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Research Paper

Model Analysis of the Concentration-Dependent Permeability of P-gp Substrates

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Purpose. Recently, it was reported that the apparent Michaelis-Menten constant ($Km_{(app)}$) of a P-glycoprotein (P-gp) substrate, defined for the extracellular substrate concentration, increases as the P-gp expression level in the cell increases. By its nature, the Km value should not depend on the level of P-gp expression. The purpose of this study is to establish a model which can estimate the Km value independent of the P-gp expression level in cells.
Methods. The previously reported concentration-dependent permeability of verapamil, quinidine, and vinblastine in MDR1-MDCKII, P-gp-highly induced Caco-2, P-gp-induced Caco-2, normal Caco-2, and MDR1-knockdown Caco-2 cells data were analyzed using a model in which the Km value was defined for the intracellular substrate concentration.
Results. The estimated Km values defined for the substrate concentration inside the cells were almost the same among various cells with different levels of P-gp expression. The estimated Vmax values were approximately proportional to the P-gp expression level.
Conclusion. The established kinetic model was found to be rational based on the results that the Km values of P-gp substrates were about the same for cells expressing various levels of P-gp, while the Vmax values were proportional to the expression levels of P-gp.

KEY WORDS: Absorption; Caco-2; Km; MDCK; P-glycoprotein.

INTRODUCTION

P-glycoprotein (P-gp), encoded in the MDR1 gene, is one of the ATP-binding cassette transporters and one of the most studied (1). P-gp is expressed in the intestine, liver, kidney, blood-brain barrier, and placenta and acts as an efflux transporter which recognizes a variety of drugs as substrates with broad specificities (2–4).

P-gp expressed in intestinal epithelial cells is reported to decrease the oral bioavailability of many P-gp substrate drugs (5–8). On the other hand, it is considered that some P-gp substrate drugs still have high bioavailability because the direct exposure of orally administrated substrates to the intestinal epithelial cells may saturate the function of P-gp. For example, talinolol, pafenolol, and celiprolol are reported to exhibit non-linear pharmacokinetics in human due to the saturation of P-gp function in the intestine (9–11). To predict the saturation of P-gp transport *in vivo*, it is necessary to estimate the Km for the P-gp substrate *in vitro*.

P-gp expressing cells, e.g. Caco-2 cells and MDR1-transfected MDCKII cells, are often used to study P-gp transport *in vitro*. Interestingly, it is reported that apparent Km values shift according to the P-gp expression level in the cells used for the transport studies (12–14). Shirasaka *et al.* conducted an absorptive (apical-to-basolateral) permeability study using 5 types of cells expressing various levels of P-gp and reported that there is a positive correlation between the P-gp expression levels and the estimated apparent Km values ($Km_{(app)}$) defined for the extracellular substrate concentration (12). Furthermore, Shirasaka *et al.* presented a methodology to predict the absorptive permeability *in vivo* based on the relationship between $Km_{(app)}$ values and P-gp expression levels (15). However, true Km values are considered to be governed solely by the interaction between substrates and P-gp and to be independent of P-gp expression levels. Such dependency of the $Km_{(app)}$ on the expression level of P-gp may have come from Shirasaka's definition of $Km_{(app)}$ for the extracellular substrate concentration. In this study, we assumed a P-gp transport model in which the Km values are defined for the intracellular substrate concentration and reanalyzed the concentration-dependent permeability to elucidate whether the Km values are independent of P-gp expression levels and also if Vmax values proportional to the P-gp expression levels can be estimated.

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MATERIALS AND METHODS

Permeability Experiments

Shirasaka *et al.* reported the concentration-dependent permeability of quinidine, verapamil, and vinblastine in an apical-to-basolateral direction using various cells (12). The permeability data obtained by Shirasaka *et al.* were used in this study. The following are brief explanations of the permeability experiments conducted by Shirasaka *et al.* The cells used, in descending order of P-gp expression level, were MDR1-expressing MDCKII cells (MDR1-MDCKII), P-gp induced Caco-2 cells from two different concentrations of vinblastine (P-gp-highly induced Caco-2 and P-gp-induced Caco-2), normal Caco-2 cells (normal Caco-2), and Caco-2 cells in which P-gp expression level was decreased by RNAi technology (MDR1-knockdown Caco-2). The cells were seeded on filter membrane inserts to form monolayers. The transport studies across the cell monolayers were performed by measuring the concentration of substrate that had appeared in the basolateral side after adding various concentrations of substrate to the apical side. The permeability (P_{app}, cm sec^{-1}) of substrates across cell monolayers was calculated using the following equation:

