DMD Fast Forward. Published on February 14, 2008 as doi:10.1124/dmd.107.020040
This article has not been copyedited and formatted. The final version may differ from this version.

DMD #20040

# SCALING OF *IN VITRO* MEMBRANE PERMEABILITY TO PREDICT P-GLYCOPROTEIN-MEDIATED DRUG ABSORPTION *IN VIVO*

Yoshiyuki Shirasaka, Yoshie Masaoka, Makoto Kataoka, Shinji Sakuma, and Shinji Yamashita

Faculty of Pharmaceutical Sciences, Setsunan University, 45-1, Nagaotoge-cho, Hirakata, Osaka 573-0101, Japan (Y.S., Y.M., M.K., S.S., S.Y.)

DMD Fast Forward. Published on February 14, 2008 as DOI: 10.1124/dmd.107.020040
This article has not been copyedited and formatted. The final version may differ from this version.



Running Title:

PREDICTION OF P-GP-MEDIATED DRUG ABSORPTION *IN VIVO*

**Correspondence:**

Shinji Yamashita, Ph.D.

Faculty of Pharmaceutical Sciences, Setsunan University

45-1 Nagaotoge-cho, Hirakata, Osaka 573-0101, Japan

Tel.: +81-72-866-3126; Fax: +81-72-866-3126

E-mail: shinji@pharm.setsunan.ac.jp

**The Number of**

Text Page: 19

Tables: 2

Figures: 5

References: 30

**The number of words in**

Abstract: 209

Introduction: 541

Materials and Methods: 1363

Results: 1001

Discussion: 1316

DMD Fast Forward. Published on February 14, 2008 as DOI: 10.1124/dmd.107.020040
This article has not been copyedited and formatted. The final version may differ from this version.



**Abbreviations:**

P-gp, P-glycoprotein; MDR, multidrug resistance; BCRP, breast cancer resistance protein; MRP, multidrug resistance-associated protein; GI, gastrointestinal; DDI, drug-drug interaction; $P_{app}$, apparent permeability; AP, apical; BL, basolateral

DMD Fast Forward. Published on February 14, 2008 as DOI: 10.1124/dmd.107.020040
This article has not been copyedited and formatted. The final version may differ from this version.



**Abstract**

In a previous study, the concentration-dependent permeability of P-gp substrate drugs, quinidine, verapamil and vinblastine, in several cell monolayers with different levels of P-gp expression was kinetically analyzed to obtain fundamental parameters for P-gp-mediated transport, $V_{max}$ and $K_{m(app)}$ values. Both $V_{max}$ and $K_{m(app)}$ values of each drug were found to show linear correlations with the expression level of P-gp. These findings imply the possibility of estimating the $V_{max}$ and $K_{m(app)}$ values of P-gp substrate drugs in the *in vivo* intestinal membrane based on the P-gp expression level. In the present study, concentration-dependent drug permeability to the rat small intestines (upper jejunum and ileum) was simulated based on $V_{max}$ and $K_{m(app)}$ values of each drug estimated from P-gp expression level in the rat small intestines. In order to validate the predictability of these procedures, drug permeability in the rat small intestines was measured by *in situ* single-pass perfusion method. It was confirmed that simulated permeability of each drug in the rat jejunum and ileum corresponded well with permeability measured by *in situ* single-pass perfusion method. This study clearly demonstrated the potential to estimate the permeability of P-gp substrate drugs in the human intestine from its P-gp expression level, and thus the possibility to predict the oral absorption of those drugs.

## Introduction

P-glycoprotein (P-gp), one of the most important efflux transporters in the field of biopharmacy, is an ATP-binding cassette (ABC) transporter encoded by the multidrug resistance 1 gene (MDR1/ABCB1). P-gp is expressed not only in cancer cells but also in many normal tissues (Loo and Clarke, 1999). So far, many reports have described the effects of P-gp on the pharmacokinetic profiles of clinically important drugs, by denaturing absorption, distribution, and excretion. For example, P-gp located in the apical domain of the enterocytes of the gastrointestinal (GI) tract limits the uptake and absorption of its substrate drugs after oral administration (Hunter and Hirst, 1997; Meijer et al., 1997; Jonker et al., 1999). Because of the great impact of P-gp on the pharmacokinetic profiles of a variety of drugs, various *in vitro* experimental techniques are now used to identify the compounds subjected to P-gp-mediated efflux (Kim et al., 1998; Polli et al., 1999; Polli et al., 2001; Susanto and Benet, 2002; Shirasaka et al., 2006). However, it is still difficult to quantitatively predict the effect of P-gp on *in vivo* intestinal absorption of such drugs.

In a previous study, we measured the apical (AP) to basal (BL) absorptive permeability of P-gp substrate drugs in several cultured cell monolayers with different expression levels of P-gp (Shirasaka et al., 2008). In all cell monolayers, AP to BL permeability of P-gp substrate drugs showed a sigmoid-type relation to their donor (AP) concentration, and reached a maximal value at the higher concentration range due to the saturation of P-gp-mediated efflux. Concentration-dependent permeability of each drug was kinetically analyzed to obtain fundamental parameters for P-gp-mediated transport,

DMD Fast Forward. Published on February 14, 2008 as DOI: 10.1124/dmd.107.020040
This article has not been copyedited and formatted. The final version may differ from this version.



$V_{max}$ and $K_{m(app)}$ values. Assuming that drug concentration in the vicinity of the drug binding site of P-gp is proportional to apical concentration, the $K_{m(app)}$ value could be regarded as an apparent affinity of the drug to P-gp, which is defined based on apical drug concentration. Interestingly, not only the $V_{max}$ value but also the $K_{m(app)}$ value was found to show linear correlation with the expression level of P-gp quantified by real-time quantitative PCR and Western blot analyses. From these results, we propose the following procedures to simulate the concentration-dependent permeability of P-gp substrate drugs in the human intestine:

1) Measure the concentration-dependent permeability of the test compound in various cell monolayers with different expression levels of P-gp (at least three kinds of cell monolayers).
2) Obtain $V_{max}$ and $K_{m(app)}$ values of the test compound in each monolayer by using kinetic models.
3) Quantify the P-gp expression level in each monolayer (protein or mRNA level) and obtain the linear regression lines to $V_{max}$ and $K_{m(app)}$ values.
4) Estimate $V_{max}$ and $K_{m(app)}$ values in the human intestinal membrane by incorporating its P-gp expression level into the linear regression line.
5) Simulate the concentration-dependent permeability of the test compound in the human intestinal membrane by using the estimated parameters.

In the present study, concentration-dependent drug permeability to the rat small intestine (upper jejunum and ileum) was simulated based on $V_{max}$ and $K_{m(app)}$ values of each drug obtained in a cell monolayer study and on P-gp expression levels in rat small intestine.

In order to validate the predictability of the above procedures, drug transport in rat small intestine was measured by *in situ* single-pass perfusion method.

