBPK analysis of intestinal absorption (Ando et al., 2015; Asano et al., 2021). These models explain the disposition of drug and intestinal contents moving through the intestine more realistically than previous models and consider the permeability of the A and B membranes separately along with the contribution of the blood flow. However, there is currently only a small amount of quantitative information regarding the differences in membrane permeability between the A and B sides of the gastrointestinal tract and in regard to the $f_{\text {ent }}$, both of which are required for precise analysis using these models.

The purpose of this study was to develop a new method for in vitro evaluation of the absorption process, including the difference in membrane permeability between the $A$ and $B$ sides and the $f_{\text {ent }}$ (Fig. $1 \mathrm{~A})$ using Caco-2 cells in which CYP3A4 and NADPH-cytochrome P450 reductase (CPR) are coexpressed using human artificial chromosome (HAC) vector technology (CYP3A4-CPR-HAC/Caco-2) (Hiratsuka et al., 2011; Takenaka et al., 2017). To achieve this, we combined a preload efflux assessment (Fig. 1B) with a conventional transcellular permeability experiment. Furthermore, to evaluate P-gp transport, we applied simultaneous and dynamic model analyses rather than the efflux ratio that has been commonly used in the past (Ozeki et al., 2015). This is because steady transcellular permeation of various drugs cannot be guaranteed under the given experimental conditions when the permeability and $f_{\text {ent }}$ differ significantly between the drugs. To verify the parameters obtained in this study, we conducted in vitro to in vivo extrapolation (IVIVE) on the oral absorption of these drugs using ATOM, considering the scaling factors. We examined the effects of each parameter and absorption control factors on absorption by simulation.

## Materials and Methods

## Reagents

All the reagents were of the highest grade of commercially available and details are described in the supplement (Supp. Methods 1.1). All the references cited in the Supplement are described in the last part of the supplement (Supp. 3).

## Descriptions of in vitro and in vivo PK parameters

Descriptions of parameters used in this study are presented in Table 1 and Table 2 for in vitro and in vivo model of ATOM, respectively.

## Transcellular permeability of A to B and B to A with CYP3A4-CPR-HAC/Caco-2 cells

Preparation of CYP3A4-CPR-HAC/Caco-2 cells and the transport assessment were performed as reported (Takenaka et al., 2017, Supp. Methods 1.2) with a slight modification for 12 drugs indicated in Table 3 (P-gp substrates: fexofenadine, digoxin, CYP3A substrates: nisoldipine, buspirone, felodipine, midazolam, terfenadine, nifedipine, sildenafil, repaglinide, P-gp and CYP3A substrates: atorvastatin, quinidine). All experiments were performed in the presence and absence of $\mathrm{P}-\mathrm{gp}$ and CYP3A4 inhibition. We examined two, three, or four drugs in combination (i.e., cocktail method) when the drug concentrations were sufficiently low to check inter-study variability (Shibata et al., 2021). The cocktail conditions and drug concentration for transport assessment are attached (Supp. Table S1). CYP3A4-CPR-HAC/Caco-2 cell monolayers were studied in both the A-to-B and B-to-A directions for the transport assessment of the 12 drugs. The culture medium was removed, and the cells were rinsed twice with transport buffer ( pH 7.4 , HBSS( + ) with 20 mM D-glucose, $4.2 \mathrm{mM} \mathrm{NaHCO}_{3}, 10 \mathrm{mM}$ HEPES, and $\left.0.5 \% \mathrm{BSA}\right)$. The cells were preincubated in the transport buffer ( $300 \mu \mathrm{~L}$ for side A and $1000 \mu \mathrm{~L}$ for side B$)$ with or without inhibitors of P-gp (valspodar, $1 \mu \mathrm{M}$ ) or CYP3A4 (ketoconazole, $1 \mu \mathrm{M}$ ) on both sides for 30 min at $37{ }^{\circ} \mathrm{C}$ in a $5 \%$ $\mathrm{CO}_{2}$ incubator. We observed that valspodar or ketoconazole selectively inhibit P-gp or CYP3A4, respectively, at these concentrations (Supp. Fig. S1). The incubations were initiated by the addition of the
substrate drugs to the A side or B side for the A to B or the B to A transport experiment, respectively. Samples on the receiver side were collected sequentially at several time points during 2 to 6 h and cryopreserved if necessary. Midazolam was also assessed with an alternative experimental condition to confirm reproducibility of the experiment (Supp. Methods 1.3). The analysis in this study is based on a model that assumes monotonous intracellular concentrations, and the number of time points were considered sufficient, unless the intracellular heterogeneous distribution is analyzed in detail.

For quinidine, nonlinearity needs to be considered because of expected low Km value for $\mathrm{P}-\mathrm{gp}$ transport. Therefore, the experiment was performed in four concentrations (1, 3.3, 10 and $33 \mu \mathrm{M}$ ). In these samples, digoxin and fexofenadine (both $1 \mu \mathrm{M}$ ) were also included to check P-gp transport. Samples on the receiver side were collected sequentially at several time points during 2 to 6 h and cryopreserved if necessary. The unbound fraction of drugs $\left(f_{u}\right)$ was experimentally determined (Supp. Methods 1.4). The samples were subjected to a measurement of drug concentrations by LC-MS/MS (Shibata et al., 2021). Details are described in the supplement (Supp. Methods 1.5 and Table S2).

## Preload efflux assessment using CYP3A4-CPR-HAC/Caco-2 cells

An overview of the preload efflux assessment is given in Fig. 1B. The monolayer cells prepared as above on 24-multiwell inserts (Greiner Bio-One GmbH, Frickenhausen, Germany; or Corning, NY, USA) were used. The culture mediums in both A side $(100 \mu \mathrm{~L})$ and B side $(600 \mu \mathrm{~L})$ were replaced with the same volumes of the incubation buffer ( $\mathrm{pH} 7.4, \operatorname{HBSS}(+)$ with 20 mM D-glucose, 4.2 mM NaHCO , and 10 mM HEPES) containing $10 \mu \mathrm{M}$ ketoconazole as P-gp and CYP3A inhibitors (Floren et al., 1997), and the cells were preincubated for 30 min . Preliminary experiments confirmed that $10 \mu \mathrm{M}$ ketoconazole inhibited P-gp transport to the same extent as $1 \mu \mathrm{M}$ valspodar (Supp. Fig. S1). Both buffers were replaced with the incubation buffer containing substrate drugs (premixed as described above), $10 \mu \mathrm{M}$ ketoconazole and $0.5 \%$ BSA. The cocktail method was also applied to the preload efflux assessment and its conditions are shown in the supplement (Supp. Table S1). The buffers were then incubated for 2 h at $37{ }^{\circ} \mathrm{C}$ in a $5 \% \mathrm{CO}_{2}$ incubator to allow sufficient uptake of the substrate drugs into the cells. The concentrations of substrate drugs were $10 \mu \mathrm{M}$. The cells were washed three times with the cold incubation buffer with ketoconazole
and BSA but without substrate drugs, and the same buffer was added to both sides to start an incubation of 2 hr at $37^{\circ} \mathrm{C}$ in a $5 \% \mathrm{CO}_{2}$ incubator. Subsequently, a portion of the buffer were sampled sequentially from both sides for up to 1 hr . The drug concentrations in the sample were measured by LC-MS/MS. In this experiment, it is more important to determine the total amount of drugs eluting on the A and B sides than to determine the details of the time course that strongly affected the membrane permeability ratio of B to A $\left(R_{B A}=P S_{B} / P_{A}\right)$ and the $f_{\text {ent }}$ in the following analysis.

## Simultaneous and dynamic model analysis with a three-compartment model

Kinetic parameters were obtained through simultaneous and dynamic model analysis with a threecompartment model consisting of A-side, the intracellular compartment, and the B-side (Fig. 1, Equations 1-5).

$$
\begin{align*}
& V_{A} \frac{d C_{A}}{d t}=-P S_{A} f_{u} C_{A}+\left(P S_{A}+P S_{P g p}\right) f_{\text {ent }} C_{\text {cell }},  \tag{Eq.1}\\
& V_{\text {cell }} \frac{d C_{\text {cell }}}{d t}=P S_{A} f_{u} C_{A}-\left(P S_{A}+P S_{P g p}+P S_{B}+C L_{M}\right) f_{\text {ent }} C_{\text {cell }}+P S_{B} f_{u} C_{B},  \tag{Eq.2}\\
& V_{B} \frac{d C_{B}}{d t}=P S_{B}\left(f_{\text {ent }} C_{\text {cell }}+f_{u} C_{B}\right),  \tag{Eq.3}\\
& P S_{P g p}=\frac{V_{\text {max, Pgp }}}{K_{m, P g p}+f_{\text {ent }} \times C_{\text {cell }}},  \tag{Eq.4}\\
& P_{a p p}=\frac{P S_{A} P S_{B}}{\left(P_{A}+P S_{B}+P_{P g p}+C_{M}\right) S A} . \tag{Eq.5}
\end{align*}
$$

A description of each parameter can be found in Table 1. SA of the insert was $0.33 \mathrm{~cm}^{2}$. The values of $\mathrm{V}_{\mathrm{A}}$, $\mathrm{V}_{\mathrm{B}}$, and $\mathrm{V}_{\text {cell }}$ were $300 \mu \mathrm{~L}, 1000 \mu \mathrm{~L}$ and $0.89 \mu \mathrm{~L}$, respectively. $\mathrm{V}_{\text {cell }}$ was calculated from SA and the height of Caco-2 cells (27.2 $\mu \mathrm{m}$, Moyes et al., 2010). For quinidine, Eq. 4 was applied to consider nonlinearity of $\mathrm{PS}_{\text {Pgp }}$. Eq. 5 was obtained from Eqs. 1 and 2 assuming the steady state.

Time courses for the drug concentration in the transcellular permeability and preload efflux assessments were fitted to models with distinct initial conditions (i.e., initial drug concentrations in the three compartments corresponding to each experiment). For each drug, the analysis was simultaneous, and all parameters were estimated from all the time course simultaneously. The initial unbound concentration in
the cells for the preload efflux assessment was considered to be the same as the unbound extracellular concentration, and thus $f_{\text {ent }}$ was naturally determined according to the $V_{\text {cell }}$ and the drug mass recovered in this experiment in the fitting analysis. That is, $f_{\text {ent }}$ and $R_{B A}$ are predominantly determined by the preload efflux assessment. For quinidine, the time courses of quinidine and coexisting digoxin with various initial concentrations of quinidine in the donor buffer were combinedly analyzed. Detail is described in the supplement (Supp. Methods 1.6). These analyses were performed using the flexible simultaneous fitting analysis function implemented in Numerical analysis program for pharmacokinetics, Napp (version 2.31 or 3.04) (Hisaka and Sugiyama, 1998).

## Analysis by ATOM

ATOM is a physiologically based intestinal absorption model that captures continuous convection and micro-mixing of contents in the lumen rather than intermittent changes divided by compartments in conventional models as previously reported (Asano et al., 2021). The effects of drug concentration, water contents, pH , and enzyme expression can be considered more accurately using this model. ATOM explains various changes based on the partial differential equations listed below but with slight modifications in this study to bridge the in vitro and in vivo observations.

$$
\begin{align*}
& \left.\frac{\partial C_{\text {lum }, \mathrm{z}}}{\partial \mathrm{t}}=\mathrm{D}_{\mathrm{z}} \frac{\partial^{2} \mathrm{C}_{\text {lum }, \mathrm{z}}}{\partial \mathrm{z}^{2}}-\mathrm{M}_{\mathrm{t}} \frac{\partial \mathrm{C}_{\text {lum }, \mathrm{z}}}{\partial \mathrm{z}}-\widehat{\mathrm{PS}}_{\mathrm{A}, \text { in }, \mathrm{z}} \frac{\mathrm{f}_{\text {lum }} \mathrm{C}_{\text {lum }, \mathrm{z}}}{\widehat{\mathrm{X}}_{\text {water }, \mathrm{z}}}+\widehat{(\widehat{P S}}_{\mathrm{A}, \text { out }, \mathrm{z}}+\widehat{\mathrm{PS}}_{\mathrm{Pgp}, \mathrm{z}}\right) \frac{\mathrm{f}_{\text {ent }} \mathrm{C}_{\text {ent }, \mathrm{z}}}{\widehat{\mathrm{~V}}_{\text {lum }, \mathrm{z}}},  \tag{Eq.6}\\
& \widehat{\mathrm{~V}}_{\text {ent,z }} \frac{\mathrm{dC}}{\text { ent }, \mathrm{z}} \mathrm{dt}=\widehat{\mathrm{PS}}_{\mathrm{A}, \text { in }, \mathrm{z}} \mathrm{f}_{\text {lum }} \mathrm{C}_{\text {lum }, \mathrm{z}}-\left(\widehat{\mathrm{PS}}_{\mathrm{A}, \text { out }, \mathrm{z}}+\widehat{\mathrm{PS}}_{\mathrm{Pgp}, \mathrm{z}}+\widehat{\mathrm{CL}}_{\mathrm{M}, \mathrm{z}}+\widehat{\mathrm{PS}}_{\mathrm{B}, \mathrm{z}}\right) \mathrm{f}_{\text {ent }} \mathrm{C}_{\mathrm{ent}, \mathrm{z}} \\
& +f_{\text {pro }} \widehat{\mathrm{PS}}_{\mathrm{B}, \mathrm{z}} \mathrm{C}_{\mathrm{pro}, \mathrm{z}},  \tag{Eq.7}\\
& \widehat{\mathrm{~V}}_{\text {pro }, \mathrm{z}} \frac{\mathrm{~d} \mathrm{C}_{\text {pro }, \mathrm{z}}}{\mathrm{dt}}=\widehat{\mathrm{PS}}_{\mathrm{B}, \mathrm{z}} \mathrm{f}_{\text {ent }} \mathrm{C}_{\text {ent }, \mathrm{z}}-\left(\widehat{\mathrm{Q}}_{\text {pro }, \mathrm{z}} / \mathrm{f}_{\mathrm{b}}+\mathrm{f}_{\text {pro }} \widehat{\mathrm{PS}}_{\mathrm{B}, \mathrm{z}}\right) \mathrm{C}_{\text {pro,z }},  \tag{Eq.8}\\
& \mathrm{F}_{\mathrm{A}} \mathrm{~F}_{\mathrm{G}}=\frac{1}{D} \iint \widehat{\mathrm{Q}}_{\mathrm{pro}, \mathrm{z}} \mathrm{C}_{\mathrm{pro,z}} d z d t, \tag{Eq.9}
\end{align*}
$$