$$P_{app} = \frac{dQ}{dt} \cdot \frac{1}{S \cdot C_a} \tag{1}$$

where Q is the amount of compound transported over time t of the experiment, and, therefore, dQ/dt is the amount of drug transported within a given time period (nmol sec^{-1}). C_a is the initial concentration of the test compound added to the apical compartment (μM), and S is the membrane surface area (cm^2).

Kinetic Analysis

Previous Model

Shirasaka *et al.* calculated the apparent Vmax and Km ($Vmax_{(app)}$, $Km_{(app)}$) using the model shown in Fig. 1A. In the model, $Vmax_{(app)}$ and $Km_{(app)}$ are described by the following equations:

$$PS_{AB} = PS_{passive} - PS_{P-gp(app)} \tag{2}$$

$$PS_{AB} \cdot C_a = PS_{passive} \cdot C_a - \frac{Vmax_{(app)} \cdot C_a^r}{Km_{(app)}^r + C_a^r} \tag{3}$$

where PS_{AB} is the permeability in the apical to basolateral direction, $PS_{passive}$ is the permeability by passive diffusion, $PS_{P\text{-}gp(app)}$ is the efflux clearance by P-gp, and r is the Hill coefficient. $Km_{(app)}$ is defined for the substrate concentration in the apical side. Not only $Vmax_{(app)}$ values but also $Km_{(app)}$ values for the all substrates increased as the P-gp expression level in the cells increased (12).

New Model

In our new models (Fig. 1B and C), the Km values are defined for the unbound substrate concentration in the cells. It was assumed that the permeability experiments were done under sink conditions. The model for apical-to-basolateral permeability (Fig. 1B) leads to the following deferential equations:

$$Vd_{cell} \frac{dC_{cell,u}}{dt} = PS_1 \cdot C_a - \left(PS_2 + PS_3 + PS_{P-gp}\right) \cdot C_{cell,u} \tag{4}$$

where $C_{cell,u}$ is the unbound concentration in the cells, Vd_{cell} is the distribution volume in the cells, PS_1 is the passive permeability across apical membrane in an apical-to-cellular direction, PS_2 is the passive permeability across the apical membrane in a cellular-to-apical direction, PS_3 is the passive permeability across the basolateral membrane in a cellular-to-basolateral direction, and $PS_{P\text{-}gp}$ is the efflux clearance by P-gp. Under steady-state conditions, the following equations can be derived.

$$C_{cell,u} = \frac{PS_1}{PS_2 + PS_3 + PS_{P-gp}} \cdot C_a \tag{5}$$

$$PS_{AB} \cdot C_a = PS_3 \cdot C_{cell,u} = \frac{PS_1 \cdot PS_3}{PS_2 + PS_3 + PS_{P-gp}} \cdot C_a \tag{6}$$

$$PS_{P-gp} = \frac{V\,max}{Km + C_{cell,u}} \tag{7}$$

The following equation can be derived by solving Eqs. 5, 6, and 7 for PS_{AB}.

$$\begin{aligned} PS_{AB} &= P_{app,AB} \cdot S \\ &= \frac{PS_3\left[PS_1C_a - (PS_2 + PS_3)K_m - V_{max} + \sqrt{\{PS_1C_a - (PS_2 + PS_3)K_m - V_{max}\}^2 + 4PS_1(PS_2 + PS_3)K_mC_a}\right]}{2(PS_2 + PS_3)C_a} \end{aligned} \tag{8}$$

In the same way, a new model for basolateral-to-apical permeability (Fig. 1C) leads to the following equation:

$$\begin{aligned} PS_{BA} &= P_{app,BA} \cdot S \\ &= PS_4 - \frac{PS_3\left[PS_4C_b - (PS_2 + PS_3)K_m - V_{max} + \sqrt{\{PS_4C_b - (PS_2 + PS_3)K_m - V_{max}\}^2 + 4PS_4(PS_2 + PS_3)K_mC_b}\right]}{2(PS_2 + PS_3)C_b} \end{aligned} \tag{9}$$

where C_b is the initial concentration of the test compound added to the basolateral compartment, and PS_4 is the passive permeability across the basolateral membrane in a basolateral-to-cellular direction.