## Materials and Methods

### Chemicals

Verapamil hydrochloride and quinidine sulfate were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Vinblastine sulfate was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). For Western blot analysis, sample buffer (pH 6.8) consisting of 62.5 mM Tris-HCl, 25% glycerol, 2% SDS and 0.01% bromophenol blue, Readygels J consisting of 7.5% resolving gels and Tris-HCl and polyvinylidene difluoride membrane (0.2 μm pore size) were purchased from Bio-Rad Laboratories (Hercules, CA). Skim milk was obtained from Becton Dickinson Bioscience (Bedford, MA). Anti-P-glycoprotein (C219) was purchased from CALBIOCHEM® (Darmstadt, Germany). Anti-GAPDH (6C5) was purchased from American Research Products, Inc. (Belmont, MA). Biotinylated Protein Ladder Detection Pack, anti-mouse IgG (H&L), HRP-Linked and Lumi GLO Chemiluminescent Substrate Kit were purchased from Cell Signaling Technology, Inc. (Danvers, MA). All other compounds and reagents were obtained from Nacalai Tesque, Inc. (Kyoto, Japan), Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Sigma Chemical Co., Bio-Rad Laboratories, or Applied Biosystems (Foster City, CA).

### *In Situ* Single-Pass Perfusion Experiment

Male Wistar rats were housed three per cage with free access to commercial chow and tap water, and were maintained on a 12 h dark/light cycle (7:00 a.m.-7:00 p.m. light) in an air-controlled room (temperature, 24.5 ± 1 ˚C; humidity, 55 ± 5 %). All

animal experimentation reported in this manuscript has been carried out in accordance with the Declaration of Helsinki and with the Guide of Setsunan University for the Care and Use of Laboratory Animals. The permeability of rat intestinal membrane was evaluated by *in situ* single-pass perfusion method. Rats (body weight, 200-250 g) fasted overnight were anesthetized with pentobarbital. The abdominal cavity was opened and an intestinal loop (length: 10 cm) was made at two regions (upper jejunum and ileum) by cannulation with a silicone tube (i.d., 3 mm), and then the intestinal contents were removed by a slow infusion of saline and air.

Following the above procedure, the test solution (phosphate-buffered solution, adjusted to pH 6.5) containing each compound and FD-4 (10 μM) was perfused with an infusion pump at a flow rate of 0.5 mL/min. The effluent was collected from 30 min after starting the perfusion to 90 min, at 10 min intervals, because steady-state absorption usually was achieved by 30 min under these conditions. Drug permeability was calculated according to the following equation:

$$P_{\mathrm{app}} = Q \cdot \frac{C_{\mathrm{in}} - C_{\mathrm{out}}}{C_{\mathrm{in}}} \cdot \frac{1}{2\pi rl} \qquad (1)$$

where $Q$ is the flow rate, and $C_{\mathrm{in}}$ and $C_{\mathrm{out}}$ are inlet and outlet drug concentrations, respectively. The effect of water transport during perfusion on $C_{out}$ was corrected using the concentration ratio of a non-absorbable marker (FD-4). $r$ and $l$ represent the radius and length of the used segment of intestine, respectively; thus, the value of $2\pi rl$ corresponds to its surface area. As a radius of each intestinal segment, the value reported by Fagerholm et al. was used (0.18 cm for jejunum and ileum) (Fagerholm et

al., 1997).

**Preparation of intestinal Brush Border Membrane Vesicles (BBMV)**

BBMV were isolated from the upper jejunum and ileum of rats by the $Ca^{2+}$ precipitation technique as described previously (Minami et al., 1993; Moore et al., 1996; Katai et al., 1999). Male Wistar rats (body weight, 200-250 g) fasted overnight were anesthetized with pentobarbital and euthanized by cervical dislocation and complete blood removal. The abdominal cavity was opened and the entire small intestine was removed and thoroughly flushed with ice-cold saline. The two regions (upper jejunum and ileum, length: 10 cm) were placed on a glass plate maintained at 4˚C and cut open; mucosa was harvested by scraping with a microscope glass slide. All subsequent procedures were performed at 4˚C. The mucosal scrapings were added to 10 mL of buffer A (10 mM Tris-HCl [pH 7.4], 50 mM mannitol, 5 mM EDTA·2Na) and centrifuged at 500 × g for 10 min. The supernatant was collected and then adjusted to 10 mM $CaCl_2$ by the addition of 100 µL of 1.0 M $CaCl_2$ with constant stirring. After stirring for 15 min at 4˚C, the solution was centrifuged at 3000 × g for 17 min to remove precipitated cellular material and organelles. The supernatant, which contained mainly brush border membranes, was then centrifuged at 35000 × g for 30 min at 4˚C. The pellet (BBM fractions) was then lysed with 100 µL of buffer L (50 mM Tris-HCl [pH 7.4], 1% Triton X-100, 1 mM DTT, protease inhibitor cocktail [Complete Mini]) and 100 µL sample buffer (62.5 mM Tris-HCl, 25% glycerol, 2% SDS, 0.01% bromophenol blue, pH 6.8) for Western blot analysis.

**Western Blot Analysis**

Briefly, BBM lysates were adjusted to a final protein concentration of 10 mg/mL in sample buffer with 5% 2-mercaptoethanol, and were loaded at a volume of 10 μL (corresponding to 100 μg of protein) on Readygels J consisting of 7.5% resolving gels with Tris-HCl. After SDS-polyacrylamide gel electrophoresis, the proteins were transferred electrophoretically onto a 0.2 μm pore polyvinylidene difluoride membrane. Blots were blocked overnight at 4˚C with Tris-buffered saline/Tween 20 (0.15 M NaCl, 0.05% Tween 20 and 20 mM Tris-HCl, pH 7.5) containing 5 mM sodium azid and 5% (w/v) skim milk, and then incubated with anti-P-glycoprotein (C219) and anti-GAPDH (6C5) for 2 h at 37˚C. Anti-mouse IgG (H&L), HRP-linked, was used as a secondary antibody. Detection was made with a Lumi GLO Chemiluminescent Substrate Kit to activate the horseradish peroxidase signal. Blots were then exposed to a cold camera imaging system (Lumino Imaging Analyzer, FAS-1000, TOYOBO Co., Ltd., Osaka, Japan). The protein level of P-gp was quantified by computer image analysis with TOYOBO image software (Gel-Pro Analyzer® Version 3.1, TOYOBO Co., Ltd., Osaka, Japan) as described previously (Shirasaka et al., 2008). Then, it was further normalized to the P-gp level per intestinal surface area (μg/cm$^2$) by using total protein amounts in the BBM fraction on 10 cm for the small intestine in each region.

**Kinetic Analysis**

In a previous report, concentration-dependent permeability of P-gp substrate drugs

DMD Fast Forward. Published on February 14, 2008 as DOI: 10.1124/dmd.107.020040
This article has not been copyedited and formatted. The final version may differ from this version.



to the cell monolayer was kinetically analyzed according to the following equation, assuming that AP to BL flux ($V_{AB}$) can be expressed as the difference between passive ($V_{PD}$) and P-gp-mediated flux ($V_{P\text{-}gp}$) (Shirasaka et al., 2008).

$$\begin{aligned} V_{AB} &= V_{PD} - V_{P-gp} \\ &= CL_{PD} \cdot C_a - \frac{V_{max} \cdot C_a{}^r}{K_{m\,(app)}{}^r + C_a{}^r} \\ &= P_{app,\,PD} \cdot S \cdot C_a - \frac{V_{max} \cdot C_a{}^r}{K_{m\,(app)}{}^r + C_a{}^r} \end{aligned} \qquad (2)$$

where $CL_{PD}$, and $P_{app,PD}$ are the permeation clearance and membrane permeability by passive diffusion, respectively, and $S$ is the surface area of the membrane. $C_a$ represents the drug concentration in the apical solution, and the Hill coefficient ($r$) was introduced to the equation to add flexibility to the fitted curve. P-gp-mediated efflux was defined by two parameters, maximal velocity of P-gp-mediated efflux ($V$max) and $K$m(app).