where distance from pylorus, z is expressed as a ratio of the hypothetical capacity volume of the lumen until the location to its overall total $(1,350 \mathrm{~mL})$ thus it ranges from 0 to 1.0 (this is because volume is more appropriate than length to consider movements of mass), $\mathrm{C}_{\text {lum, } z}$ is luminal concentration at z based on the
capacity volume, and $\mathrm{C}_{\text {ent, }}$ and $\mathrm{C}_{\mathrm{pro,z}}$ are concentrations in the epithelial cells and lamina propria at z , respectively. $D_{z}$ and $M_{t}$ represent the dispersion constant at $z$ and flow rate at time $t$ after oral administration of dose $D$, respectively. $\widehat{V}_{\text {ent }, \mathrm{z}}$ and $\widehat{V}_{\text {pro,z }}$ are volumes of epithelial cells and lamia propria at z. $\widehat{\mathrm{X}}_{\text {water,z }}$ represents the hypothetical concentration of inflating water based on the capacity volume at z . By assuming $\widehat{X}_{\text {water,z }}$, the luminal drug concentrations subjected to absorption are determined by the local water contents, not by the capacity volume. The total water volume in the small intestine is assumed to be approximately 70 mL at steady state based on the observed in vivo water contents in the intestine. In ATOM, $\widehat{X}_{\text {water }, \mathrm{z}}$ is calculated assuming a separate partial differential equation regulated also by $D_{z}$ and $M_{t}$. Unbound fraction in the lumen, $\mathrm{f}_{\text {lum }}$, was assumed to be 1 , and unbound fraction in the lamina propria, $\mathrm{f}_{\text {pro }}$, was assumed to be the same as that in the plasma. The hats attached to PS, CL, V, X, and Q representing in vivo parameter. The distribution of $\widehat{\mathrm{PS}}_{\mathrm{Pgp}, \mathrm{z}}$ and $\widehat{\mathrm{CL}}_{\mathrm{M}, \mathrm{z}}$ were set to decrease or increase along the location as reported (Asano et al., 2021). The intestinal distribution of $\widehat{\mathrm{PS}}_{\mathrm{A}, \mathrm{in}, \mathrm{z}}, \widehat{\mathrm{PS}}_{\mathrm{A}, \mathrm{out}, \mathrm{z}}, \widehat{\mathrm{PS}}_{\mathrm{B}, \mathrm{z}}$ and $\widehat{\mathrm{Q}}_{\text {pro,z }}$ were calculated considering changes in the boundary length of the cross-section caused by location-dependent changes in radius, plicae, villi and microvilli expansion. $\widehat{\mathrm{Q}}_{\mathrm{proz}, \mathrm{z}}$ and $\mathrm{f}_{\mathrm{b}}$ are local blood flow to the lamia propria at z and unbound fraction in the blood, respectively. The relationships between in vitro and in vivo parameters are defined in equations 10-14.

$$
\begin{align*}
& \widehat{\mathrm{PS}}_{\mathrm{A}, \mathrm{in}, \mathrm{z}}=\mathrm{R}_{\mathrm{HH}, \mathrm{z}} \mathrm{R}_{\mathrm{BL}, \mathrm{apical}, \mathrm{z}} \mathrm{PS}_{\mathrm{A}},  \tag{Eq.10}\\
& \widehat{\mathrm{PS}}_{\mathrm{A}, \mathrm{out}, \mathrm{z}}=\mathrm{R}_{\mathrm{BL}, \mathrm{apical}, \mathrm{z}} \mathrm{PS}_{\mathrm{A}},  \tag{Eq.11}\\
& \int_{0}^{1} \widehat{\mathrm{PS}}_{\mathrm{Pgp}, \mathrm{z}} \mathrm{dz}=\mathrm{SF}_{\mathrm{Pgp}} \mathrm{PS}_{\mathrm{Pgp}} \int_{0}^{1} \mathrm{R}_{\mathrm{BL}, \text { apical, } \mathrm{Z}} \mathrm{dz}  \tag{Eq.12}\\
& \widehat{\mathrm{PS}}_{\mathrm{B}, \mathrm{z}}=\mathrm{R}_{\mathrm{BL}, \text { basal,z }} \mathrm{PS}_{\mathrm{B}},  \tag{Eq.13}\\
& \int_{0}^{1} \widehat{\mathrm{CL}}_{\mathrm{M}, \mathrm{z}} d z=\mathrm{SF}_{\mathrm{CYP} 3 \mathrm{~A} 4} \mathrm{R}_{\mathrm{CYP} 3 \mathrm{~A} 4} \mathrm{CL}_{\mathrm{M}}, \tag{Eq.14}
\end{align*}
$$

where $\mathrm{R}_{\mathrm{HH}, \mathrm{z}}$ represents an adjustment constant for uncharged fraction of drug at z from the value of the in vitro experiment (performed at pH 7.4 ) that was calculated using the Henderson-Hasselbalch equation. Drug specific values for pKa and $\mathrm{f}_{\mathrm{b}}$ are listed in the supplement (Supp. Table S3). $\mathrm{R}_{\mathrm{BL}, \text { apical,z }}$ and $\mathrm{R}_{\mathrm{BL}, \text { basal,z }}$
represent the ratios between the boundary length of the cross-section of the lumen at $z$ to the surface area in Caco-2 experiments for the A and B membranes, respectively. $\mathrm{R}_{\mathrm{CYP} 3 \mathrm{~A} 4}$ represents the ratio of in vitro to in vivo CYP3A4 expression. $\mathrm{SF}_{\mathrm{Pgp}}$ and $\mathrm{SF}_{\mathrm{CYP3A}}$ are scaling factors for $\mathrm{PS}_{\mathrm{Pgp}}$ and $\mathrm{CL}_{\mathrm{M}}$ in vitro and in vivo, respectively, and they were calculated sequentially by fitting to observed $F_{A} F_{G}$. Although the present analysis generally assumes linear conditions, the $\mathrm{K}_{\mathrm{m}}$ of P -gp or CYP3A4 for quinidine is small and is already nonlinear at clinical doses (Maeda and Sugiyama, 2011; Maeda et al., 2011). Therefore, the analysis was performed in a nonlinear fashion for quinidine. Detail is described in the supplement (Supp. Methods 1.7).

## Data availability

Napp (beta version) is available from web site of Clinical Pharmacology and Pharmacometrics Laboratory, Chiba University (https://www.p.chiba-u.jp/lab/cpp/en/index.html). The essential part of ATOM source code is provided (Supp. 2). All the raw data of in vitro experiments would be provided upon request.

## Results

## In vitro assessment using CYP3A4-CPR-HAC/Caco-2 cells

First, the permeability of both A to B and B to A directions were assessed using CYP3A4-CPR-HAC/Caco-2 cells for 12 representative substrates of P-gp and CYP3A4, and concentration changes in the receiver buffer were evaluated (Fig. 2). The permeability of P-gp substrates (fexofenadine, digoxin, atorvastatin, and quinidine at lower concentrations) was higher for the $B$ to $A$ direction than it was for the A to B , and the difference was diminished in the presence of the P -gp inhibitor or at higher concentrations. For varying initial concentrations of quinidine, a change in the efflux transport of coexisting digoxin was observed in the A membrane (Fig. 2M). Unexpectedly, however, only the A to B permeability of coexisting fexofenadine was nearly constant (Fig. 2N), probably due to the combined effects of suppression of input and output transports across the A membrane. For nisoldipine, which is the compound most susceptible to CYP3A4 metabolism in this study, apparent permeability was increased in the presence of CYP3A4 inhibitor in both A to B and B to A directions (Fig. 2E). For all CYP3A4 substrates (nisoldipine, midazolam, nifedipine, buspirone, felodipine, terfenadine, sildenafil atorvastatin, and repaglinide), peaks of their metabolites or presumed metabolites were detected by LC-MS/MS on both the donor and receiver sides which disappeared in the presence of CYP3A4 inhibitors (data not shown). However, for drugs other than nisoldipine, no significant change in permeability was observed upon CYP3A4 inhibition. Therefore, it is likely that these drugs were metabolized by CYP3A4 in the cells under the conditions of this experiment, but the change in permeability was not detected due to insufficient sensitivity. A nearly identical permeability assessment for midazolam was also performed with different inhibitors of P-gp and CYP3A4, and the reproducibility was confirmed (Table 3 and Supp. Fig. S2).

Next, the cells were preloaded with these drugs from both the A and B sides for a sufficient time under conditions P-gp and CYP3A4 were completely inhibited, and then efflux to both sides of the cells were assessed after expedite washing and replacement with blank buffers (Fig. 3). While the efflux profiles of sides A and B were similar for some drugs, the efflux from side B was more extensive than that from
side A for fexofenadine, digoxin, atorvastatin, midazolam, and repaglinide. For felodipine and nisoldipine, the efflux from side A was higher than was that from side B.

It is important that the preloading is performed in a manner that allows substrate drugs to be taken up sufficiently by the cells and reach a steady state. To achieve this, inhibition of P-gp was necessary, and the validity of the preloading time was confirmed according to simulation analysis (Supp. Fig. S3). In these experiments, $0.5 \%$ bovine serum albumin (BSA) was added to the buffer to prevent adsorption. The free fraction $\left(f_{u}\right)$ was evaluated using the equilibrium dialysis method to correct binding (Table 3).

## Parameter estimation for all elementary processes involved in absorption

Results from transcellular permeation (Fig. 2 ) and preload efflux experiments (Fig. 3) were analyzed using simultaneous dynamic model analysis (Fig. 1A) to obtain the A and B side membrane exchange permeabilities $\left(\mathrm{PS}_{\mathrm{A}}, \mathrm{PS}_{\mathrm{B}}\right)$, efflux by P -gp $\left(\mathrm{PS}_{\mathrm{Pgp}}\right)$, metabolic clearance $\left(\mathrm{CL}_{\mathrm{M}}\right)$, and unbound fraction in the enterocytes $\left(\mathrm{f}_{\text {ent }}\right)$ for all drugs (Table 3). We did not use efflux ratios for this analysis, as the permeability and $f_{\text {ent }}$ values appeared to be significantly different among the drugs and the appropriate incubation time to achieve steady transcellular permeation would be very difficult to verify, as demonstrated by a posthoc simulation (Fig. 4A). In the dynamic model analysis, the efflux ratio changes time-dependently and is meaningless. For CYP3A4 substrates other than nisoldipine, the intrinsic clearances reported from microsomal incubations of human intestine (Gertz et al., 2010) were converted to estimated $\mathrm{CL}_{\mathrm{M}}$ under the conditions of this experiment using nisoldipine as a standard. The membrane permeability from the A side was composed of $\mathrm{PS}_{\mathrm{A}}$ and $\mathrm{PS}_{\mathrm{Pgp}}$, while the equivalent permeation in both directions was assumed for the B side. This model explained the observations satisfactorily, and a model assuming different inflow and outflow velocities for the B side did not improve the correlation between the data and predictions. For midazolam, changing the inhibitors or other conditions did not essentially change the results (Table 3).

The $\mathrm{PS}_{\mathrm{A}}$ obtained by the analysis was the largest for felodipine and the smallest for fexofenadine, with a difference of more than 700 -fold (Table 3 ). The $B$ to A membrane permeability ratio $\left(\mathrm{R}_{\mathrm{BA}}=\right.$ $\mathrm{PS}_{\mathrm{B}} / \mathrm{PS}_{\mathrm{A}}$ ) was 0.39 for felodipine. In contrast, digoxin, repaglinide, fexofenadine, and atorvastatin
exhibited higher permeability from the $B$ side ( $3.44,2.39,2.27$, and 1.90 , respectively). Therefore, the $R_{B A}$ differed by approximately 8.8 -fold depending on the drug used in this study. The $f_{\text {ent }}$ was the highest for digoxin and the lowest for felodipine, with a difference of more than 3,000-fold. Clear correlation between $f_{u}, f_{\text {ent }}$, and $\log D$ values was not found in this study. Digoxin and fexofenadine were included at low concentrations in the quinidine evaluation to confirm P-gp activity, but fexofenadine was not included in the kinetic analysis because of unexpected profile of its A to B permeability. The nonlinearities observed with quinidine and digoxin were excellently explained by the model with $\mathrm{K}_{\mathrm{m}}$ of $0.077 \mu \mathrm{M}$.