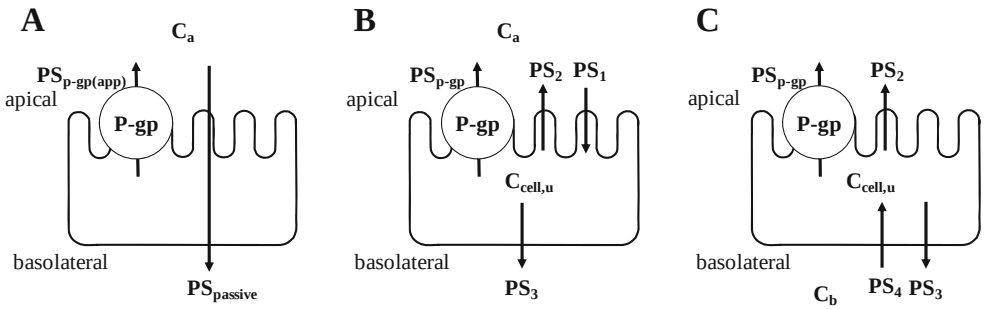

Fig. 1. Schematic diagram illustrating the PS products for the penetration of P-gp substrates across the cell monolayer. **A**, a previous model for apical-to-basolateral permeability considering only apical (donor) concentration; **B**, a new model for apical-to-basolateral permeability considering intracellular unbound concentration as the determiner of Km; **C**, a new model for basolateral-to-apical permeability.

First, simulations of the concentration dependent permeability were performed under the conditions of S=1 (cm^2), $PS_1/S=PS_2/S=PS_3/S=PS_4/S=2$ ($x10^{-5}$cm sec^{-1}), Km= 0.5 (μM), and Vmax/S=1, 3, 10, 30, 100 ($x10^{-5}$μM cm sec^{-1}) using Eqs. 8 and 9. Second, the apical-to-basolateral permeability data obtained by Shirasaka *et al.* were fitted to Eq. 8 and the Km values and Vmax values were optimized. The fitting was performed using the numerical mode of kinetic analysis software SAAMII version 1.2 (SAAM Institute, University of Washington). Passive permeability was assumed to be the same for each substrate ($PS_1=PS_2=PS_3$).