In the present study, based on Eq. (2), the concentration-dependent permeability of three drugs (quinidine, verapamil and vinblastine) in rat small intestine was simulated from both *in vitro* and *in situ* data. Because the $r$ values for all drugs were close to 1.0 in all cell monolayers in our previous study, the $r$ value was fixed to 1.0 in all simulations. In addition, kinetic parameters for the concentration-dependent permeability of three drugs in rat intestinal membrane, $V_{max}$ and $K_{m(app),}$ were obtained by fitting the experimental data of *in situ* single-pass perfusion to the Eq. (2) (the $r$ value was fixed to 1.0) by the nonlinear least-squares method using the MULTI program (Yamaoka K et al., 1981).

**Analytical Methods**

The concentration of drugs in perfusate samples was analyzed with the reversed-phase HPLC system (LC-20AD Shimadzu Co., Kyoto, Japan) equipped with LCMS detector (LCMS-2010A, Shimadzu Co., Kyoto, Japan). Mercury MS (Luna 5μ C18, 10 × 4.0 mm, Phenomenex, CA) was used as an analytical column, and the mobile phase was composed of 0.1% formic acid in water and acetonitrile. Selected ion monitoring was used to detect protonated molecules of quinidine (m/z 325.10 [+]), verapamil (m/z 455.25 [+]), and vinblastine (m/z 811.60 [+]). In some perfusate samples, the concentration of drugs was analyzed with the reversed-phase HPLC system (LC-10AT Shimadzu Co., Kyoto, Japan) equipped with a variable wavelength ultraviolet detector (SPD-10AV, Shimadzu Co., Kyoto, Japan). The column (J'sphere ODS-H80 75 mm × 4.6 mm, YMC, Japan) was used with a mobile phase consisting of 50 mM phosphate buffer (pH 2.5) and acetonitrile. Quinidine and verapamil were quantified at wavelengths of 230 and 235 nm, respectively.

For the measurement of total protein, the absorbance of the cell suspension was photometrically measured at a wavelength of 562 nm by UV-visible spectrophotometer (UV-1650PC, Shimazu Company, Kyoto, Japan).

## Results

### Expression Level of P-gp in Rat Intestinal Membrane

In a previous report, concentration-dependent permeability of P-gp substrate drugs, quinidine, verapamil and vinblastine, in several cell monolayers with different levels of P-gp expression was kinetically analyzed according to Eq (2), assuming that AP to BL flux ($V_{AB}$) can be expressed as the difference between passive ($V_{PD}$) and P-gp-mediated flux ($V_{P\text{-}gp}$) (Shirasaka et al., 2008). In Eq. (2), *K*m(app) value represents the donor concentration of the drug at which the decreased permeability by P-gp-mediated efflux (*P*app(max) - *P*app(min)) became half of its maximal value (Figs. 1A and B); therefore, *K*m(app) value could be regarded as the apparent affinity of drugs to P-gp. Based on Eq. (2), kinetic parameters for the concentration-dependent permeability of all three drugs, $V_{max}$ and $K_{m(app)}$ values, were obtained (Shirasaka et al., 2008). It was demonstrated that both $V_{max}$ and $K_{m(app)}$ values of each drug are the proportional function of P-gp expression level (Figs. 2A and B). Since $K_{m(app)}$ value was defined based on the apical concentration of drugs, this parameter can be regarded as a hybrid parameter of the real affinity of drugs to P-gp ($K_m$) and the ratio of drug concentration in the apical solution ($C_a$) and in the vicinity of the drug binding site of P-gp ($C_{bind}$). If P-gp activity is high enough, the substrate drug that diffused to the vicinity of the drug binding site of P-gp from the apical solution would be pumped out efficiently, and thus $C_{bind}$ is kept low even when the $C_a$ becomes high. Therefore, in cells that highly express P-gp, higher $C_a$ is required to saturate P-gp, and then $K_{m(app)}$ values increase depending on the P-gp expression level.

DMD Fast Forward. Published on February 14, 2008 as DOI: 10.1124/dmd.107.020040
This article has not been copyedited and formatted. The final version may differ from this version.



These findings imply the possibility of estimating the $V_{max}$ and $K_{m(app)}$ values of P-gp substrate drugs in the *in vivo* intestinal membrane from its P-gp expression level. In order to simulate the concentration-dependent permeability of P-gp substrate drugs in the rat small intestine, P-gp expression levels in the rat small intestine (upper jejunum and ileum) were first determined by Western blot analysis (Fig. 3A). P-gp expression levels in the jejunum and ileum quantified by computer image analysis were 77.7 ± 6.9 and 231.5 ± 36.1 μg/cm$^2$ (mean ± SE), respectively, indicating that P-gp expression level in the ileum was approximately 3-fold higher than that in the jejunum.

**Estimation of $V_{max}$ and $K_{m(app)}$ Values of P-gp Substrate Drugs in Rat Intestinal Membrane**

Quinidine was used as an example to explain the process of estimating $V_{max}$ and $K_{m(app)}$ values in the rat intestinal membrane (Figs. 4A and B, and Table 1).

Expression levels of P-gp in the rat small intestine were incorporated in *in vitro* relation between the $V_{max}$ value and P-gp expression level ($Y = 3.61\times10^{-8} X$) (Fig. 4A). Then, $V_{max}$ values of quinidine in rat small intestines were obtained as $0.280\times10^{-5}$ and $0.835\times10^{-5}$ μmol/min/cm$^2$ in the jejunum and ileum, respectively, and the results are summarized in Table 2, where the $V_{max}$ value is expressed as a unit of μmol/min/10 cm gut.

In the case of the $K_{m(app)}$ value, passive diffusivity of drugs to the vicinity of the drug binding site of P-gp affects the relation between the $K_{m(app)}$ and P-gp expression levels because $K_{m(app)}$ is a hybrid parameter of real affinity ($K_m$) and the diffusive

DMD Fast Forward. Published on February 14, 2008 as DOI: 10.1124/dmd.107.020040
This article has not been copyedited and formatted. The final version may differ from this version.



process to the drug binding site of P-gp. In order to observe differences in diffusivity between cell monolayers and the rat small intestine, passive permeability of drugs to rat small intestines were measured by adding P-gp inhibitor (alprenolol) to the perfusion medium. In Table 1, passive permeability ($P_{app,PD}$) of quinidine to rat jejunum and ileum was compared with that to Caco-2 monolayers. Quinidine showed 3.5-4.0 times higher $P_{app,PD}$ to rat small intestines than to Caco-2 monolayers. In the process of estimating the $K_{m(app)}$ value in rat intestines from the relation between $K_{m(app)}$ in cell monolayers and P-gp expression level, the difference in passive permeability was used as the scaling factor by dividing the slope in Fig. 2B by the ratio of $P_{app,PD}$ ($P_{app,PD(rat)}/P_{app,PD(Caco-2)}$) (Fig. 4B). This process of *in vitro-in vivo* scaling is reasonable because, for example, 4-times higher diffusivity has the same effect on $C_{bind}$ with the condition that $C_a$ increased 4 times (if diffusivity is the same). In Fig. 4B ($Y = 1.21\times10^{-2} X + 0.584$ in jejunum and $Y = 1.10\times10^{-2} X + 0.584$ in ileum), $K_{m(app)}$ values of quinidine in the rat jejunum and ileum were estimated as 1.53 and 3.13 μM, respectively (Table 2).