## Simulation of absorption profiles of substrate drugs of P-gp and CYP3A4 by ATOM

ATOM takes into account various changes in the small intestine along length from the pylorus such as in radius and surface area, in the pH of the lumen, in the density of villi, and in the expressions of CYP3A4 and P-gp (Fallingborg et al., 1989; Willmann et al., 2004; Bruyère et al., 2010). The absorption profile of digoxin was simulated using the parameters that were obtained using ATOM. In the presence of P-gp activity, digoxin was retained in the lumen and migrated into the lower intestine (Fig. 5A. 5C), and rapid absorption from the upper intestine was estimated in the absence of P-gp activity (Fig. 5B, 5D). The major absorption site was shifted in response to the inhibition of digoxin. (Fig. 5E). In contrast, for CYP3A4 substrates, the absorption site was not altered by inhibition (Supp. Fig. S4). It was estimated that the absorption time of felodipine with the lowest estimated $f_{\text {ent }}$ value was altered within hours depending on $\mathrm{f}_{\text {ent }}$ (Fig. 4B). These simulations suggest that the in vitro parameters affect the in vivo absorption profile.

## Prediction of in vivo overall intestinal availability $\left(F_{A} F_{G}\right)$ and its influencing factors

Scaling factors for $\mathrm{CL}_{\mathrm{M}}$ and $\mathrm{PS}_{\text {Pgp }}$ in vitro and in vivo $\left(\mathrm{SF}_{\mathrm{CYP3A4}}, \mathrm{SF}_{\mathrm{Pgp}}\right)$ were defined as the activity ratios based on the CYP3A4 expression and surface area of apical membrane, respectively, between in vitro and in vivo, and the values of 1.43 and 18.9 were obtained from observed overall intestinal availability $\left(\mathrm{F}_{\mathrm{A}} \mathrm{F}_{\mathrm{G}}\right)$ for CYP3A4 substrate and P-gp substrate drugs, respectively.

Using these SFs in ATOM, the relationships between estimated and observed $\mathrm{F}_{\mathrm{A}} \mathrm{F}_{\mathrm{G}}$ for 12 substrate drugs of P-gp and CYP3A4 were obtained. The accuracy of the prediction was within the range of
$\pm 0.2$ for 10 of the 12 drugs evaluated (Fig. 6). For quinidine, in vitro experiments could predict $F_{A} F_{G}$ in the range of 0.1-100 mg with satisfactory accuracy considering nonlinearity. On the other hand, the observed values for buspirone tended to be overestimated, while those for terfenadine tended to be underestimated.

Since it was observed that the $\mathrm{R}_{\mathrm{BA}}$ varied in the range of at least 0.39 to 3.44 depending on the drug used in this study, we simulated the effect of this range of variation on $\mathrm{F}_{\mathrm{A}} \mathrm{F}_{\mathrm{G}}$ (Fig. 6). The simulation suggested that $F_{A} F_{G}$ tended to decrease as $R_{B A}$ increased, and this change was particularly pronounced for drugs with low $\mathrm{F}_{\mathrm{A}} \mathrm{F}_{\mathrm{G}}$. We also simulated $\mathrm{F}_{\mathrm{A}} \mathrm{F}_{\mathrm{G}}$ of these drugs under different assumptions of intestinal location dependence of P-gp and CYP3A expressions and the intestinal blood flow (Supp. Fig S5A and S5B). However, the changes were minor compared to those caused by changes in $R_{B A}$.

## Discussion

In this study, the role of basal membrane permeability in the absorption process was evaluated by using a new in vitro method and analyzed it with an advanced intestinal PBPK model. Our results for fexofenadine are consistent with those report by Sugano et al., who evaluated permeabilities of the A and B side membrane using the asymmetry of P-gp activity, with a greater permeability from the B-side (Sugano et al., 2011). We revealed that it is inappropriate to assume that the membrane permeabilities of the A and $B$ sides are the same for various drugs. The simulation indicated that $F_{A} F_{G}$ was decreased for both CYP3A4 and P-gp substrate drugs when the membrane permeability from the B side was decreased (Fig. 6).

Digoxin and atorvastatin with $\mathrm{R}_{\mathrm{BA}}$ values that were estimated to be greater than 1.0 in this study have been reported as substrates of the organic solute transporter OST $\alpha / \beta$ (Wang et al., 2001; Seward et al., 2003; Beaudoin et al., 2020) that is highly expressed in the small intestine and facilitates the diffusion of various drugs on the B membrane in both directions. Its substrate recognition has been reported to be similar to the organic anion transporting polypeptide (OATP) (Wang et al., 2001; Ballatori et al., 2005; Beaudoin et al., 2020). Accordingly, fexofenadine and repaglinide with $\mathrm{R}_{\mathrm{BA}}$ values that were also evaluated to be greater than 1.0 are substrates of OATP (Shimizu et al., 2005; Kalliokoski and Niemi, 2009), and thus might also be substrates of OST $\alpha / \beta$. Additionally, multidrug resistance-associated protein 3 (MRP3) on the B-membrane is also suspected to be involved in causing larger $R_{B A}$ of its substrate, fexofenadine, but this cannot be simply interpreted because it does not function as an exchange transporter (Ming et al., 2011).

Fexofenadine and quinidine are known to be substrates for OATP and OCTN, respectively, on the A-membrane (Yabuuchi et al., 1999; Shimizu et al., 2005); however, the extent of their contribution is unknown. Moreover, it is unknown if many drugs are substrates (or inhibitors) of transporters on the Bside. The difference in permeability between membranes A and B that was observed in this study may be, at least for some drugs, due to unknown contribution of some transporter.

Side A possesses microvilli that results in an extremely enlarged surface area. Side B may be affected by the permeability of the lateral membrane. These physical differences may not only affect membrane permeability but also diffusion rate near the membrane and binding to macromolecules that
affect diffusion rate, ultimately also causing apparent differences in permeability between the A and B sides without transporter contribution.

In the present study, including the A-to-B and B-to-A permeation and preload efflux experiments, linear kinetics were assumed with the exception of quinidine to avoid excessive complexity. It is noteworthy that this study evaluated the $\mathrm{K}_{\mathrm{m}}$ of quinidine while taking into account the contribution of intracellular binding and the permeability of the B-side, thus resulting in a significantly lower value than that previously reported (Takano et al., 2016), and this was used to predict in vivo dose dependency that resulted in a clear improvement in accuracy, particularly at the lower dose range where prediction accuracy has been reported to be poor (Ando et al., 2017). The unexpected profile of A to B permeability of fexofenadine in the quinidine combination experiment might be due to inhibition by quinidine of an unidentified uptake transporter on the A membrane.

For appropriate preload-efflux assessments, the preloading time should be sufficient for the medium and intracellular drug concentrations to reach equilibrium. In the present study, based on the parameters obtained in the preliminary study, the necessary preloading time was confirmed by modeling analysis (Supp. Fig. S3). In addition, conditions should be set at both the A and B membranes where intracellular metabolic activities and transport, which may result in concentration gradients of the free drug, are sufficiently inhibited. Although conditions of sufficient inhibition were used for P-gp and CYP3A4 in this study, care should be taken when using primary cultured human intestinal cells (Kasendra et al., 2018) or iPS cells (Kabeya et al., 2020), as these cells exhibit numerous types of activity.

The profile of drug absorption was simulated by ATOM based on the parameters obtained from the in vitro experiments. For P-gp substrates, changes in absorption time and the disappearance of the double peak phenomenon have been reported to be associated with the inhibition of P-gp (Thomas and Aldous, 1973; Davies et al., 2010; Wada et al., 2013; Li et al., 2016). These issues may become an interesting theme for future applications of analysis using ATOM. For both P-gp and CYP3A4, simulations with uniform distribution in the gastrointestinal tract revealed that $F_{A} F_{G}$ tended to be increased for many drugs (Supp. Fig. S5A), which is consistent with the results reported by Watanabe et al. using a tube model (Watanabe et al., 2013).

To predict in vivo $\mathrm{F}_{\mathrm{A}} \mathrm{F}_{\mathrm{G}}$ in ATOM, we set minimum SFs for each CYP3A4 and P-gp activity; for which $\mathrm{SF}_{\text {CYP3A4 }}$ was 1.42 , based on enzyme expression, which is close to 1 , indicating that ATOM appropriately accounts for CYP3A4 activity. However, we did not set an SF for $\mathrm{R}_{\mathrm{BA}}$ since it may differ between in vitro and in vivo, in which case the $\mathrm{SF}_{\text {CYP3A4 }}$ may need to be adjusted. As $\mathrm{SF}_{\text {Pgp }}$ is based on the apical membrane area, the difference in activity between Caco-2 cells and the human intestine may be the cause this high value of 18.9.

Using the above $\mathrm{SFs}, \mathrm{F}_{\mathrm{A}} \mathrm{F}_{\mathrm{G}}$ was predicted with satisfactory accuracy for 10 of the 12 drugs evaluated in this study. However, the prediction accuracy for terfenadine and buspirone was insufficient. Among the drugs evaluated in this study, the dissociation constant of these two compounds is close to the pH of the small intestine (Kornhuber et al., 2008; Karlsson et al., 2013), and the instability of dissociation in the gastrointestinal tract may have affected the prediction accuracy. Terfenadine is a drug with a very low unbound fraction in the microsomal system ( $\mathrm{f}_{\text {umic }}$ ) (Gertz et al., 2008), making it difficult to assess CYP3A4 clearance. A large CL, about half that of nisoldipine, has been reported (Gertz et al., 2010). However, there was no apparent change in permeability in CYP3A4-expressing Caco-2 cells (Fig. 2I). For terfenadine, there is a discrepancy between the metabolic activity of previous microsomal experiments and the present cellular experiment (Supp. Fig. S6), which may be the reason for the poor predictability of in vivo $\mathrm{F}_{\mathrm{A}} \mathrm{F}_{\mathrm{G}}$. Buspirone was also reported as an outlier among 15 CYP 3 A substrate drugs in a $\mathrm{F}_{\mathrm{G}}$ prediction study (Nishimuta et al. 2011). In the future, intestinal clearance mechanisms other than CYP3A4 may also need to be pursued for buspirone.

A number of limitations and issues may restrict the reliability of the present analysis. First, the mechanism of the relatively high permeability of the B membrane must be identified. Further understanding of precise mechanisms of the membrane permeation of drugs, including the contribution of transporters other than P-gp even in the A membrane, as well as the contribution of the paracellular pathway, is also needed. If it is mediated by a transporter, the molecular species, its expression and distribution in vitro and in vivo, and the mechanism of its regulation need to be clarified. The $\mathrm{K}_{\mathrm{m}}$ of P-gp transport for quinidine evaluated in this study was lower than previously reported. Therefore, it is possible that $\mathrm{K}_{\mathrm{m}}$ of the other P-gp substrates, when evaluated more accurately considering intracellular
concentrations, are also lower than previously reported, and therefore, some of experiments in this study would have been performed at inappropriately high concentrations. Next, the binding targets of drugs in the epithelial cells should be elucidated, which causes significant differences in the $f_{\text {ent }}$ between drugs. Although this study minimizes the number of scaling factors, there remains still a risk that the fit may appear better than it actually is due to overfitting. Future studies with validation data sets may be needed to fully prove the validity of the method proposed in this study. Finally, accurate information regarding the gastrointestinal tract is critical for the success of ATOM prediction. Therefore, the size, water distribution, viscosity and osmotic pressure in the lumen, density and distribution of villi and microvilli, blood flow, and their changes in sex, age, ethnicity, and pathological conditions should be further elucidated.

We believe that permeability of the A membrane is of course important as the dominant factor in absorption. In addition to that, this study experimentally showed for the first time that permeability of the B membrane should not be ignored, and demonstrated that the usefulness of new approaches, including the preload efflux assessment, simultaneous and dynamic model analysis, and absorption prediction by ATOM. On the other hand, there were still only a limited number of applications. It is necessary to accumulate many further objective results to determine the preciseness and practicality of the method proposed here.

## Authorship Contributions

Participated in research design: Yoshitomo, Sato, Hisaka.

Conducted experiments: Yoshitomo, Asano, Hozuki, Tamemoto, Shibata, Hashimoto, Takahashi, Sasaki, Ozawa, Kageyama, Iijima.

Contributed new reagents or analytic tools: Yoshitomo, Asano, Kazuki, Hisaka.

Performed data analysis: Yoshitomo, Asano, Hisaka.

Wrote or contributed to the writing of the manuscript: Yoshitomo, Asano, Sato, Hisaka.

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## Footnotes

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Figure legends

Fig. 1 Three-compartment kinetic model for analysis of in vitro experiment (Panel A) and the concept of preload efflux method (Panel B) for assessment of necessary parameters for prediction of intestinal absorption. $\mathrm{PS}_{\mathrm{A}}$ is permeability surface area product assuming passive process for both directions, and $\mathrm{PS}_{\mathrm{Pgp}}$ represents efflux by P-gp at apical (A) side. $\mathrm{PS}_{\mathrm{B}}$ is permeability surface area product assuming passive or exchange transport at basal (B) side. $\mathrm{CL}_{\mathrm{M}}$ represents intrinsic metabolic clearance by CYP3A4. $\mathrm{V}_{\mathrm{A}}, \mathrm{V}_{\mathrm{B}}$, and $\mathrm{V}_{\text {cell }}$ are distribution volumes of buffers at the A and B sides and cells, respectively.

Fig 2. Changes in normalized drug concentration in receiver buffer in the permeation experiment using CYP3A4-CPR-HAC/Caco-2 cells. Apical (A) to basal (B) and B to A in panels A-N represent the permeation of each direction for the drug shown. The black, red and blue symbols and lines represent measurements and fitting lines by the model (except for panel N ) in the absence of P-gp and CYP3A4 inhibitor, in the presence of P-gp inhibitors, and in the presence of CYP3A4 inhibitor, respectively. In panels $\mathrm{D}, \mathrm{M}$, and N , closed square, open circle, closed circle and open square represent initial quinidine concentrations of $1,3.3,10$, and $33 \mu \mathrm{M}$, respectively. Each point represents the mean $\pm$ standard deviation of 21 samples for midazolam and three samples for the other drugs.