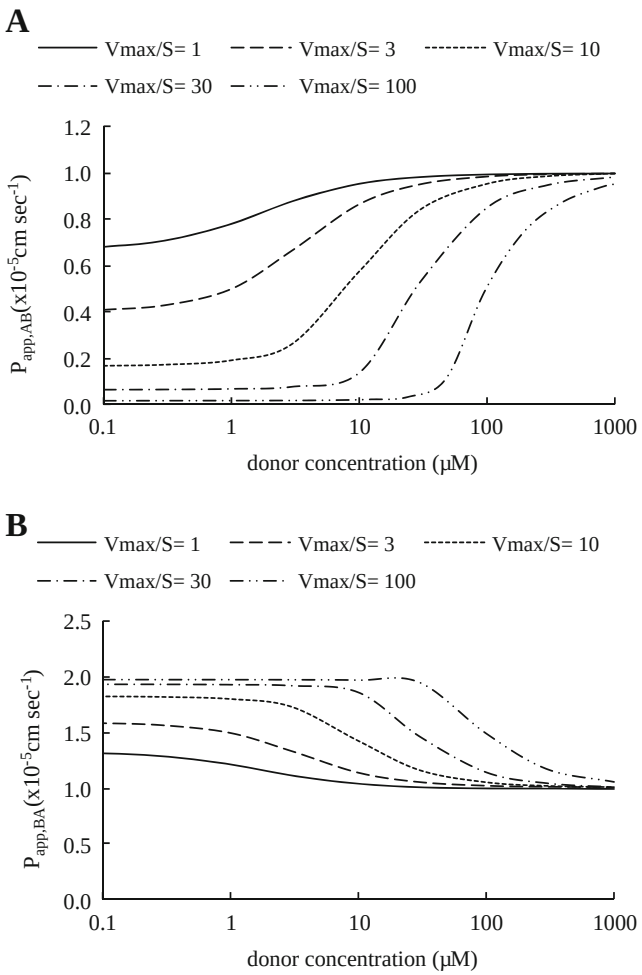

Fig. 2. Simulation of concentration-dependent apical-to-basolateral permeability (**A**) and basolateral-to-apical permeability (**B**) with the new model. $PS_1/S=PS_2/S = PS_3/S = PS_4/S = 2$ ($x10^{-5}$cm sec^{-1}), Km= 0.5 (μM), and Vmax/S = 1, 3, 10, 30, 100 ($x10^{-5}$μM cm sec^{-1}).

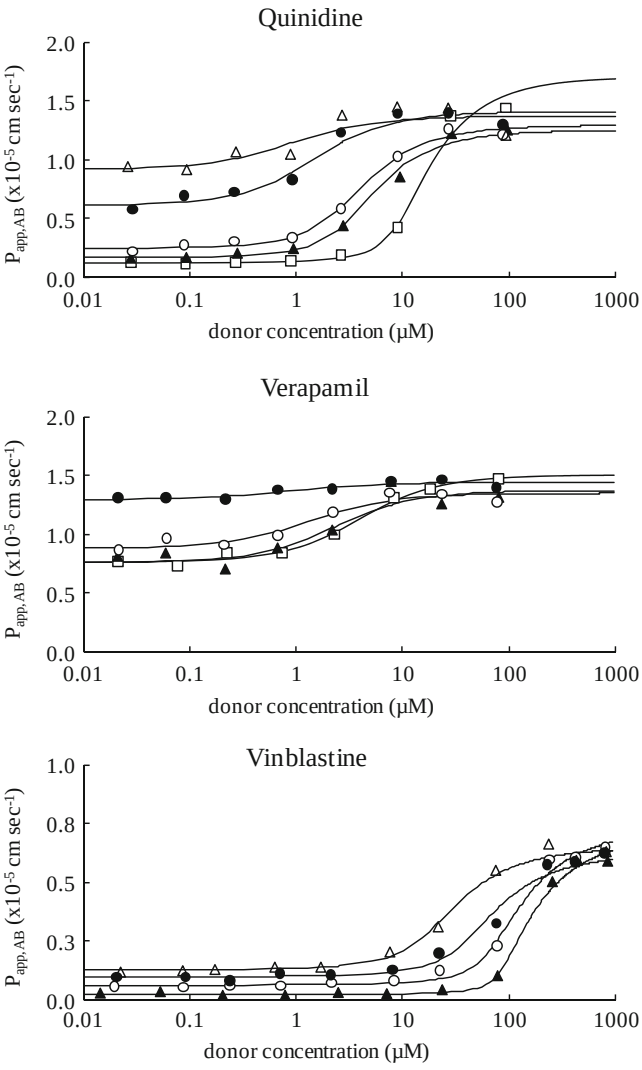

Fig. 3. Effect of P-gp expression level on the concentration dependency of absorptive (apical-to-basolateral) permeability of three P-gp substrate drugs (*quinidine*, *verapamil*, and *vinblastine*) in normal Caco-2 cells, (●); P-gp-induced Caco-2 cells, (○); P-gp-highly induced Caco-2 cells, (▲); MDR1-knockdown Caco-2 cells, (△); MDR1-MDCKII cells, (□).

Table I. Kinetic Parameters (PS_1/S, Km, Vmax/S) of P-gp-Mediated Transport for Quinidine, Verapamil, and Vinblastine in Various Cell Monolayers