For verapamil and vinblastine, the same procedures were used to estimate $V_{max}$ and $K_{m(app)}$ values in rat intestines, and the results are summarized in Table 2.

**Simulation of the Concentration-Dependent Permeability of P-gp Substrate Drugs in Rat Intestinal Membrane**

By incorporating $V_{max}$ and $K_{m(app)}$ values of quinidine estimated in Figs. 4A and B as well as $P_{app,PD}$ measured by single-pass perfusion method in Table 1 to Eq. (2), the concentration-dependent permeability of quinidine in the rat jejunum and ileum was

DMD Fast Forward. Published on February 14, 2008 as DOI: 10.1124/dmd.107.020040
This article has not been copyedited and formatted. The final version may differ from this version.



simulated (Figs. 5A and B, solid line). In order to validate the accuracy of the simulation, the permeability of quinidine in rat small intestines was measured by *in situ* single-pass perfusion methods with different luminal concentrations (Figs. 5A and B, open squares). As shown in Figs. 5A and B, simulated permeability in the rat jejunum and ileum coincided well with the permeability measured by *in situ* single-pass perfusion method. In Table 2, predicted and experimentally obtained parameters, $V_{max}$ and $K_{m(app)}$ values, are summarized for P-gp-mediated transport of quinidine. These parameters were found to differ slightly between predicted and experimental values.

The same procedures were used to simulate the concentration-dependent permeability of verapamil and vinblastine in rat small intestines (Fig. 5C-F and Table 2). It was confirmed that the simulated permeability of verapamil and vinblastine in the rat jejunum and ileum also coincided well with the permeability obtained by *in situ* single-pass perfusion method. The permeability of vinblastine showed strong dependency on its luminal concentration while that of verapamil was rather insensitive to the change in the concentration (Fig. 5C-F).

**Discussion**

In a previous report, we have proposed a new method to simulate the concentration-dependent permeability of P-gp substrate drugs in the human intestine from *in vitro* data of cultured cell monolayers such as Caco-2 cells (Shirasaka et al., 2008). In this study, the appropriateness of our method was assessed by simulating the permeability of three P-gp substrates to the rat small intestine. Although it may come under intense scrutiny that permeability of P-gp substrates to the rat small intestine was predicted from *in vitro* study using cell lines expressing human P-gp, several reports have shown the good correlation between the functional activity of human MDR1 and that of rat mdr1a/1b. Takeuchi *et al.* established the LLC-PK1 cell lines which stably express various MDR1 (derived from human, monkey, canine, rat, and mouse) to investigate species differences in P-gp-mediated efflux activity (Takeuchi et al., 2006). When the permeability ratios (BL to AP/AP to BL) of P-gp substrate drugs were compared among these cell lines, a good correlation was observed between human and rat MDR1 (both MDR1a and 1b). Particularly, three P-gp substrates, quinidine, verapamil and vinblastine, which were used in this study, contributed to a good correlation. By using the series of same cell lines, Katoh *et al.* examined the species differences in maximal activity ($V_{max}/K_m$) of P-gp to transport its substrate drugs and clarified that maximal activity of rat MDR1b correlated well with that of human MDR1 (Katoh et al., 2006). In addition, apparent $K_m$ value of quinidine to rat MDR1a was reported to be approximately 5 μM (Müller et al., 1994) and corresponded well with that to human MDR1 in other reports (5.42 μM by Adachi et al., 2001; 7.88 μM by

Shirasaka et al., 2006). Based on these facts, it is possible to validate our method by simulating the concentration-dependent permeability of three P-gp substrate drugs to rat small intestine and, then by comparing the results of simulation with the experimentally measured permeability of those drugs.

The expression level of P-gp in the rat ileum was revealed to be approximately 3-fold higher than that in the jejunum (Figs. 3A and B), consisting with recent reports. Cao *et al.* reported that P-gp expression gradually increased along the rat GI tract from the duodenum to the colon, and was 2.7-fold higher in the ileum than in the jejunum (Cao et al., 2005). Tian *et al.* also clarified that P-gp expression in the ileum was 2.31-fold higher than that in the jejunum (Tian et al., 2002).

By incorporating the expression level of P-gp in the rat jejunum and ileum into the *in vitro* correlation lines in Fig. 2, both $V_{max}$ and $K_{m(app)}$ values of three P-gp substrates in rat small intestines were estimated. It has already been demonstrated that the $K_{m(app)}$ value increases depending on the P-gp expression level because the degree of P-gp activity affects the $C_{bind}$ (Shirasaka et al., 2008). In addition, it is reasonable to consider that the $K_{m(app)}$ value decreases with increasing $P_{app,PD}$ because the passive diffusivity of drugs to the vicinity of the drug binding site of P-gp affects the $C_{bind}$, thus affecting the relation between $K_{m(app)}$ and P-gp expression levels. In this study, to estimate the $K_{m(app)}$ value in rat small intestines, differences in the diffusivity between cell monolayers and rat small intestinal epithelium should be taken into account. In order to estimate the $K_{m(app)}$ value in rat small intestines, the relation to the P-gp expression level in Fig. 2B was corrected by dividing the slope by the ratio of passive permeability

DMD Fast Forward. Published on February 14, 2008 as DOI: 10.1124/dmd.107.020040
This article has not been copyedited and formatted. The final version may differ from this version.



($P_{app,PD(rat)}/P_{app,PD(Caco-2)}$) in Table 1 (Fig. 4B). Although the differences in the lipid composition of the cellular membrane between Caco-2 cells and rat enterocytes are not clear, it is reasonable to consider that these epithelial membranes showed almost the same passive diffusivity to small and lipophilic molecules such as the drugs used in this study; therefore, the differences in passive permeability between Caco-2 cell monolayers and rat small intestines shown in Table 1 might correspond to differences in the effective surface area of drug diffusion, caused by villous structures of the intestinal membrane. Our method to correct of the relation between P-gp expression levels and $K_{m(app)}$ by the passive permeability of drugs enables the comparison of drug movement in the same area of the membrane (per cell).