Fig. 3. Drug efflux from preloaded CYP3A4-CPR-HAC/Caco-2 cells to the apical (A) and basal (B) sides. In panels A-L, the black and green symbols and solid lines represent measurements and fitting lines in the A and B sides, respectively, for the drug shown. The dotted black line represents simulated amount change in the cells by the model. Each point represents the mean $\pm$ standard deviation of three samples.

Fig. 4 Simulation of the effects of varying drug unbound fraction in enterocytes ( $\mathrm{f}_{\mathrm{ent}}$ ) on transcellular permeation assessment in vitro (A) and cumulative absorption profile in vivo (B) for felodipine. In Panel (A), solid and broken lines represent A to B and B to A assessments, respectively. Lines in gray, green, blue and orange represent $f_{\text {ent }}$ of $0.0003,0.0006,0.0015$ and 0.003 , respectively.

Fig. 5 Simulation of in vivo intestinal absorption profile of digoxin by ATOM with parameters estimated from in vitro experiments. The small intestine was divided into 40 segments hypothetically by volume in this simulation. Migrated drug to the lumen of the segments with normal P-gp activity (A, gray) and without P-gp activity (B, orange). Distribution of drug in the epitherial cells in the segments with normal Pgp activity (C, gray) and without P-gp activity (D, orange). Cumulative absorptions to the portal blood were shown by blue lines in panels (C) and (D). Small numbers in panels (A) to (D) represent the number of the segment from upstream. Contributions of each segment to the cumulative absorption with normal P-gp activity and without P-gp activity were shown in gray and orange, respectively, in (E).

Fig 6. Relationships between observed and predicted overall intestinal availability ( $\mathrm{F}_{\mathrm{A}} \mathrm{F}_{\mathrm{G}}$ ) by ATOM with parameters estimated from in vitro experiments. It represents simulated effects by varying permeability ratio of basal and apical membranes $\left(\mathrm{R}_{\mathrm{BA}}\right)$ on predictions of $\mathrm{F}_{\mathrm{A}} \mathrm{F}_{\mathrm{G}}$. Red, blue, and orange symbols indicate P-gp substrates, CYP3A4 substrates, and those serve as both, respectively. The solid and dotted lines represent the unity and error range within $\pm 0.2$-fold. The vertical bar lengths were determined based on the highest (3.44) and the lowest $\mathrm{R}_{\mathrm{BA}}(0.39)$ observed in this study. $\mathrm{PS}_{\mathrm{A}}$ was fixed to the observed value and $\mathrm{PS}_{\mathrm{B}}$ was varied to adjust $\mathrm{R}_{\mathrm{BA}}$. The small numbers identify drugs in panel (A); fexofenadine 1, digoxin 2, terfenadine 3 , nisoldipine 4 , felodipine 5 , midazolam 6 , buspirone 7 , nifedipine 8 , sildenafil 9 , repaglinide 10 , atorvastatin 11 , quinidine $0.1 \mathrm{mg} \mathrm{12-1}$, quinidine $1 \mathrm{mg} \mathrm{12-2} ,\mathrm{quinidine} 10 \mathrm{mg} \mathrm{12-3}$ and quinidine 100 mg 12-4.

Table 1. Descriptions of in vitro parameters in this study

| parameters | description |
| :---: | :---: |
| $\mathrm{PS}_{\text {A }}$ | $\mathrm{PS}^{\mathrm{a}}$ in both directions at the A- membrane |
| PS ${ }_{\text {B }}$ | $\mathrm{PS}^{\mathrm{a}}$ in both directions at the B-membrane |
| PS $\mathrm{P}_{\text {Pg }}$ | PS ${ }^{\text {a }}$ for efflux transport by P-gp |
| $\mathrm{K}_{\mathrm{m}, \mathrm{Pgp}}$ | $\mathrm{K}_{\mathrm{m}}$ of transport by P-gp |
| $\mathrm{V}_{\text {max,Pgp }}$ | $\mathrm{V}_{\text {max }}$ of transport by P-gp |
| $\mathrm{CL}_{\mathrm{M}}$ | metabolic clearance by CYP3A4 |
| $\mathrm{P}_{\text {app }}$ | apparent permeability |
| $\mathrm{f}_{\text {ent }}$ | unbound fractions in the cells |
| $\mathrm{f}_{\mathrm{u}}$ | unbound fractions in the incubation buffer |
| SA | surface area (without microvilli expansion) |
| $\mathrm{V}_{\text {A }}$ | the volumes of the incubation buffers at the A-side |
| $\mathrm{V}_{\text {cell }}$ | the volumes of the cells |
| $\mathrm{V}_{\mathrm{B}}$ | the volumes of the incubation buffers at the B-side |

[^0]Table 2. Description of in vivo parameters used for ATOM analysis

| parameters | description |
| :---: | :---: |
| Clum,z | luminal concentration at z based on the capacity volume |
| Cent,z | concentrations in the epithelial cells at z |
| Cpro,z | concentrations in the lamina propria at z |
| Dz | the dispersion constant at z |
| Mt | flow rate at time t after dose |
| $\widehat{X}_{\text {water, }}$ | the volume of inflating water at z |
| $\widehat{\mathrm{V}}_{\text {ent,z }}$ | the volume of inflating epithelial cells at z |
| $\widehat{\mathrm{V}}_{\text {pro,z }}$ | the volume of inflating lamia propria at z |
| $\mathrm{f}_{\text {lum }}$ | Unbound fraction in the lumen |
| $\mathrm{f}_{\text {pro }}$ | Unbound fraction in the lamina propria |
| $\widehat{\mathrm{Q}}_{\text {pro,z }}$ | local blood flow to the lamia propria at z |
| $\mathrm{f}_{\mathrm{b}}$ | unbound fraction in the blood |

Table 3. Parameters of elementary process in absorption determined by transcellular permeation and preload efflux experiments using CYP3A4-CPR-HAC/Caco-2 cells.

| Class | Drug | $\mathbf{P S}_{\mathbf{P g p}}{ }^{\text {a, b }}$ | $\mathbf{C L}_{\mathbf{M}}{ }^{\text {a, } \mathrm{c}}$ | $\mathbf{P S}_{\mathbf{A}}{ }^{\text {a, d }}$ | $\mathbf{P S}_{\mathbf{B}}{ }^{\text {a, e }}$ | $\mathbf{R}_{\mathbf{B A}}{ }^{\text {f }}$ | $\mathrm{f}_{\text {ent }}{ }^{\text {g }}$ | $\mathrm{f}_{\mathrm{u}}{ }^{\text {b }}$ | $\mathbf{P}_{\text {app }}{ }^{\text {i }}$ |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| P-gp | fexofenadine | $\begin{gathered} \hline 0.020 \\ (0.0052) \end{gathered}$ | $\mathrm{NA}^{\mathrm{j}}$ | $\begin{gathered} 0.0019 \\ (0.00020) \end{gathered}$ | $\begin{gathered} 0.0043 \\ (0.00063) \end{gathered}$ | 2.27 | $\begin{gathered} 0.40 \\ (0.027) \end{gathered}$ | $\begin{aligned} & \hline 0.49 \\ & (0.30) \end{aligned}$ | 0.26 |
|  | digoxin | $\begin{gathered} 0.15 \\ (0.032) \end{gathered}$ | NA | $\begin{gathered} 0.011 \\ (0.0010) \end{gathered}$ | $\begin{gathered} 0.038 \\ (0.016) \end{gathered}$ | 3.44 | $\begin{gathered} 0.98 \\ (0.12) \end{gathered}$ | $\begin{gathered} 0.88 \\ (0.15) \end{gathered}$ | 1.8 |
| $\begin{gathered} \text { P-gp + } \\ \text { CYP3A } \end{gathered}$ | atorvastatin | $\begin{aligned} & \hline 0.53 \\ & (0.11) \end{aligned}$ | 0.0041 | $\begin{gathered} \hline 0.030 \\ (0.0026) \end{gathered}$ | $\begin{gathered} \hline 0.057 \\ (0.0082) \end{gathered}$ | 1.90 | $\begin{gathered} \hline 0.017 \\ (0.00074) \end{gathered}$ | $\begin{gathered} 0.16 \\ (0.072) \end{gathered}$ | 2.3 |
|  | quinidine | $\begin{gathered} \text { Km: } 0.077^{\mathrm{k}} \\ (0.034) \\ \text { Vmax: } 0.17^{1} \\ (0.40) \end{gathered}$ | 0.000076 | $\begin{gathered} 0.11 \\ (0.0099) \end{gathered}$ | $\begin{gathered} 0.12 \\ (0.010) \end{gathered}$ | 1.06 | $\begin{gathered} 0.0046 \\ (0.00032) \end{gathered}$ | $\begin{aligned} & 0.59 \\ & (0.37) \end{aligned}$ | 4.6 |
| CYP3A | nisoldipine | NA | $\begin{aligned} & \hline 1.16 \\ & (0.21) \end{aligned}$ | $\begin{gathered} 0.37 \\ (0.032) \end{gathered}$ | $\begin{gathered} 0.22 \\ (0.056) \end{gathered}$ | 0.60 | $\begin{gathered} \hline 0.0035 \\ (0.00040) \end{gathered}$ | $\begin{gathered} 0.15 \\ (0.088) \end{gathered}$ | 39 |
|  | buspirone | NA | 0.033 | $\begin{gathered} 0.21 \\ (0.018) \end{gathered}$ | $\begin{gathered} 0.21 \\ (0.027) \end{gathered}$ | 0.96 | $\begin{gathered} 0.10 \\ (0.0025) \end{gathered}$ | $\begin{gathered} 0.78 \\ (0.20) \end{gathered}$ | 82 |
|  | felodipine | NA | 0.35 | $\begin{gathered} 1.42 \\ (0.058) \end{gathered}$ | $\begin{aligned} & 0.56 \\ & (0.14) \end{aligned}$ | 0.39 | $\begin{gathered} 0.0003 \\ (0.000035) \end{gathered}$ | $\begin{aligned} & 0.032 \\ & (0.016) \end{aligned}$ | 287 |
|  | dazolam |  | 0.10 | $\begin{gathered} 0.16 \\ (0.016) \end{gathered}$ | $\begin{gathered} 0.21 \\ (0.021) \end{gathered}$ | 1.38 | $\begin{gathered} 0.015 \\ (0.00084) \end{gathered}$ | 0.10 | 60 |
|  | midazolam | NA | 0.10 | $\begin{aligned} & 0.19^{\mathrm{m}} \\ & (0.024) \end{aligned}$ | $\begin{aligned} & 0.26^{\mathrm{m}} \\ & (0.031) \end{aligned}$ | $1.37{ }^{\text {m }}$ | $\begin{gathered} 0.024^{\mathrm{m}} \\ (0.00084) \end{gathered}$ | (0.080) | $76^{\text {m }}$ |
|  | terfenadine | NA | 0.50 | $\begin{gathered} 0.039 \\ (0.0015) \end{gathered}$ | $\begin{aligned} & 0.042 \\ & (0.005) \end{aligned}$ | 1.06 | $\begin{gathered} 0.0036 \\ (0.00011) \end{gathered}$ | $\begin{gathered} 0.42 \\ (0.22) \end{gathered}$ | 2.4 |
|  | nifedipine | NA | 0.033 | $\begin{gathered} 0.19 \\ (0.0077) \end{gathered}$ | $\begin{gathered} 0.17 \\ (0.020) \end{gathered}$ | 0.91 | $\begin{gathered} 0.017 \\ (0.00046) \end{gathered}$ | $\begin{gathered} 0.31 \\ (0.081) \end{gathered}$ | 69 |
|  | sildenafil | NA | 0.017 | $\begin{gathered} 0.079 \\ (0.0030) \end{gathered}$ | $\begin{aligned} & 0.091 \\ & (0.014) \end{aligned}$ | 1.15 | $\begin{gathered} 0.053 \\ (0.0019) \end{gathered}$ | $\begin{gathered} 0.94 \\ (0.073) \end{gathered}$ | 32 |
|  | repaglinide | NA | 0.0071 | $\begin{gathered} 0.21 \\ (0.0088) \end{gathered}$ | $\begin{gathered} 0.51 \\ (0.060) \end{gathered}$ | 2.39 | $\begin{gathered} 0.021 \\ (0.00042) \end{gathered}$ | $\begin{aligned} & 0.071 \\ & (0.032) \end{aligned}$ | 124 |

The values in parenthesis represent the estimated standard deviation obtained by the fitting analysis. ${ }^{\text {a }}$ Unit is $\mathrm{mL} / \mathrm{h} / 2.5 \times 10^{5}$ cells. ${ }^{\mathrm{b}}$ Permeability surface area product (PS) for efflux by P-gp. ${ }^{\mathrm{c}}$ Intrinsic metabolic clearance. The value for nisoldipine was obtained from the fitting analysis. Other values were calculated from microsomal incubations (Gertz et al., 2010) using nisoldipine as a standard. ${ }^{\text {d }}$ PS of A membrane for both-direction permeation. ${ }^{\mathrm{e}}$ PS of B-membrane for both-direction permeation. ${ }^{\mathrm{f}}$ The membrane permeability ratio of $B$ membrane to A membrane $\left(R_{B A}\right) .{ }^{g}$ Unbound fraction in the enterocytes obtained from the fitting analysis. ${ }^{\text {h }}$ Unbound fraction in the incubation buffer containing $0.5 \%$ bovine serum albumin obtained experimentally. ${ }^{i}$ Apparent permeability estimated from $\mathrm{PS}_{\mathrm{Pgp}}, \mathrm{CL}_{\mathrm{M}}, \mathrm{PS}_{\mathrm{A}}$ and $\mathrm{PS}_{\mathrm{B}}$ with

Eq. 5. Unit is $\mathrm{cm} / \mathrm{sec} \times 10^{-6} .{ }^{\mathrm{j}}$ Not applicable. ${ }^{\mathrm{k}}$ Unit is $\mu \mathrm{M}$. ${ }^{1}$ Unit is $\mathrm{nmol} / \mathrm{h} / 2.5 \times 10^{5}$ cells. ${ }^{\mathrm{m}}$ Evaluated in the alternative experiment (Supp. Methods 1.3).