	P-gp level [a]	PS_1/S [b]	Km [b]	$Km_{(app)}$ [a]	Vmax/S [b]	$Vmax_{(app)}$/S [a]
	μg cm^{-2}	x10^{-5}cm sec^{-1}	μM	μM	x10^{-5}μM cm sec^{-1}	x10^{-5}μM cm sec^{-1}
Quinidine						
MDR1-MDCKII	359.6	3.40±0.26	0.339±0.033	16.4	29.09±4.03	22.1
P-gp-highly induced Caco-2	191.0	2.50±0.13	0.199±0.023	8.13	6.37±0.91	10.5
P-gp-induced Caco-2	103.7	2.58±0.16	0.234±0.040	6.20	5.13±1.06	6.8
Normal Caco-2	26.8	2.82 ± 0.14	0.230±0.079	1.69	1.70±0.61	1.3
MDR1-knockdown Caco-2	8.71	2.74±0.16	0.253±0.238	0.61	0.68±0.64	0.5
Verapamil						
MDR1-MDCKII	359.6	3.01 ± 0.10	0.760±0.166	2.85	4.53±1.19	2.1
P-gp-highly induced Caco-2	191.0	2.75±0.20	0.495±0.325	2.07	2.21±1.58	1.4
P-gp-induced Caco-2	103.7	2.70±0.09	0.384±0.177	1.66	1.10±0.53	0.8
Normal Caco-2	26.8	2.88±0.04	0.370±0.324	1.01	0.24±0.21	0.2
MDR1-knockdown Caco-2	8.71	ND	ND	ND	ND	ND
Vinblastine						
MDR1-MDCKII	359.6	ND	ND	ND	ND	ND
P-gp-highly induced Caco-2	191.0	1.39±0.19	1.37±0.24	323.4	113.2±23.8	196.8
P-gp-induced Caco-2	103.7	1.44±0.14	2.98±0.42	149.0	88.0±19.3	83.1
Normal Caco-2	26.8	1.23±0.11	3.10±0.63	80.7	40.0±11.5	35.6
MDR1-knockdown Caco-2	8.71	1.29±0.05	1.74±0.18	29.9	18.1±2.3	14.7

[a] Data from Shirasaka *et al.* (12).
[b] Errors of optimized values are presented as SE. ND, not determined.

RESULTS

Simulated Permeability with the New Model

The concentration-dependent absorptive permeability was simulated using Eq. 8 derived by the new model shown in Fig. 1B. Only Vmax values varied according to the differences in the P-gp expression levels of cells, whereas the other parameters (Km and PS) were fixed. The simulated relationships between the donor concentration of substrates and $P_{app,AB}$ are shown in Fig. 2A. The simulated absorptive (apical-to-basolateral) permeability (Fig. 2A) well resembled the dependency of permeability on the apical substrate concentration observed by Shirasaka *et al.* (12). Under the linear conditions in which the apical (donor) concentration of substrate was sufficiently low, the $P_{app,AB}$ values ($P_{app,AB,low}$) decreased as the Vmax values increased (Fig. 2A). Under conditions of the saturated P-gp in which the apical (donor) concentration of substrate was sufficiently high, the $P_{app,AB}$ values ($P_{app,AB,high}$) were the same regardless of the Vmax values (Fig. 2A). With the definition of the $Km_{(app)}$ values as the apical (donor) concentration showing the average P_{app} values of $P_{app,AB,low}$ and $P_{app,AB,high}$, the $Km_{(app)}$ values increased as the Vmax values increased.

The concentration-dependent basolateral-to-apical permeability was simulated using Eq. 9 derived by the new model shown in Fig. 1C. The simulated relationships between the donor concentration of substrates and $P_{app,BA}$ are shown in Fig. 2B. The $Km_{(app)}$ values also increased as the Vmax values increased.

Kinetic Analysis on P-gp-Mediated Transport of P-gp Substrate Drugs

The permeability data of quinidine, verapamil, and vinblastine obtained by Shirasaka *et al.* were analyzed using the new model shown in Fig. 1B. The fitted curves for the concentration-dependent permeability for each substrate in each cell monolayer are shown in Fig. 3. The fitted curves well described the observed permeability. The optimized parameters (PS_1/S, Km, Vmax/S) and the $Km_{(app)}$ and $Vmax_{(app)}$/S values reported by Shirasaka *et al.* are shown in Table I. The Km values for quinidine ranged from 0.199 to 0.339 μM among the cells. The Km values for verapamil and vinblastine ranged from 0.370 to 0.760 μM and from 1.37 to 3.10 μM,