By incorporating these parameters of rat intestinal P-gp flux and measured $P_{app,PD}$ into Eq. (2), the concentration-dependent permeability of three drugs in the rat jejunum and ileum was simulated (Fig. 5A-F, solid line). As a consequence, it was confirmed that the simulated permeability of all three drugs in the rat jejunum and ileum corresponded well with permeability measured by *in situ* single-pass perfusion method, suggesting that our procedures to simulate the permeability of P-gp substrate drugs *in vivo* were successful (Fig. 5A-F, open squares); however, in the ileum, simulated permeability of quinidine in the lower concentration range deviated slightly from the measured permeability. One of the plausible explanations for this inconsistency is that, because several CYP enzymes were reported to be expressed in the intestinal epithelial layer, CYP-mediated metabolism of drugs during the intestinal absorption process might affect the permeability measured by *in situ* single-pass perfusion method (Ching et al.,

1995; Tracy et al., 1999; Galetin and Houston, 2006). In addition, the effects of other ABC transporters, such as BCRP and MRP2, are possible factors in the deviation (Englund et al., 2006; Han and Sugiyama, 2006). Han *et al.* revealed that the expression level of BCRP in the ileum was about twice as high as that in the duodenum and jejunum (Han and Sugiyama, 2006).

The present study indicated that the effect of P-gp on intestinal absorption of quinidine and vinblastine was significantly greater in the ileum than in the jejunum. This fact implies that regional differences in the expression level of P-gp affects the intestinal absorption of P-gp substrate drugs. However, in the case of verapamil, the impact of P-gp on its absorption in both jejunum and ileum was almost negligible. This might be reasoned by its high permeability (high $P_{app,PD}$), high affinity to P-gp (low $K_m$), and/or low $V_{max}$ compared with other drugs, as shown in Fig. 2B (Borgnia et al., 1996; Adachi et al., 2001; Troutman and Thakker, 2003; Shirasaka et al., 2006). Ogihara *et al.* investigated the influence of P-gp on the intestinal absorption of verapamil in each intestinal segment in wild-type and mdr1a/1b gene-deficient mice, whereas no significant effects of P-gp on the absorption of verapamil were observed in all intestinal segments (Ogihara et al., 2006). These results also suggested that the effect of P-gp on *in vivo* intestinal absorption of its substrate drugs varied widely among drugs.

In this study, rat intestinal permeability of P-gp substrate drugs were simulated from both *in vitro* and *in situ* data. Since the ratios of passive permeability ($P_{app,PD(rat)}/P_{app,PD(Caco-2)}$) in Table 1 were considerably tight range (3.59-14.3), it might be possible to evaluate $P_{app,PD(rat)}$ only from the *in vitro* study, if the relation of passive

permeability in rat intestine and in Caco-2 monolayers is analyzed precisely. This is the issue of future project to develop a method for simulating the concentration-dependent permeability of P-gp substrate drugs to animal and also to human small intestine by knowing only the intestinal P-gp expression levels.

In conclusion, in this study, the concentration-dependent permeability of P-gp substrate drugs to the intestinal membrane was successfully simulated from the *in vitro* study with a cell culture system. This method enables quantitative evaluation of the effect of P-gp on the *in vivo* oral absorption of its substrate drugs. Regional differences in the intestinal absorption of P-gp substrate drugs are possible to be simulated from the differences in P-gp expression levels. Since the expression level of P-gp in the human intestinal membrane can be quantified by using intestinal tissue samples obtained by biopsy, in the near future, a study of human intestine will be undertaken and presented in the next report.

**References**

Adachi Y, Suzuki H, and Sugiyama Y (2001) Comparative studies on in vitro methods for evaluating in vivo function of MDR1 P-glycoprotein. *Pharm Res* **18**:1660-1668.

Borgnia MJ, Eytan GD., and Assaraf YG (1996) Competition of hydrophobic peptides, cytotoxic drugs, and chemosensitizers on a common P-glycoprotein pharmacophore as revealed by its ATPase activity. *J Biol Chem* **271**:3163-3171.

Cao X, Yu LX, Barbaciru C, Landowski CP, Shin HC, Gibbs S, Miller HA, Amidon GL, and Sun D (2005) Permeability dominates in vivo intestinal absorption of P-gp substrate with high solubility and high permeability. *Mol Pharm* **2**:329-340.

Ching MS, Blake CL, Ghabrial H, Ellis SW, Lennard MS, Tucker GT, and Smallwood RA (1995) Potent inhibition of yeast-expressed CYP2D6 by dihydroquinidine, quinidine, and its metabolites. *Biochem Pharmacol* **50**:833-837.

Englund G., Rorsman F, Ronnblom A, Karlbom U, Lazorova L, Grasjo J, Kindmark A, and Artursson P (2006) Regional levels of drug transporters along the human intestinal tract: co-expression of ABC and SLC transporters and comparison with Caco-2 cells. *Eur J Pharm Sci* **29**:269-277.

Fagerholm U, Lindahl A, and Lennernäs H (1997) Regional intestinal permeability in rats of compounds with different physicochemical properties and transport mechanisms. *J Pharm Pharmacol* **49**:687-690.

Galetin A and Houston JB (2006) Intestinal and hepatic metabolic activity of five cytochrome P450 enzymes: impact on prediction of first-pass metabolism. *J Pharmacol Exp Ther* **318**:1220-1229.

Han Y and Sugiyama Y (2006) Expression and regulation of breast cancer resistance protein and multidrug resistance associated protein 2 in BALB/c mice. *Biol Pharm Bull* **29**:1032-1035.

Hunter J and Hirst BH (1997) Intestinal secretion of drugs: The role of P-glycoprotein and related drug efflux systems in limiting oral drug absorption. *Adv Drug Del Rev* **25**:129-157.

Jonker JW, Wagenaar E, van Deemter L, Gottschlich R, Bender HM, Dasenbrock J, and Schinkel AH (1999) Role of blood-brain barrier P-glycoprotein in limiting brain accumulation and sedative side-effects of asimadoline, a peripherally acting analgaesic drug. *Br J Pharmacol* **127**:43-50.

Katai K, Miyamoto K, Kishida S, Segawa H, Nii T, Tanaka H, Tani Y, Arai H, Tatsumi S, Morita K, Taketani Y, and Takeda E (1999) Regulation of intestinal Na+-dependent phosphate co-transporters by a low-phosphate diet and 1,25-dihydroxyvitamin D3. *Biochem J* **343**:705-712.

Katoh M, Suzuyama N, Takeuchi T, Yoshitomi S, Asahi S, and Yokoi T (2006) Kinetic analyses for species differences in P-glycoprotein-mediated drug transport. *J Pharm Sci* **95**:2673-2683.

Kim RB, Fromm MF, Wandel C, Leake B, Wood AJ, Roden DM, and Wilkinson GR (1998) The drug transporter P-glycoprotein limits oral absorption and brain entry of HIV-1 protease inhibitors. *J Clin Invest* **101**:289-294.

Loo TW and Clarke DM (1999) Molecular dissection of the human multidrug resistance P-glycoprotein. *Biochem Cell Biol* **77**:11-23.

Meijer DK, Smit JW, and Muller M (1997) Hepatobiliary elimination of cationic drugs: the role of P-glycoprotein and other ATP-dependent transporters. *Adv Drug Del Rev* **25**:159-200.

Minami H, Kim JR, Tada K, Takahashi F, Miyamoto K, Nakabou Y, Sakai K, and Hagihira H (1993) Inhibition of glucose absorption by phlorizin affects intestinal functions in rats. *Gastroenterology* **105**:692-697.

Moore AC, Gugger ET, and Erdman JW (1996) Brush border membrane vesicles from rats and gerbils can be utilized to evaluate the intestinal uptake of all-trans and 9-cis beta-carotene. *J Nutr* **126**:2904-2912.