Fig. 1

## A.



1. Preload drugs
into the cells
2. Wash and
replace with
blank buffers

3. Collect samples over time from both sides

Fig. 2



Fig. 4


Fig. 5


Fig. 6


## Supplementary file(figures and tables)

Article's Title;
Significance of basal membrane permeability of epithelial cells in predicting intestinal drug absorption

Authors;
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Journal Title;
Drug Metabolism and Disposition

## Contents

Supplemental Fig. S1
Supplemental Fig. S2
Supplemental Fig. S3
Supplemental Fig. S4
Supplemental Fig. S5
Supplemental Fig. S6


Supplemental Fig. S1 Demonstrations of inhibition of CYP3A4 (Panels A and B) and P-gp (Panels C and D) by ketoconazole. In Panels A and B, results from A to B permeability assessment of midazolam $(5 \mu \mathrm{M})$ are shown. In the presence of ketoconazole ( 1 and $10 \mu \mathrm{M}$ ) and valspodar ( $1 \mu \mathrm{M}$ ) (indicated by open diamond, square and closed triangle, respectively), the time course of midazolam was unaltered from the control (closed circle) in Panel A, but the generated hydroxymidazolam disappeared completely in the presence of ketoconazole in Panel B. Panels C and D represent A to B and B to A assessments of fexofenadine ( $10 \mu \mathrm{M}$ ). Compared with the control (closed circle), $10 \mu \mathrm{M}$ ketoconazole (open square) demonstrated similar degree of inhibition as $1 \mu \mathrm{M}$ valspodar (closed triangle).


Supplemental Fig. S2 Results of midazolam permeation experiments performed using CYP3A4-CPR-HAC/Caco-2 cells by alterative transport assessment method (Supp. 1.3). Panel A represents the A to B and panel B represents the B to A. The black, red and blue symbols and lines represent measurements and fitting lines by the model in the absence of P-gp nor CYP3A4 inhibitor, in the presence of P-gp inhibitor, and in the presence of CYP3A4 inhibitor, respectively.


Supplemental Fig. S3 Simulation of pre-incubation time necessary for preload efflux method. The intracellular concentrations were simulated in the presence (black) and absence (red) of P-gp and CYP3A inhibitors for each drug. For quinidine, initial buffer concentrations are $1,3.3,10$ and $33 \mu \mathrm{M}$ for purple, blue, red, and black lines, respectively. For terfenadine, blue dotted line represents time course with CYP3A activity of $1 / 10$ compared with that estimated from microsomal experiment considering possible overestimation of CYP3A activity.


Supplemental Fig. S4 Simulation of in vivo intestinal absorption profile of midazolam by ATOM with parameters estimated from in vitro experiments. Migrated drug to hypothetical segments of the lumen with normal CYP3A activity (A) and without CYP3A activity (B). Distribution of drug to segments of the enterocytes and cumulative absorption with normal CYP3A activity (C) and without CYP3A activity (D). Cumulative absorption from each intestinal segment (E). The small intestine was divided into 40 segments hypothetically in this simulation.

## A. expression distribution of P-gp and CYP3A


B. gastrointestinal blood flow


Supplemental Fig. S5 Relationships between observed and simulated overall intestinal availability $\left(\mathrm{F}_{\mathrm{A}} \mathrm{F}_{\mathrm{G}}\right)$ by ATOM. In panel (A), the simulated differences between the physiological and hypothetical monotonous distribution for both P-gp and CYP3A4 expressions were shown. Note that larger $\mathrm{F}_{\mathrm{A}} \mathrm{F}_{\mathrm{G}}$ was always estimated for the monotonous distribution. In panel (B), changes with half ( $0.9 \mathrm{~L} / \mathrm{h}$ ) or doubled ( $3.6 \mathrm{~L} / \mathrm{h}$ ) intestinal blood flow were simulated compared with the normal flow ( $1.8 \mathrm{~L} / \mathrm{h}$ ).
However, changes were small and hidden by the size of marks in most cases. Red, blue, and orange symbols indicate P-gp substrates, CYP3A4 substrates, and those serve as both, respectively. The solid and dotted lines represent the unity and error range within $\pm 0.2$-fold. The small numbers identify drugs in panel (A); fexofenadine 1 , digoxin 2, terfenadine 3 , nisoldipine 4 , felodipine 5 , midazolam 6 , buspirone 7 , nifedipine 8 , sildenafil 9 , repaglinide 10 , atorvastatin 11 , quinidine $0.1 \mathrm{mg} 12-1$, quinidine $1 \mathrm{mg} 12-$ 2 , quinidine 10 mg 12-3 and quinidine 100 mg 12-4.


Supplemental Fig. S6 Simulation of apparent changes in the permeability assessment in CYP3A4-CPR-HAC/Caco-2 cells due to CYP3A metabolic clearance estimated from the microsomal experiments. Black and red lines represent A to B and B to A experiments, respectively. Solid and broken lines represent in the presence and absence of a CYP3A4 inhibitor, respectively. Please note significant changes are estimated to be appear for both nisoldipine and terfenadine, whereas as shown in Fig. 2 in the main text, noticeable changes were observed only for nisoldipine. Therefore, the metabolic clearance of terfenadine obtained from the microsomal experiment does not explain results of the cell experiment in this study.

Supplemental Table S1. Cocktail conditions for transport and efflux experiments

| experiment | P-gp inhibitor | CYP3A <br> inhibitor | Cocktail condition | drugs |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| transport | Valspodar$(1 \mu \mathrm{M})$ | Ketoconazole <br> ( $1 \mu \mathrm{M}$ ) | 1 | nifedipine ( $5 \mu \mathrm{M}$ ) | digoxin( $10 \mu \mathrm{M})$ | midazolam ( $5 \mu \mathrm{M}$ ) |  |
|  |  |  | 2 | buspirone ( $5 \mu \mathrm{M}$ ) | midazolam ( $5 \mu \mathrm{M}$ ) |  |  |
|  |  |  | 3 | fexofenadine (10 $\mu \mathrm{M})$ | atorvastatin ( $20 \mu \mathrm{M}$ ) | midazolam ( $5 \mu \mathrm{M}$ ) |  |
|  |  |  | 4 | felodipine ( $5 \mu \mathrm{M}$ ) | terfenadine ( $5 \mu \mathrm{M}$ ) | midazolam ( $5 \mu \mathrm{M}$ ) |  |
|  |  |  | 5 | nisoldipine ( $5 \mu \mathrm{M}$ ) | repaglinide ( $5 \mu \mathrm{M}$ ) | midazolam ( $5 \mu \mathrm{M}$ ) |  |
|  |  |  | 6 | sildenafil ( $5 \mu \mathrm{M}$ ) | midazolam ( $5 \mu \mathrm{M}$ ) |  |  |
|  |  |  | 7 | quinidine ( $1,3.3,10$ <br> and $33 \mu \mathrm{M}$ ) | digoxin ( $1 \mu \mathrm{M}$ ) | fexofenadine ( $1 \mu \mathrm{M}$ ) |  |
|  | Elacridar $(0.5 \mu \mathrm{M})$ | 1-ABT(1 mM ) | Supp Method 1.3 | midazolam ( $3 \mu \mathrm{M}$ ) |  |  |  |
| efflux | Ketoconazole$(10 \mu \mathrm{M})$ | Ketoconazole$(10 \mu \mathrm{M})$ | 1 | midazolam ( $10 \mu \mathrm{M}$ ) | felodipine ( $10 \mu \mathrm{M}$ ) | fexofenadine ( $10 \mu \mathrm{M}$ ) |  |
|  |  |  | 2 | terfenadine ( $10 \mu \mathrm{M}$ ) | sildenafil ( $10 \mu \mathrm{M}$ ) |  |  |
|  |  |  | $3$ | atorvastatin (10 $\mu \mathrm{M}$ ) | nifedipine ( $10 \mu \mathrm{M}$ ) | buspirone ( $10 \mu \mathrm{M}$ ) | quinidine ( $10 \mu \mathrm{M}$ ) |
|  |  |  | 4 | digoxin ( $10 \mu \mathrm{M}$ ) |  |  |  |
|  |  |  | 5 | repaglinide ( $10 \mu \mathrm{M}$ ) | nisoldipine ( $10 \mu \mathrm{M}$ ) |  |  |

Supplemental Table S2. MRM transitions of compounds measured by LC-MS/MS in this study. All compounds were measured in a positive mode.

| Compound | Q1 | Q3 |
| :---: | :---: | :---: |
| fexofenadine | 502.3 | 466.1 |
| digoxin | 781.3 | 97.0 |
| midazolam | 326.0 | 291.2 |
| 1'-hydroxymidazolam | 341.9 | 202.9 |
| nifedipine | 347.3 | 314.9 |
| buspirone | 386.2 | 122 |
| felodipine | 384.2 | 352.0 |
| terfenadine | 472.3 | 436.2 |
| sildenafil | 474.9 | 311.1 |
| nisoldipine | 389.0 | 315.0 |
| repaglinide | 453.2 | 230.1 |
| atorvastatin | 559.2 | 440.2 |
| quinidine | 325.1 | 306.9 |
| diazepam (IS) | 193.1 |  |
| sulfaphenazole (IS) ${ }^{\text {a }}$ | 284.9 | 158.7 |

[^1]Supplemental Table S3. Drug-specific parameters used for the analysis of $\mathrm{F}_{\mathrm{A}} \mathrm{F}_{\mathrm{G}}$ by ATOM.

|  | $\mathrm{pKa}_{\text {acid }}{ }^{\text {c }}$ | pKabase ${ }^{\text {c }}$ | $\mathrm{fb}^{\text {A }}$, C | Dose (mg) p.o. | Observed $\mathrm{F}_{\mathrm{A}} \mathrm{F}_{\mathrm{G}}{ }^{\text {c }}$ |
| :---: | :---: | :---: | :---: | :---: | :---: |
| fexofenadine | $4.04{ }^{\text {a }}$ | $9.01{ }^{\text {a }}$ | $0.28^{\text {B, c }}$ | 0.1 | $0.46{ }^{\text {f }}$ |
| digoxin | $14^{\text {b) }}$ | $1^{\text {b }}$ | $0.71{ }^{\text {b }}$ | 0.75 | $0.62^{\text {f }}$ |
| atorvastatin | $4.33{ }^{\text {a }}$ | $14^{\text {a) }}$ | $0.02^{\text {B, d) }}$ | N/A | $0.14{ }^{\text {g }}$ |
|  |  |  |  | 0.1 | $0.19{ }^{\text {f }}$ |
| quinidine | $13.89^{\text {a }}$ | $9.05^{\text {a }}$ | $0.26{ }^{\text {e }}$ | 1 | $0.24{ }^{\text {f }}$ |
|  |  |  |  | 10 | $0.34{ }^{\text {e }}$ |
|  |  |  |  | 100 | $0.48{ }^{\text {e }}$ |
| nisoldipine | $11.2{ }^{\text {a }}$ | $0.68{ }^{\text {a }}$ | $0.003{ }^{\text {e) }}$ | N/A | $0.12{ }^{\text {h }}$ |
| buspirone | $14^{\text {a) }}$ | $7.19^{\text {a }}$ | $0.05{ }^{\text {e }}$ | N/A | $0.11^{\text {i }}$ |
| felodipine | $10.95^{\text {a }}$ | $0.51^{\text {a }}$ | $0.004^{\text {e }}$ | 2.5 | $0.24{ }^{\text {f }}$ |
| midazolam | $14^{\text {b) }}$ | $4.57^{\text {b }}$ | $0.056^{\text {b) }}$ | 6 | $0.40^{\text {f }}$ |
| terfenadine | $13.2{ }^{\text {a }}$ | $9.02{ }^{\text {a }}$ | $0.03{ }^{\text {B, d) }}$ | N/A | $0.40^{\text {j }}$ |
| nifedipine | $11.06^{\text {a }}$ | $1.28{ }^{\text {a }}$ | $0.044^{\text {e }}$ | 10 | $0.70^{\text {g }}$ |
| sildenafil | $9.38{ }^{\text {a }}$ | $7.6^{\text {a }}$ | $0.04{ }^{\text {e }}$ | 50 | $0.75{ }^{\text {f }}$ |
| repaglinide | $3.68{ }^{\text {a }}$ | $4.82^{\text {a }}$ | $0.015^{\text {e }}$ | N/A | $0.94{ }^{\text {h) }}$ |