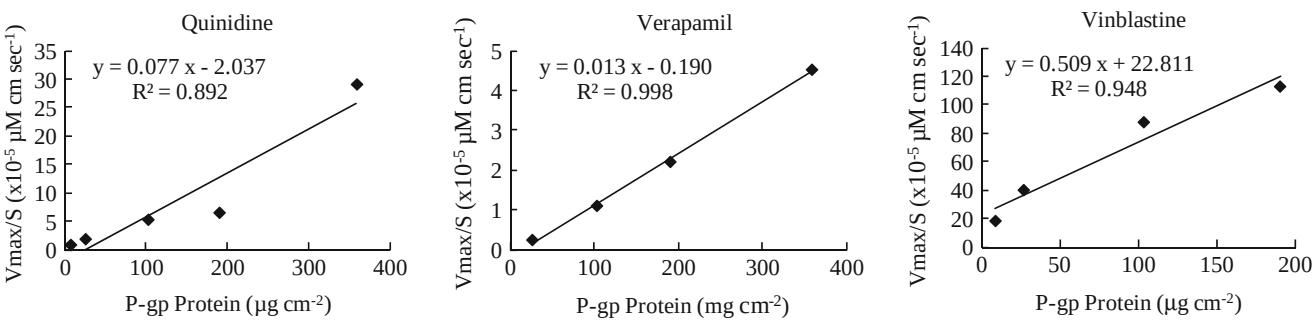

Fig. 4. Correlation between P-gp expression level and Vmax value of three P-gp substrates, *quinidine*, *verapamil*, and *vinblastine*.

respectively. On the other hand, the $Km_{(app)}$ values for the three substrates greatly depended on the P-gp expression level (Table I). The relationship between the Vmax/S values and P-gp expression levels is shown in Fig. 4. The Vmax/S values were directly proportional to the P-gp expression levels. The coefficient of determination (R^2) ranged from 0.892 to 0.998. The passive permeability (PS_1/S) for each substrate was comparable among the cells (Table I).

DISCUSSION

The simulation using the new model (Fig. 1B) was carried out by varying the Vmax values with a constant Km value (Fig. 2A) and elicited the same pattern of concentration-dependent permeability observed by Shirasaka *et al*. The $Km_{(app)}$ values increased as the Vmax values increased. And the reanalysis of the experimental data using the new model revealed that the Km values defined for the intracellular concentration were almost the same among the cells expressing various levels of P-gp for each substrate. These results support the idea that the Km values defined for the intracellular concentration are governed only by the interaction between the substrates and P-gp and are not dependent on the expression level of P-gp. It also makes sense that the Vmax values were directly proportional to the expression level of P-gp.

In addition to Shirasaka *et al*., Korjamo *et al.* performed absorptive permeability experiments for quinidine using three types of cells, Caco-2 cells, hPXR-expressing Caco-2 cells, and MDR1-expressing MDCK cells, and reported that the apparent Km values correlated with the P-gp levels in the cells used (13). Korjamo *et al.* also performed permeability experiments in the basolateral-to-apical direction and reported that the apparent Km values shifted according to the P-gp level. The shift in the apparent Km values estimated by the permeability experiments in a basolateral-to-apical direction can be explained by our new model (Fig. 2B). The apparent Km values for P-gp substrates differ among the reports. As shown in the present study, the variability in the P-gp level at least partly contributes to the variability in the reported apparent Km values. However, the interpretation should be made cautiously, because complexity due to variation in passive permeability between cell types and variation in the efficiency of the efflux transporter in different membrane environment might also contribute to the variability in the reported apparent Km values.

To predict the effect of P-gp-mediated transport on the absorption in human, development of a method which can correct the Km values obtained from different laboratories is necessary. The use of standard compounds such as quinidine and vinblastine might help such standardization.

CONCLUSIONS

Shirasaka *et al*. measured and reported the concentration-dependent permeability of verapamil, quinidine, and vinblastine in cells with different levels of P-gp expressions and found that the $Km_{(app)}$ values positively correlated with the P-gp expression levels (12). In this report, the permeability data were reanalyzed using a model in which the Km values were defined for intracellular concentration. The calculated Km values for each substrate were comparable among the cells expressing different levels of P-gp. The calculated Vmax values were directly proportional to the P-gp expression levels and thus indicate the validity of the new model.

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