Müller M, Mayer R, Hero U, Keppler D (1994) ATP-dependent transport of amphiphilic cations across the hepatocyte canalicular membrane mediated by mdr1 P-glycoprotein. *FEBS Lett* 343:168-172.

Ogihara T, Kamiya M, Ozawa M, Fujita T, Yamamoto A, Yamashita S, Ohnishi S, and Isomura Y (2006) What kinds of substrates show P-glycoprotein- dependent intestinal absorption? Comparison of verapamil with vinblastine. *Drug Metab Pharmacokinet* **21**:238-244.

Polli JW, Jarrett JL, Studenberg SD, Humphreys JE, Dennis SW, Brouwer KR, and Woolley JL (1999) Role of P-glycoprotein on the CNS disposition of amprenavir (141W94), an HIV protease inhibitor. *Pharm Res* **16**:1206-1212.

Polli JW, Wring SA, Humphreys JE, Huang L, Morgan JB, Webster LO, and Serabjit-Singh CS (2001) Rational use of in vitro P-glycoprotein assays in drug discovery. *J Pharmacol Exp Ther* **299**:620-628.

Shirasaka Y, Onishi Y, Sakurai A, Nakagawa H, Ishikawa T, and Yamashita S (2006) Evaluation of human P-glycoprotein (MDR1/ABCB1) ATPase activity assay method by comparing with in vitro transport measurements: Michaelis-Menten kinetic analysis to estimate the affinity of P-glycoprotein to drugs. *Biol Pharm Bull* **29**:2465-2471.

Shirasaka Y, Kawasaki M, Sakane T, Omatsu H, Moriya Y, Nakamura T, Sakaeda T, Okumura K, Langguth P, and Yamashita S (2006) Induction of Human P-Glycoprotein in Caco-2 cells: Development of a Highly Sensitive Assay System for P-Glycoprotein-Mediated Drug Transport. *Drug Metab Pharmacokinet* **21**:414-423.

Shirasaka Y, Sakane T, and Yamashita S (2008) Effect of P-glycoprotein expression levels on the concentration-dependent permeability of drugs to the cell membrane. *J Pharm Sci* **97**:553-565.

Susanto M and Benet LZ (2002) Can the enhanced renal clearance of antibiotics in cystic fibrosis patients be explained by P-glycoprotein transport? *Pharm Res* **19**:457-462.

Takeuchi T, Yoshitomi S, Higuchi T, Ikemoto K, Niwa S, Ebihara T, Katoh M, Yokoi T, and Asahi S (2006) Establishment and characterization of the transformants stably-expressing MDR1 derived from various animal species in LLC-PK1. *Pharm Res* **23**:1460-1472.

Tian R, Koyabu N, Takanaga H, Matsuo H, Ohtani H, and Sawada Y (2002) Effects of grapefruit juice and orange juice on the intestinal efflux of P-glycoprotein

substrates. *Pharm Res* **19**:802-809.

Tracy TS, Korzekwa KR, Gonzalez FJ, and Wainer IW (1999) Cytochrome P450 isoforms involved in metabolism of the enantiomers of verapamil and norverapamil. *Br J Clin Pharmacol* **47**:545-552.

Troutman MD and Thakker DR (2003) Novel experimental parameters to quantify the modulation of absorptive and secretory transport of compounds by P-glycoprotein in cell culture models of intestinal epithelium. *Pharm Res* **20**:1210-1224.

Yamaoka K, Tanigawara Y, Nakagawa T, and Uno T (1981) A pharmacokinetic analysis program (multi) for microcomputer. *J Pharmacobiodyn* **4**:879-885.

DMD Fast Forward. Published on February 14, 2008 as DOI: 10.1124/dmd.107.020040
This article has not been copyedited and formatted. The final version may differ from this version.



**Legends for figures**

**Figure 1.** Kinetic model of AP to BL transport of P-gp substrate. (A) AP to BL flux of drugs can be determined by subtraction of P-gp-mediated transport, $V_{P\text{-}gp}$, from passive transport, $V_{PD}$. (B) $K_{m(app)}$ value represents the donor concentration of the drug at which the decreased permeability by P-gp-mediated efflux ($P_{app(max)} - P_{app(min)}$) became half of its maximal value. Scheme was reproduced with permission from the publishing journal and authors; Shirasaka, et al., *J Pharm Sci*. 97:553-565 (2008).

**Figure 2.** Correlation between P-gp expression level and kinetic parameters ($V_{max}$ and $K_{m(app)}$ values) of three P-gp substrates, quinidine, verapamil and vinblastine. P-gp expression level was quantified by Western blot analysis. (A) Correlation between $V_{max}$ value and P-gp expression level. (B) Correlation between $K_{m(app)}$ value and P-gp expression level. Filled triangles, quinidine; filled squares, verapamil; filled circles, vinblastine. Data were derived with permission from the publishing journal and authors; Shirasaka, et al., *J Pharm Sci.* 97:553-565 (2008).

**Figure 3.** Western blot analysis of P-gp in rat small intestines (jejunum and ileum). Western blotting was performed in loading 100 μg of BBM lysates prepared from rat small intestines (lane 1, the molecular mass of standards; lane 2, blank; lane 3, 5, and 7, jejunum; lane 4, 6, and 8, ileum). P-gp and GAPDH was detected using monoclonal anti-P-gp (C219) and monoclonal anti-GAPDH (6C5), respectively. Expression levels of P-gp were quantified using computer image analysis.

**Figure 4.** Estimation of $V_{max}$ and $K_{m(app)}$ values of quinidine in rat small intestines (jejunum and ileum). Expression level of P-gp in rat small intestines (77.7 μg/cm$^2$ for jejunum and 231.5 μg/cm$^2$ for ileum) was incorporated into the *in vitro* relationship between $V_{max}$ and P-gp expression levels (A), and the *in vitro* relationship between $K_{m(app)}$ and P-gp expression levels (B).

**Figure 5.** Simulation of concentration-dependent permeability of three P-gp substrates, quinidine, verapamil and vinblastine, in rat small intestines (jejunum and ileum). $P_{app}$ of quinidine (A and B), verapamil (C and D) and vinblastine (E and F) to rat small intestines in two regions (A, C and E, jejunum; B, D and F, ileum) was simulated by using estimated $K_{m(app)}$ and $V_{max}$ values and measured $P_{app,PD}$ (solid line). Experimental $P_{app}$ to rat small intestines was obtained by *in situ* single-pass perfusion method (open squares). Data are represented as the means ± SE (n = 3).