${ }^{\text {A }}$ The unbound fraction in the blood
${ }^{B}$ Obtained from $f_{b}=f_{p} / R_{B}$. $f_{p}$ is the unbound fraction of plasma, and $R_{B}$ is the plasma-to-blood ratio.
${ }^{\text {C }}$ Obtained from the literature; a) Drugbank. Available at: https://go.drugbank.com. Accessed February 10, 2021., b) Asano et al., 2021, c) Ito and Houston, 2004, d) Gertz et al., 2010, e) Ando et al., 2015, f) Takano et al., 2016, g) Tachibana et al., 2012, h) Varma et al., 2010, i) Nishimuta et al., 2011, j) Gertz et al., 2010

## Supplementary file

DMD-AR-2022-000907

Article's Title;
Significance of basal membrane permeability of epithelial cells in predicting intestinal drug absorption

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Journal Title;
Drug Metabolism and Disposition

## Contents of Supplement

## 1. Supporting Methods

### 1.1 Reagents

1.2 Preparation and culture of CYP3A4-CPR-HAC/Caco-2 cells
1.3 Alternative transport assessment method for midazolam with CYP3A4-CPR-

HAC/Caco-2 cells under similar condition with the previous report
1.4 Measurement of unbound fraction in the transport buffer
1.5 Measurement of drug concentrations by LC-MS/MS
1.6 Analysis of in vitro nonlinear pharmacokinetics for quinidine
1.7 Analysis of nonlinear pharmacokinetics by ATOM for quinidine
2. The essential part of source code of ATOM
3. Reference for Supplementary file

## 1. Supporting Methods

### 1.1 Reagents

Fexofenadine, digoxin, nifedipine, nisoldipine, felodipine, sildenafil, repaglinide, atorvastatin, quinidine and 1-aminobenzotriazole (1-ABT) were purchased from Tokyo Chemical Industry (Tokyo, Japan). Midazolam, lucifer yellow CH dipotassium salt, and ketoconazole were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Buspirone was purchased from Abcam (Cambridge, UK). Sulfaphenazole, terfenadine and 1'hydroxymidazolam were purchased from Cayman Chemical (Ann Arbor, MI, USA). Valspodar was purchased from Toronto Research Chemicals (North York, Ontario, Canada). BSA was purchased from Sigma-Aldrich (St. Louis, MO, USA). Elacridar was purchased from SigmaAldrich (FUJIFILM Wako Pure Chemical Corporation).

### 1.2 Preparation and culture of CYP3A4-CPR-HAC/Caco-2 cells

Using the Cre/loxP system, the CYP3A4 and CPR genes were inserted into the HAC vector in CHO cells, and then the CYP3A4-CPR-HAC vector was transferred into Caco-2 cells by microcell-mediated chromosome transfer (Hiratsuka et al., 2011; Takenaka et al., 2017). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, $4.5 \mathrm{~g} / \mathrm{L}$ glucose) containing $10 \%$ fetal bovine serum, $1 \%$ nonessential amino acids, $1 \%$ L-alanyl-L-glutamine, 50 or $100 \mathrm{U} / \mathrm{mL}$ penicillin, 50 or $100 \mu \mathrm{~g} / \mathrm{mL}$ streptomycin, and $2 \mu \mathrm{~g} / \mathrm{mL}$ blastcidin S. Cells were seeded at a density of $2.5 \times 10^{4}$ cells/well or $4.0 \times 10^{4}$ cells/well on 24 -multiwell inserts (Greiner Bio-One GmbH, Frickenhausen, Germany; or Corning, NY, USA) and cultured for 21-23 days by changing the culture medium every 2 or 3 days. The cells were incubated at $37^{\circ} \mathrm{C}$ in a $5 \%$ $\mathrm{CO}_{2}$ incubator. To check the condition of the cells, trans-epithelial electrical resistance (TEER) measurement using Millicell-ERS (Millipore) was performed to confirm $\geq 300 \Omega \cdot \mathrm{~cm}^{2}$ or permeability test of lucifer yellow was performed after the transport experiments to confirm a permeability ratio of $<2 \%$ compared with the added amount.

### 1.3 Alternative transport assessment method for midazolam with CYP3A4-CPR-

## HAC/Caco-2 cells under similar condition with the previous report ${ }^{\text {a }}$

The choice of inhibitors, incubation time and substrate concentration are modified in the alternative method. CYP3A4-CPR-HAC/Caco-2 cell monolayers were studied in both A to B and B to A directions for transport assessment of midazolam. The culture medium was removed and the cells were rinsed twice with the transport buffer ( $\mathrm{pH} 7.4, \operatorname{HBSS}(+)$ with 20 mM D-glucose, $4.2 \mathrm{mM} \mathrm{NaHCO} 3,10 \mathrm{mM}$ HEPES, and $0.5 \% \mathrm{BSA})$. The cells were preincubated in the transport buffer ( $100 \mu \mathrm{~L}$ for A side and $600 \mu \mathrm{~L}$ for B side) added with or without P-gp or CYP3A4 inhibitors in both sides as needed for 60 min at $37^{\circ} \mathrm{C}$ in a $5 \% \mathrm{CO}_{2}$ incubator. We used elacridar $(0.5 \mu \mathrm{M})$ as P-gp inhibitor and 1-ABT ( 1 mM ) as CYP3A inhibitor. We checked beforehand that the P-gp inhibitors did not inhibit CYP3A4 noticeably and vice versa in these concentrations. The incubation was started by an addition of the substrate drugs. The midazolam concentrations were $3 \mu$ M. Samples on the receiver side were collected sequentially at several time points during 0.5 to 2 h and cryopreserved if necessary. The samples were subjected to a measurement of drug concentrations by LC-MS/MS.
${ }^{\text {a) }}$ Takenaka T, Kazuki K, Harada N, Kuze J, Chiba M, Iwao T, Matsunaga T, Abe S, Oshimura M, and Kazuki Y (2017) Development of Caco-2 cells co-expressing CYP3A4 and NADPH-cytochrome P450 reductase using a human artificial chromosome for the prediction of intestinal extraction ratio of CYP3A4 substrates. Drug Metab Pharmacokinet 32:61-68.

### 1.4 Measurement of unbound fraction in the transport buffer

The unbound fraction of drugs ( $f_{u}$ ) in the transport buffer containing $0.5 \%$ BSA were measured referring to the report (Klotz et al., 1946). The cellulose tubes containing 3 mL of the $0.5 \%$ BSA and the transport buffer containing the substrates was placed in a bottle containing 5 mL of the transport buffer containing substrates without $0.5 \%$ BSA and shaken at $37^{\circ} \mathrm{C}$ for 24 h . After 24 h , the outer solution was sampled and 5 times the volume of acetonitrile was added, and the tubes were centrifuged at $15,000 \times \mathrm{g}$ for 15 min . The supernatant was measured by LC-MS/MS under the analysis conditions described below.

### 1.5 Measurement of drug concentrations by LC-MS/MS

In the following, method I was applied for measurements of drugs described in the main text and method II was applied for measurement of midazolam described in Supporting Method 1.3. MRM conditions are shown in Supplemental Table S1.

Method I: The samples were added with 5 times volume of acetonitrile, mixed, and then centrifuged at $15,000 \times \mathrm{g}$ for 15 min to collect the supernatant. The LC-MS/MS (LC: Shimadzu, MS/MS: SCIEX QTRAP4500) measurements were performed with the following elution condition: the mobile phases were $0.1 \%$ formic acid as solution A and $0.1 \%$ formic acid in acetonitrile as solution B. Regarding gradient conditions, $5 \%$ ( $0.00 \sim 0.30 \mathrm{~min}$ ), $5 \% \sim 95 \%$ ( $0.30 \sim 2.00 \mathrm{~min}$ ), $95 \%$ ( $2.00 \sim 4.00 \mathrm{~min}$ ), $95 \sim 5 \%$ ( $4.00 \sim 4.10 \mathrm{~min}$ ), and $5 \%(4.10 \sim 7.00 \mathrm{~min}$ ) of solution B were applied as time programs. For digoxin, $5 \%$ ( $0.00 \sim 0.50 \mathrm{~min}$ ), $5 \sim 95 \%$ ( $0.50 \sim 2.00 \mathrm{~min}$ ), $95 \%(2.00 \sim 5.00 \mathrm{~min}), 95 \sim 5 \%(5.00 \sim 5.50 \mathrm{~min})$, and $5 \%(5.50 \sim 9.00 \mathrm{~min})$ of solution B were applied as time programs. The temperatures in the column and autosampler were $40^{\circ} \mathrm{C}$ and $10^{\circ} \mathrm{C}$, respectively. The flow rate and injection volume were set at $0.2 \mathrm{~mL} / \mathrm{min}$ and $5 \mu \mathrm{~L}$, respectively. A Kinetex EVO C18 column (Phenomenex, $2.6 \mu \mathrm{~m}, 100 \mathrm{~mm} \times 2.1$ mm ) and a Kinetex EVO C18 guard cartridge (Phenomenex, $2.6 \mu \mathrm{~m}, 2.1 \mathrm{~mm}$ ) were used in the measurements. A Porpshell 120 column (Agilent Technologies, $2.7 \mu \mathrm{~m}, 100 \mathrm{~mm} \times 2.1 \mathrm{~mm}$ ) was used in analysis for quinidine. As the internal standard (IS), $50 \mathrm{ng} / \mathrm{mL}$ diazepam was used.

Method II: The samples were subjected to solid-phase extraction with an Oasis HLB $30 \mu \mathrm{~m}$ $\mu$ Elution plate (Waters), and the recovery solution was analyzed by LC-MS/MS (Shimadzu, SCIEX, 4000 QTRAP). The measurement conditions were as follows: The mobile phases were 5 mM ammonium formate/formic acid (1000:1, $\mathrm{v} / \mathrm{v})$ as solution A and methanol as solution B. Regarding gradient conditions, the initial mobile phase composition was maintained at 5\% solvent B for $30 \mathrm{~s}(0.00 \sim 0.50 \mathrm{~min})$, from $5 \% \sim 95 \%$ in $1 \mathrm{~min}(0.50 \sim 1.50 \mathrm{~min})$, and held for 72 s at $95 \%(1.50 \sim 2.70 \mathrm{~min})$ before re-equilibration to $5 \%$ in $72 \mathrm{~s}(2.80 \sim 4.00 \mathrm{~min})$. The temperatures for the column and autosampler were $40^{\circ} \mathrm{C}$ and $10^{\circ} \mathrm{C}$, respectively. The flow rate
and injection volume were set to $0.5 \mathrm{~mL} / \mathrm{min}$ and $20 \mu \mathrm{~L}$, respectively. A Kinetex $2.6 \mu \mathrm{~m} \mathrm{C} 8$ column (Phenomenex, $3.5 \mu \mathrm{~m}, 2.1 \mathrm{~mm} \times 50 \mathrm{~mm}$ ) was used for the analysis. Sulfaphenazole at $1 \mu \mathrm{~g} / \mathrm{mL}$ was used as the internal standard.

### 1.6 Analysis of in vitro nonlinear pharmacokinetics for quinidine

In this analysis, all concentrations, $\mathrm{C}_{\mathrm{A}}, \mathrm{C}_{\text {cell, }}$, and $\mathrm{C}_{\mathrm{B}}$, were converted to normalized concentrations, $\mathrm{C}_{\mathrm{A}, \mathrm{N}}, \mathrm{C}_{\mathrm{cell}, \mathrm{N}}$ and $\mathrm{C}_{\mathrm{B}, \mathrm{N}}$, respectively, by dividing by the initial concentration in the donner compartment, $\mathrm{C}_{\mathrm{D}, 0}$. Eqs. 1~3 need not be changed, simply replacing C with $\mathrm{C}_{\mathrm{N}}$, while Eq. 4 needs to be modified to Eq. S1.

$$
\begin{equation*}
\mathrm{PS}_{\mathrm{Pgp}}=\frac{\mathrm{V}_{\mathrm{max}, \mathrm{Pgp}}}{\mathrm{~K}_{\mathrm{m}, \mathrm{Pgp}}+\mathrm{f}_{\mathrm{ent}} * \mathrm{C}_{D, 0} \mathrm{C}_{\text {cell, }}} . \tag{Eq.S1}
\end{equation*}
$$

For coexisting digoxin, we assumed that due to competitive inhibition, $\mathrm{PS}_{\mathrm{Pgp}}$ is determined by the intracellular concentration of quinidine with Eq. S2.

$$
\begin{equation*}
\mathrm{PS}_{\mathrm{Pgp}, \mathrm{dg}}=\frac{\mathrm{R}_{\mathrm{Pgp}, \mathrm{dg}}}{1+\mathrm{f}_{\mathrm{ent}, \mathrm{qn}} * \mathrm{C}_{D, 0, q n} \mathrm{C}_{\text {cell, }, \mathrm{qn}} / \mathrm{K}_{\mathrm{m}, \mathrm{Pgp}, \mathrm{qn}}}, \tag{Eq.S2}
\end{equation*}
$$

where $\mathrm{R}_{\mathrm{Pgp}}$ is the ratio of $\mathrm{PS}_{\text {Pgp }}$ to that of quinidine at the lowest concentration of the drugs, and the subscripts of qn and dg refer to the parameters of quinidine and digoxin, respectively. In this analysis, $\mathrm{R}_{\mathrm{BA}}$ and $\mathrm{f}_{\text {ent }}$ of quinidine were determined beforehand by a fitting analysis of data from the preload efflux experiment of quinidine, and those of digoxin were those obtained from the evaluation of digoxin alone. The time courses of quinidine and digoxin for A to B and B to A permeability assessments with four initial concentrations of quinidine were simultaneously subjected to a fitting analysis by Napp to obtain $\mathrm{PS}_{\mathrm{A}}$ of the drugs, $\mathrm{R}_{\mathrm{BA}} \mathrm{K}_{\mathrm{m}}$, $\mathrm{V}_{\text {max }}$, and $\mathrm{f}_{\text {ent }}$ of quinidine. Since all the concentrations of quinidine at the lowest initial donor concentration $(1 \mu \mathrm{M})$ were below the detection limit, 14 time courses were used for the analysis.

### 1.7 Analysis of nonlinear pharmacokinetics by ATOM for quinidine

The basic equations of ATOM (Eq. 6 and 7 in the main text) are replaced with Eqs. S3 and S4, respectively, when nonlinearity is considered for activities of P-gp and CYP3A4. Please note only nonlinearity of P-gp was considered for quinidine.

$$
\begin{gather*}
\frac{\partial \mathrm{C}_{\mathrm{lum}, \mathrm{z}}}{\partial \mathrm{t}}=\mathrm{D}_{\mathrm{z}} \frac{\partial^{2} \mathrm{C}_{\mathrm{lum}, \mathrm{z}}}{\partial \mathrm{z}^{2}}-\mathrm{M}_{\mathrm{t}} \frac{\partial \mathrm{C}_{\mathrm{lum}, \mathrm{z}}}{\partial \mathrm{z}}-\widehat{\mathrm{PS}}_{\mathrm{A}, \mathrm{in}, \mathrm{z}} \frac{\mathrm{f}_{\mathrm{lum}} \mathrm{C}_{\mathrm{lum}, \mathrm{z}}}{\widehat{\mathrm{X}}_{\text {water }, \mathrm{z}}} \\
+\left(\widehat{(P S}_{\mathrm{A}, \mathrm{out}, \mathrm{z}}+\frac{\widehat{\mathrm{V}}_{\text {max, Pgp, } \mathrm{z}}}{\mathrm{~K}_{\mathrm{m}, \mathrm{Pgp}}+\mathrm{f}_{\mathrm{ent}} \mathrm{C}_{\mathrm{ent}, \mathrm{z}}}\right) \frac{\mathrm{f}_{\mathrm{ent}} \mathrm{C}_{\mathrm{ent}, \mathrm{z}}}{\widehat{\mathrm{~V}}_{\mathrm{lum}, \mathrm{z}}} \tag{Eq.S3}
\end{gather*}
$$

$$
\begin{align*}
& \widehat{\mathrm{V}}_{\text {ent }, \mathrm{z}} \frac{\mathrm{dC}_{\text {ent } z}}{\mathrm{dt}}=\widehat{\mathrm{PS}}_{\mathrm{A}, \mathrm{in}, \mathrm{z}} \mathrm{f}_{\text {lum }} \mathrm{C}_{\text {lum }, \mathrm{z}} \\
& -\left(\widehat{\mathrm{PS}}_{\mathrm{A}, \text { out }, \mathrm{Z}}+\frac{\widehat{\mathrm{V}}_{\text {max, Pgp,z }}}{\mathrm{K}_{\mathrm{m}, \mathrm{Pgp}}+\mathrm{f}_{\text {ent }} \mathrm{C}_{\text {ent }, \mathrm{Z}}}+\frac{\widehat{\mathrm{V}}_{\text {max }, \mathrm{CYP} 3 \mathrm{~A}, \mathrm{Z}}}{\mathrm{~K}_{\mathrm{m}, \mathrm{CYP} 3 \mathrm{~A} 4}+\mathrm{f}_{\text {ent }} \mathrm{C}_{\text {ent }, \mathrm{Z}}}+\widehat{\mathrm{PS}}_{\mathrm{B}, \mathrm{z}}\right) \mathrm{f}_{\text {ent }} \mathrm{C}_{\text {ent }, \mathrm{Z}}+\mathrm{f}_{\text {pro }} \widehat{\mathrm{PS}}_{\mathrm{B}, \mathrm{Z}} \mathrm{C}_{\text {pro, }} \tag{Eq.S4}
\end{align*}
$$

$\widehat{V}_{\text {max, Pgp,Z }}$ and $\widehat{V}_{\text {max,CYP3A4,Z }}$ are obtained by Eq. S5 and S6.

$$
\begin{align*}
& \widehat{\mathrm{V}}_{\text {max }, \mathrm{Pgp}, \mathrm{z}}=\frac{\mathrm{V}_{\text {max }, \mathrm{Pgp}}}{\mathrm{PS}_{\mathrm{Pgp}}} \widehat{\mathrm{PS}}_{\mathrm{Pgp}, \mathrm{z}},  \tag{Eq.S5}\\
& \widehat{\mathrm{~V}}_{\text {max }, \mathrm{CYP} 3 \mathrm{~A} 4, \mathrm{z}}=\frac{\mathrm{V}_{\text {max }, \mathrm{CYP} 3 \mathrm{~A} 4}}{\mathrm{CL}_{\mathrm{M}}} \widehat{\mathrm{CL}}_{\mathrm{M}, \mathrm{z}}, \tag{Eq.S6}
\end{align*}
$$

where $\mathrm{K}_{\mathrm{m}, \text { Pgp }}, \mathrm{V}_{\text {max,Pgp }}, \mathrm{K}_{\mathrm{m}, \text { CYP3A4 }}$, and $\mathrm{V}_{\text {max, CYP3A4 }}$ represent the Michaelis-Menten constants for in vitro activities of P-gp and CYP3A4, respectively).

## 2. The essential part of source code of ATOM



Source code of ATOM in Objective-C

This file is compiled by XCode (v13.2.1) to make bundle and then dynamically linked to Napp.

This file contains the essential part of ATOM
by A. Hisaka and A. Yoshitomo, Jan, 2022

```
***********************************************************************************
******************************/
#import "Napp.h" // header file for interface to Napp
#define Nseg 40
// number of segments for finite difference method for calculation of partial differential
equation
// This number is adjustable (ca 20-80)
#define DNseg 40
#define Fupper (3.0/DNseg)
#define Fmiddle (11.0/DNseg)
#define Flower (18.0/DNseg)
#define Fileum (8.0/DNseg)
#define F_jejunum (20.0/DNseg)
#define LUMEN_W 0
#define LUMEN (Nseg) // 1... Nseg-1
#define ENT (Nseg * 2) // Nseg ... 2 * Nseg-1
#define LAMINA (Nseg * 3) // 2 * Nseg ... 3* Nseg-1
#define A_BASE 0.005
@interface Atom1c: NSObject {
    real *prms, volOfSeg, total_api_surf_area_ent, radAtSeg[Nseg], lenAtSeg[Nseg],
        vEntAtSeg[Nseg], vLamAtSeg[Nseg], qLamAtSeg[Nseg], pH_lum[Nseg],
        cl_Cyp3a[Nseg], psA_in[Nseg], psA_out_pass[Nseg], psA_Pgp[Nseg], psB_in[Nseg],
        psB_out[Nseg];
    real psA_total, psPgp_total, clCyp3a_total, psB_total;
    id delegate;
}
@end
// This is a list of compartments
enum {ESO_W = Nseg * 4, STOMACH_W, CAECUM_W, FECES_W, ESO, STOMACH,
```

CAECUM, FECES, ABSORP, PORT, LIVER, BLOOD, PERI, Pcnt_in_stomach,
Pcnt_in_jejunum, Pcnt_in_ileum, Pcnt_in_colon, Pcnt_in_whole_lumen, FA, FAFG, FG, TOTAL_W, Plasma_conc_uM, AbsFromSeg\};
// This is a list of parameters
enum \{PSa_in_Caco2, PSa_out_Caco2, PSb_in_Caco2, PSb_out_Caco2, CLm_Caco2, KmCyp3a_Caco2, PSpgp_Caco2, KmPgp_Caco2, Fent, SF_api_passive, SF_baso, SF_CYP3A, SF_pgp, Dose, MW, pKa_acid, pKa_base, pH_Caco2, insert_Caco2, ME_Caco2, pH_lum_0, pHgrad, PE, VE_0, VE_Z, ME, Tot_CYP3A, CYP3A_grad, Pgp_grad, Ves, Vstm, Vpv, Vliver, Vb, LenOfSI, RadAtSeg_0, RadGradient, Tent, Hvilli, A, B, C, D, E, F, Tlag, Ka, Kg, Krec, lag_rec, fb, Rb, Qpv, Qh, Qha, Q_lam, Kp_liver, CLint_h, K12, K21, Sec_W_Stm, Sec_W_SI, Sec_Wfe_SI, T_fe, UpSecRatio, Sec_W_Col, Kw_stm, Kw_SI, Kw_col, Vw0_esp, Vw0_stm, Vw0_up, Vw0_mid, VwO_low, Vw0_il, Vw0_col
\};

- (BOOL)crossDiscontinuityAtIndex:(int)idx \{ // to set initial conditions int i, n1, n2, n3;
real tot = 0.0;
if(idx) return NO;
// initial conditions for water volume
[delegate setValue:prms[Vw0_esp] / prms[Ves] at:ESO_W];
[delegate setValue:prms[Vw0_stm] / prms[Vstm] at:STOMACH_W];
[delegate setValue:prms[Vw0_col] at:CAECUM_W];

```
n1 = Fupper * Nseg;
\(\mathrm{n} 2=(\) Fupper + Fmiddle) * Nseg;
n3 = (Fupper + Fmiddle + Flower) * Nseg;
for ( \(\mathrm{i}=0\); \(\mathrm{i}<\) Nseg; \(\mathrm{i}++\) ) \{
    real vw;
    if(i<n1) \{
        vw = prms[Vw0_up] / (Fupper * Nseg);
    \} else if( \(\mathrm{i}<\mathrm{n} 1+1\) ) \{
        vw \(=\) prms[Vw0_up] * (Fupper * Nseg - i) / (Fupper * Nseg);
        vw += prms[Vw0_mid] * (1.0 - Fupper * Nseg + i) / (Fmiddle * Nseg);
    \} else if(i<n2) \{
        vw = prms[VwO_mid] / (Fmiddle * Nseg);
    \} else if( \(\mathrm{i}<\mathrm{n} 2+1\) ) \{
        vw \(=\) prms[Vw0_mid] * ((Fupper + Fmiddle) * Nseg - i) / (Fmiddle * Nseg);
        vw += prms[Vw0_low] * (1.0 - (Fupper + Fmiddle) * Nseg + i) / (Flower * Nseg);
    \} else if(i<n3) \{
        vw = prms[Vw0_low] / (Flower * Nseg);
    \} else if( \(\mathrm{i}<\mathrm{n} 3+1\) ) \{
        vw \(=\) prms[Vw0_low] * ((Fupper + Fmiddle + Flower) * Nseg - i) / (Flower * Nseg);
        vw += prms[Vw0_il] * (1.0-(Fupper + Fmiddle + Flower) * Nseg + i) / (Fileum * Nseg);
    \} else \{
```

```
        vw = prms[VwO_il] / (Fileum * Nseg);
        }
        [delegate setValue:vw at: LUMEN_W + i];
        tot += vw;
    }
    // set dose in esophagus
    [delegate setValue:prms[Dose] / prms[Ves] at:ESO];
    return YES;
}
- (void)preparativeCalculation \{
    // set variables independent of time for preparation of the differential equations
    int i;
    real pgp_0, pgp_prev, pgp_curr, cyp3a4_0, cyp3a4_curr, cyp3a4_prev, pH_adj0, pH_adj,
        len_prev, rad_prev, total_vol, villi_ex, sc, bas_surf_area_ent,
        api_surf_area_ent;
    total_api_surf_area_ent = psA_total = psB_total
    = psPgp_total = clCyp3a_total = 0.0;
    radAtSeg[Nseg - 1] = prms[RadAtSeg_0] - prms[RadGradient] * prms[LenOfSI];
    total_vol = 1.0 / 3.0 * PI * prms[LenOfSI] * (pow(prms[RadAtSeg_0], 2.0)
            + prms[RadAtSeg_0] * radAtSeg[Nseg - 1] + pow(radAtSeg[Nseg - 1], 2.0));
volOfSeg = total_vol / Nseg;
total_api_surf_area_ent = len_prev = 0.0;
cyp3a4_0 = cyp3a4_prev = (1.0 - prms[CYP3A_grad]) * prms[Tot_CYP3A] / prms[LenOfSI];
pgp_0 = pgp_prev = (1.0 - prms[Pgp_grad]) / prms[LenOfSI];
rad_prev = prms[RadAtSeg_0];
pH_adj0 = 1.0 + pow(10.0, prms[pH_Caco2] - prms[pKa_acid])
            + pow(10.0, prms[pKa_base] - prms[pH_Caco2]);
for(i = 0; i < Nseg; i++) {
    radAtSeg[i] = pow(pow(rad_prev, 3.0) - 3.0 * prms[RadGradient] * volOfSeg / PI, 1.0 /
3.0);
    lenAtSeg[i] = (prms[RadAtSeg_0] - radAtSeg[i]) / prms[RadGradient];
    sc = lenAtSeg[i] / prms[LenOfSI];
    villi_ex = prms[VE_0] - (prms[VE_O] - prms[VE_Z]) * sc;
    pH_lum[i] = prms[pH_lum_0] + prms[pHgrad] * sc;
    bas_surf_area_ent = PI * (rad_prev + radAtSeg[i]) * pow(pow(rad_prev - radAtSeg[i], 2.0)
                + pow(lenAtSeg[i] - len_prev, 2.0), 0.5) * prms[PE] * villi_ex;
    api_surf_area_ent = bas_surf_area_ent * prms[ME];
    total_api_surf_area_ent += api_surf_area_ent;
    qLamAtSeg[i] = prms[Q_lam] * api_surf_area_ent;
    vEntAtSeg[i] = bas_surf_area_ent * prms[Tent];
    vLamAtSeg[i] = bas_surf_area_ent * prms[Hvilli] / villi_ex - vEntAtSeg[i];
```

```
    sc = 2.0 * lenAtSeg[i] / (prms[LenOfSI] * prms[LenOfSI]);
    cyp3a4_curr = cyp3a4_0 + sc * prms[CYP3A_grad] * prms[Tot_CYP3A];
    pgp_curr = pgp_0 + sc * prms[Pgp_grad];
    pH_adj = pH_adj0 / (1.0 + pow(10.0, pH_lum[i] - prms[pKa_acid])
            + pow(10.0, prms[pKa_base] - pH_lum[i]));
    sc = api_surf_area_ent * prms[SF_api_passive] / (prms[insert_Caco2] *
prms[ME_Caco2]);
    psA_in[i] = pH_adj * sc * prms[PSa_in_Caco2];
    psA_out_pass[i] = sc * prms[PSa_out_Caco2];
    sc = bas_surf_area_ent * prms[SF_baso] / prms[insert_Caco2];
    psB_out[i] = sc * prms[PSb_out_Caco2];
    psB_in[i] = sc * prms[PSb_in_Caco2];
    sc = (lenAtSeg[i] - len_prev) / 2.0;
    psA_Pgp[i] = sc * (pgp_prev + pgp_curr);
    cl_Cyp3a[i] = sc * (cyp3a4_prev + cyp3a4_curr);
        rad_prev = radAtSeg[i];
        len_prev = lenAtSeg[i];
        cyp3a4_prev = cyp3a4_curr;
        pgp_prev = pgp_curr;
    }
    sc = total_api_surf_area_ent / (prms[insert_Caco2] * prms[ME_Caco2]);
    for(i = 0; i < Nseg; i++) {
        psPgp_total += psA_Pgp[i] *= sc * prms[SF_pgp] * prms[PSpgp_Caco2];
        psA_total += psA_in[i];
        clCyp3a_total += cl_Cyp3a[i] *= prms[SF_CYP3A] * prms[CLm_Caco2];
        psB_total += psB_out[i];
        qLamAtSeg[i] /= total_api_surf_area_ent; // blood flow in lamina propria
    }
}
```