**Table 1. Passive permeability ($P_{app,PD}$) of quinidine, verapamil and vinblastine in Caco-2 monolayers and rat small intestine (jejunum and ileum).**

| | | Cell monolayers | Rat small intestine | |
|---|---|---|---|---|
| | | | Jejunum | Ileum |
| Quinidine | $P_{app,PD}$ (×10$^{-5}$ cm/sec) | 1.41 ± 0.03 | 5.06 ± 0.27 | 5.60 ± 0.75 |
| | Ratio (rat/Caco-2) | _ | 3.59 | 3.97 |
| Verapamil | $P_{app,PD}$ (×10$^{-5}$ cm/sec) | 1.37 ± 0.03 | 8.76 ± 1.85 | 10.6 ± 0.71 |
| | Ratio (rat/Caco-2) | _ | 6.39 | 7.74 |
| Vinblastine | $P_{app,PD}$ (×10$^{-5}$ cm/sec) | 0.56 ± 0.01 | 5.04 ± 1.36 | 8.02 ± 1.13 |
| | Ratio (rat/Caco-2) | _ | 8.98 | 14.3 |

$P_{app,PD}$ to rat small intestines was measured by *in situ* single-pass perfusion method with P-gp inhibitor (alprenolol) to the perfusion medium. Data are represented as the means ± SE (n = 3). Ratio (rat/Caco-2) represents the ratio of passive permeability in Caco-2 monolayers ($P_{app,PD(Caco-2)}$) and in rat small intestines ($P_{app,PD(rat)}$), i.e. $P_{app,PD(rat)}/P_{app,PD(Caco-2)}$.

**Table 2 Comparison between predicted and experimental kinetic parameters ($K_{m(app)}$、$V_{max}$) for P-gp-mediated transport of quinidine, verapamil and vinblastine.**

| | | Jejunum | | | Ileum | | |
|---|---|---|---|---|---|---|---|
| | | $V_{max}$ ($\times 10^{-5}$ μmol/min/10cm gut) | $K_{m(app)}$ (μM) | $V_{max}/K_{m(app)}$ ($\times 10^{-5}$ L/min /10cm gut) | $V_{max}$ ($\times 10^{-5}$ μmol/min/10cm gut) | $K_{m(app)}$ (μM) | $V_{max}/K_{m(app)}$ ($\times 10^{-5}$ L/min /10cm gut) |
| Quinidine | Predicted | 3.13 | 1.53 | 2.05 | 9.35 | 3.13 | 2.99 |
| | Experimental | 1.08 | 0.60 | 1.80 | 1.70 | 0.70 | 2.42 |
| Verapamil | Predicted | 0.33 | 1.05 | 0.31 | 0.98 | 1.15 | 0.85 |
| | Experimental | 0.69 | 0.87 | 0.80 | 1.50 | 0.82 | 1.83 |
| Vinblastine | Predicted | 51.3 | 33.6 | 1.53 | 153 | 45.1 | 3.39 |
| | Experimental | 38.6 | 23.1 | 1.67 | 111 | 32.9 | 3.37 |

$V_{max}$ and $K_{m(app)}$ values were expressed as units of μmol/min/10 cm gut and μM, respectively. Maximal activity ($V_{max}/K_{m(app)}$) for P-gp mediated-transport is expressed in units of L/min/10 cm gut.

**Figure 1.**

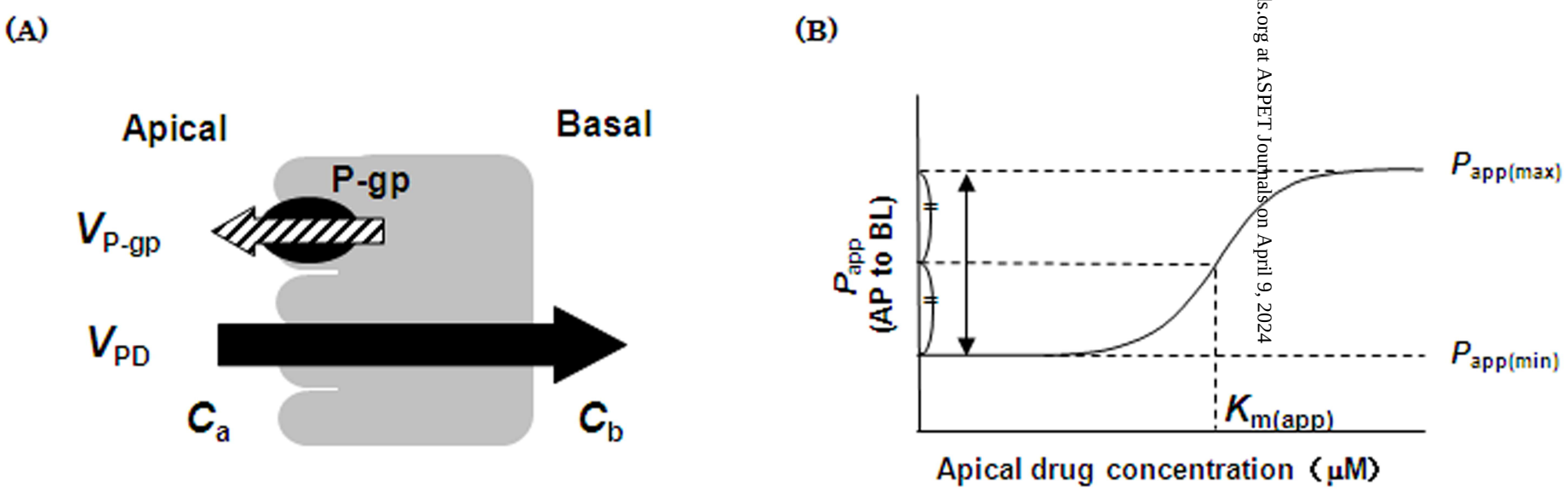
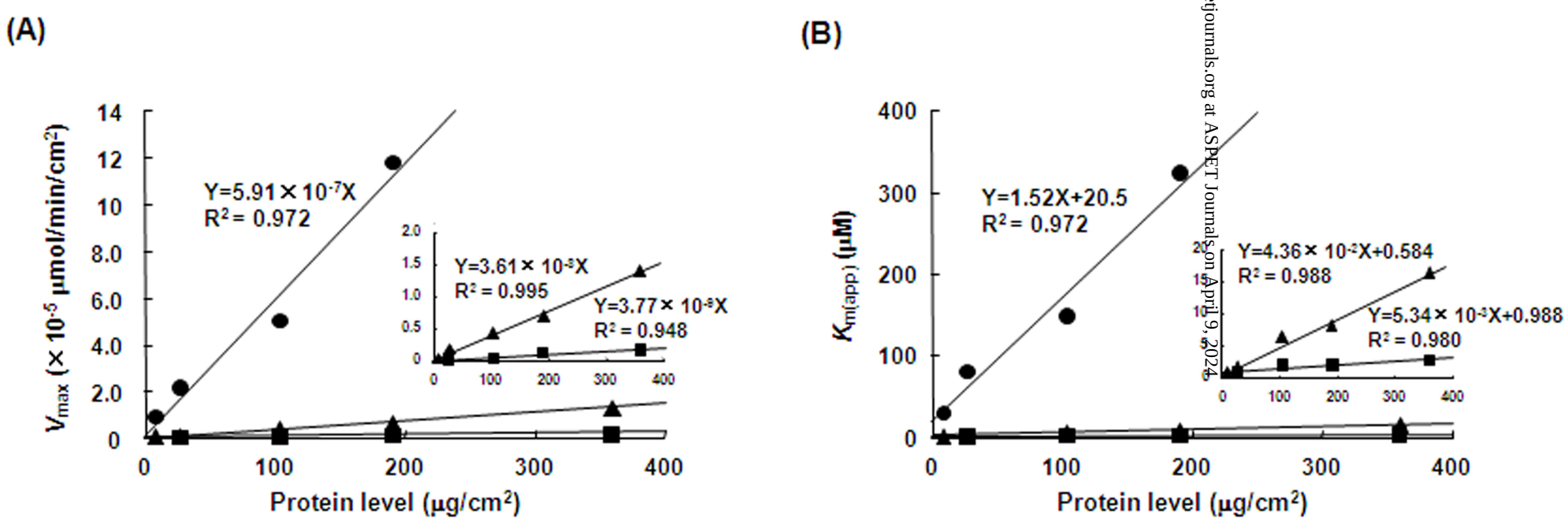