- (void)rkfEvaluateDifferentials:(real*)di fromValues:(const real*)v atTime:(real)t \{ // This is the main body of partial differential equations.
int i, j, k;
real $a, b, q, f p$, secw 0 , secw, $c[5][5]$;
$q=\operatorname{prms}[C] ;$
$q^{*}=1.0-\operatorname{prms}[\mathrm{D}]{ }^{*} \exp \left(-\left(\mathrm{pow}(\mathrm{fabs}(\mathrm{t}-\mathrm{prms}[\mathrm{Tlag}]), \operatorname{prms}[\mathrm{F}]) /\left(2.0^{*} \operatorname{pow}(\mathrm{prms}[\mathrm{E}]\right.\right.\right.$, prms[F]))));
b = q / volOfSeg;
$\operatorname{di}[A B S O R P]=0.0 ;$

```
    secw = ((t < prms[T_fe]) ? prms[Sec_Wfe_SI]: prms[Sec_W_SI]) * DNseg;
    secw0 = secw * prms[UpSecRatio];
    secw *= (1.0 - prms[UpSecRatio]) / Nseg;
    fp = prms[fb] / prms[Rb];
    for(i = -2; i < Nseg; i++) {
    a = i <= 0 ? lenAtSeg[0] / 2.0: (lenAtSeg[i - 1] + lenAtSeg[i]) / 2.0;
    a = prms[A] * exp(-prms[B] * a) + A_BASE;
    a *= b * Nseg;
    c[0][4] = -(a + b) / AA;
    c[1][4] = a / BB + b / CC
    c[2][4] = -a * DD; // - ke,
    c[3][4] = a / BB - b / CC;
    c[4][4] = (b - a) / AA;
    if(i >= 0) {
        real conc_f_ent, conc_lamina, flx, flx_from_lamina, cc1, cc2;
        conc_f_ent = prms[Fent] * v[ENT + i] / vEntAtSeg[i];
        flx = prms[KmPgp_Caco2] == 0.0 ? psA_Pgp[i]: psA_Pgp[i] / (prms[KmPgp_Caco2] +
        conc_f_ent);
        flx = -(flx + psA_out_pass[i]) * conc_f_ent;
        flx += psA_in[i] * v[LUMEN + i] / v[LUMEN_W + i];
        if(i== 0) {
            cc1 = -c[0][2]+c[2][2]+c[3][2]+c[4][2];
            cc2 = c[3][3] + c[4][3];
            di[LUMEN_W] = (cc1 - prms[Kw_SI]) * v[LUMEN_W] + cc2 * v[LUMEN_W + 1] +
c[4][4]
    *v[LUMEN_W + 2]
                + secw0 + secw;
        di[LUMEN] = cc1 * v[LUMEN] + cc2 * v[LUMEN + 1] + c[4][4] * v[LUMEN + 2] - flx;
    } else if(i == 1) {
        cc1 = c[0][1] + c[1][1];
        di[LUMEN_W + 1] = cc1 * v[LUMEN_W] + (c[2][2] - prms[Kw_SI]) * v[LUMEN_W + 1]
            +c[3][3]*v[LUMEN_W + 2] + c[4][4] * v[LUMEN_W + 3] + secw;
        di[LUMEN + 1] = cc1 *v[LUMEN] + c[2][2] * v[LUMEN + 1] + c[3][3] * v[LUMEN + 2]
            +c[4][4] * v[LUMEN + 3] - flx;
    } else if(i < Nseg - 2) { // (3-38)
    int ii = LUMEN_W + i, jj = LUMEN + i;
    di[ii] = c[0][0]*v[ii-2] +c[1][1] * v[ii - 1] + (c[2][2] - prms[Kw_SI])*v[ii]
            +c[3][3] * v[ii + 1] + c[4][4] * v[ii + 2] + secw;
        di[j]] = c[0][0] * v[jj - 2] + c[1][1] *v[jj - 1] +c[2][2] * v[jj]
            +c[3][3] * v[jj + 1] + c[4][4] * v[jj + 2] - flx;
    } else if(i == Nseg - 2) {
    cc1 = c[3][3] + c[4][3];
    di[LUMEN_W + Nseg - 2] = c[0][0] * v[LUMEN_W + Nseg - 4] + c[1][1] * v[LUMEN_W
    + Nseg - 3] + (c[2][2] - prms[Kw_SI]) * v[LUMEN_W + Nseg - 2] + cc1
        * v[LUMEN_W + Nseg - 1] + secw;
    di[LUMEN + Nseg - 2] = c[0][0] * v[LUMEN + Nseg - 4] + c[1][1] * v[LUMEN + Nseg - 3]
            +c[2][2]*v[LUMEN + Nseg - 2] + cc1 * v[LUMEN + Nseg - 1] - flx;
```

```
    } else {// i== Nseg - 1
    cc1 = c[1][1] + c[4][1];
    cc2 = c[2][2] + c[3][2];
    di[LUMEN_W + Nseg - 1] = c[0][0] * v[LUMEN_W + Nseg - 3] + cc1 * v[LUMEN_W
                + Nseg - 2] + (cc2 - prms[Kw_SI]) * v[LUMEN_W + Nseg - 1] + secw;
    di[LUMEN + Nseg - 1] = c[0][0] * v[LUMEN + Nseg - 3] + cc1 * v[LUMEN + Nseg - 2]
            + cc2 * v[LUMEN + Nseg - 1] - flx;
    }
    flx -= prms[KmCyp3a_Caco2] == 0.0 ?
    cl_Cyp3a[i] * conc_f_ent: cl_Cyp3a[i] * conc_f_ent / (prms[KmCyp3a_Caco2]
                + conc_f_ent);
    conc_lamina = v[LAMINA + i] / vLamAtSeg[i];
    flx_from_lamina = fp * psB_in[i] * conc_lamina;
    flx_from_lamina -= psB_out[i] * conc_f_ent;
    di[ENT + i] = flx_from_lamina + flx;
    di[ABSORP] += di[AbsFromSeg + i] = conc_lamina * qLamAtSeg[i] / fp;
    di[LAMINA + i] = - di[AbsFromSeg + i] - flx_from_lamina;
    }
    for(j = 0; j < 4; j++) for(k = 0; k < 5; k++) c[k][j] = c[k][j + 1];
}
// Water volume in the esophagus
di[ESO_W] = -prms[Ka] * v[ESO_W];
// Water volume in stomach
di[STOMACH_W] = (prms[Ka] * v[ESO_W] * prms[Ves] + prms[Sec_W_Stm]) / prms[Vstm]
            - (prms[Kg] + prms[Kw_stm]) * v[STOMACH_W];
di[LUMEN_W] += prms[Kg] * v[STOMACH_W] * prms[Vstm];
// Concentration of substrate in the esophagus
di[ESO] = -prms[Ka] * v[ESO];
// Concentration of substrate in the stomach
di[STOMACH] = prms[Ka] * v[ESO] * prms[Ves] / prms[Vstm] - prms[Kg] * v[STOMACH];
di[LUMEN] += prms[Kg] * v[STOMACH] * prms[Vstm];
// Cumulative amount of substrate in the portal vein
di[PORT] = di[ABSORP] - prms[Qpv] * v[PORT] / prms[Vpv];
// Water volume in the caecum/colon
di[CAECUM_W] = ((9.0 * v[LUMEN_W + Nseg] - v[LUMEN_W + Nseg - 1]) / 8.0)
    * q / volOfSeg - prms[Kw_col] * v[CAECUM_W] + prms[Sec_W_Col];
// Water volume in feces (assumed to be 0)
di[FECES_W] = prms[Krec] * v[CAECUM_W];
```

```
    // Amount of substrate in the caecum/colon
    di[CAECUM] = ((9.0*v[LUMEN + Nseg - 1] - v[LUMEN + Nseg - 2]) / 8.0) * q / volOfSeg;
    if(t > prms[lag_rec]) {
        di[CAECUM_W] -= prms[Krec] * v[CAECUM_W];
        di[CAECUM] -= prms[Krec] * v[CAECUM];
        di[FECES] = prms[Krec] * v[CAECUM]; // Amount of substrate in feces (assumed to be 0)
    }
    // Concentration of substrate in the liver
    di[LIVER] = (prms[Qpv] * v[PORT] / prms[Vpv] - prms[Qh] * v[LIVER] / prms[Kp_liver]
            * prms[Rb] - prms[fb] * prms[Rb] / prms[Kp_liver] * prms[CLint_h] * v[LIVER] +
prms[Qha]
            * v[BLOOD]) / prms[Vliver];
    // Concentration of substrate in the blood
    di[BLOOD] = (prms[Qh] * v[LIVER] / prms[Kp_liver] * prms[Rb] - prms[Qha] * v[BLOOD]
        - prms[K12] * v[BLOOD] * prms[Vb] + prms[K21] * v[PERI]) / prms[Vb];
    // Amount of substrate in the peripheral compartment
    di[PERI] = prms[K12] * v[BLOOD] * prms[Vb] - prms[K21] * v[PERI];
}
- (void)rkfEvaluateValues:(real*)vals atTime:(real)t;
// This is for calculation of compartments not included in the partial differential equations
int i, bd;
real total_jejunum, total_ileum, total_w;
//\% of dose in stomach (for estimation of 99mTc-DTPA distribution)
vals[Pcnt_in_stomach] = vals[STOMACH] * prms[Vstm] / prms[Dose];
//\% of dose in jejunum (for estimation of 99mTc-DTPA distribution)
total_jejunum = total_w = 0.0;
bd = F jejunum * Nseg;
for ( \(\mathrm{i}=0 ; \mathrm{i}<\mathrm{bd} ; \mathrm{i}++\) ) \{
total_jejunum += vals[LUMEN +i];
total_w += vals[LUMEN_W +i];
\}
vals[Pcnt_in_jejunum] = total_jejunum / prms[Dose];
//\% of dose in ileum (for estimation of 99mTc-DTPA distribution)
total_ileum = 0.0;
for(i = bd; i < Nseg; i++) \{
total_ileum += vals[LUMEN + i];
total_w += vals[LUMEN_W +i];
\}
vals[Pcnt_in_ileum] = total_ileum / prms[Dose];
```

```
    //% of dose in colon (for estimation of 99mTc-DTPA distribution)
    vals[Pcnt_in_colon] = vals[CAECUM] / prms[Dose];
    //% of dose in whole lumen
    vals[Pcnt_in_whole_lumen] = vals[Pcnt_in_jejunum] + vals[Pcnt_in_ileum]
        + vals[Pcnt_in_colon];
    //FA (substrate)
    vals[FA] = 1 - vals[Pcnt_in_whole_lumen] - vals[Pcnt_in_stomach];
    //FAFG (substrate)
    vals[FAFG] = vals[ABSORP] / prms[Dose];
    //FG (substrate)
    vals[FG] = vals[FAFG] / vals[FA];
    vals[TOTAL_W] = total_w + vals[ESO_W] * prms[Ves] + vals[STOMACH_W] * prms[Vstm]
    + vals[CAECUM_W];
//change from blood to plasma concentration and unit conversion from ug/mL to umol/L vals[Plasma_conc_uM] = vals[BLOOD] / prms[MW] / prms[Rb] * 1000;
}
@end
```


## 3. Reference for Supplementary file (text, Figure, and Tables)

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[^0]:    ${ }^{\text {a }}$ permeability surface area product

[^1]:    a internal standard