**Figure 2.**



# Figure 3.

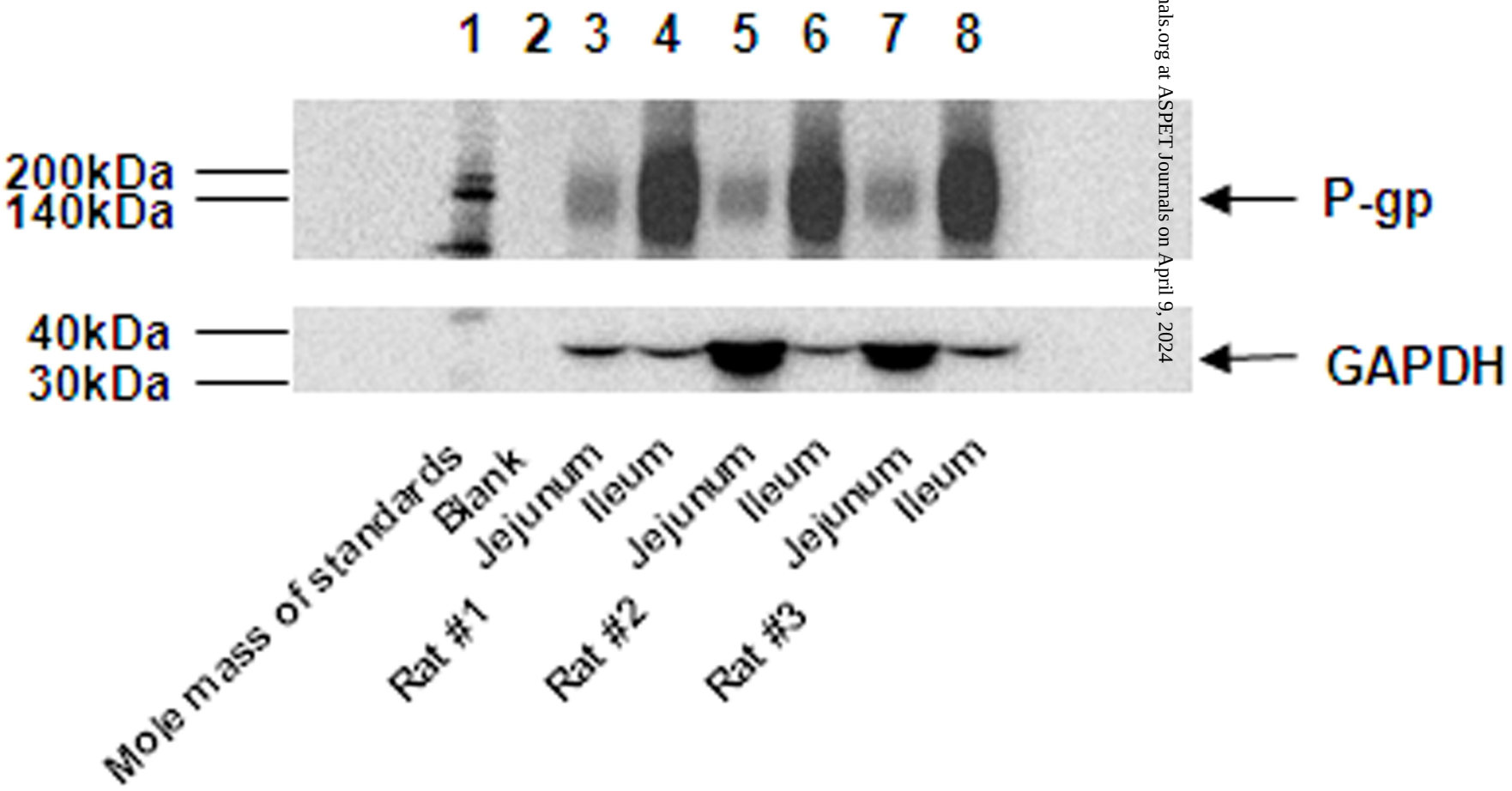
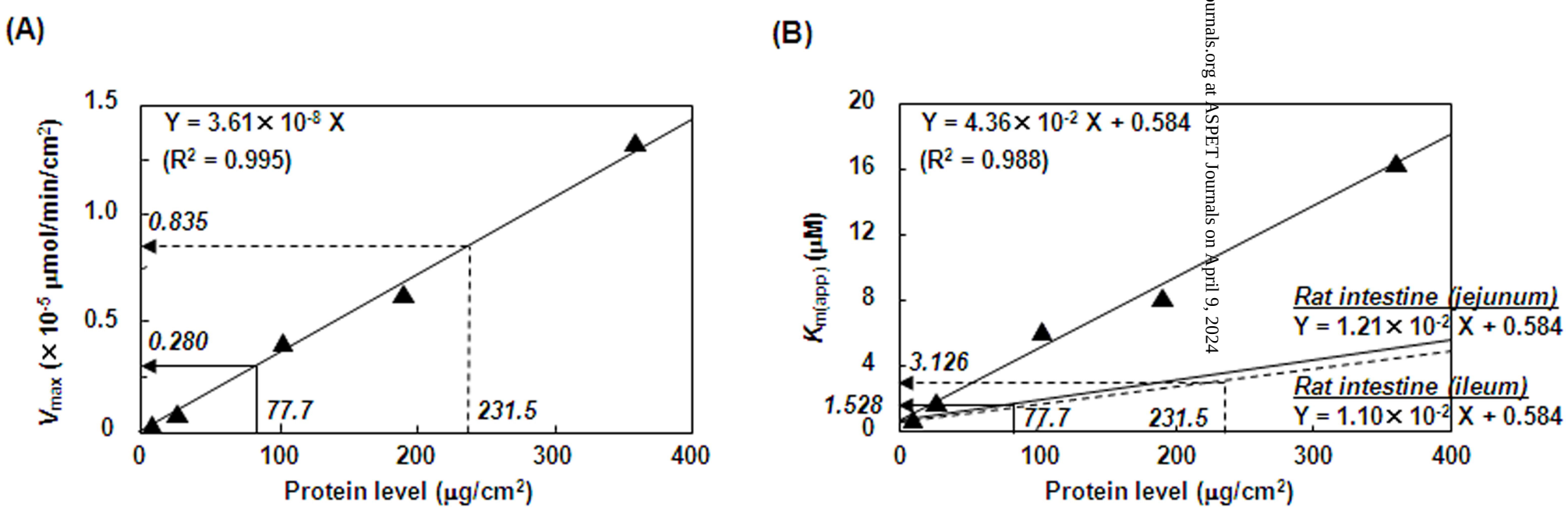


**Figure 4.**



DMD Fast Forward. Published on February 14, 2008 as DOI: 10.1124/dmd.107.020040
This article has not been copyedited and formatted. The final version may differ from this version.

**Figure 5.**