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What Kinds of Substrates Show P-Glycoprotein-Dependent Intestinal Absorption? Comparison of Verapamil with Vinblastine

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Summary: The influence of P-glycoprotein (P-gp) on intestinal absorption of drugs was investigated by comparison of the uptakes of two P-gp substrates, verapamil and vinblastine, using intestinal segments of wild-type and mdr1a/1b gene-deficient (mdr1a/1b$^{-/-}$) mice, and Caco-2 cells. When [^{3}H]vinblastine was injected into intestinal segments of wild-type mice, vinblastine was absorbed from duodenum and ileum, but not from jejunum. This difference among intestinal regions could not be explained by segmental differences of mdr1a mRNA expression. In Caco-2 cells, it was found that vinblastine had a high value of efflux/influx ratio (an index of affinity for P-gp) of 12.1, and a low permeability of less than 1×10^{-6} cm/sec. The corresponding values for verapamil were 4.9 and 10.6×10^{-6} cm/sec, respectively. After oral administration of [^{3}H]vinblastine to mice, the maximum concentration (C_{max}) and the area under the plasma concentration time-curve from time 0 to 24 hr (AUC_{0-24hr}) for mdr1a/1b$^{-/-}$ mice were 1.5 times greater than those for wild-type mice, while these parameters were not significantly different between the two strains in the case of [^{3}H]verapamil. Therefore, P-gp substrates may be classified into at least two types, *i.e.*, verapamil-type, for which the intestinal absorption is unaffected by P-gp, and vinblastine-type, for which the intestinal absorption is influenced by P-gp. Vinblastine-type P-gp substrates, with low permeability and high affinity for P-gp, would be unfavorable candidates for oral drugs.

Key words: P-glycoprotein; intestinal absorption; efflux transporter; vinblastine; verapamil

Introduction

The drug efflux transporter P-glycoprotein (P-gp, mdr1, ABCB1) is expressed not only in drug-resistant tumor cells, but also in many normal tissues with excretory functions, including the bile canalicular membrane of hepatocytes, the luminal membrane of endothelial cells in the blood-brain barrier, the epithelial apical membrane of the intestine, and renal proximal tubules. Thus, P-gp may play an important role in the barrier function of these tissues against xenobiotic drugs. Studies of clinical drug-drug interactions (DDI) involving P-gp have focused mainly on central nervous system (CNS) toxicities arising from the translocation of excessive amounts of P-gp substrates to the brain.[1)] Candidate drugs in development are often examined to see whether they are P-gp substrates or not, from the viewpoints of potential DDI, and drug distribution to the CNS. Moreover, DDI involving renal clearance *via* P-gp has been observed clinically.[2)] In contrast, there have been few clinical reports of serious DDI involving intestinal absorption. Further, the absorption rates of various P-gp substrates after oral administration are good in humans. For example, the absorption rates of digitoxin, quinidine, digoxin, nifedipine, amiodarone, propranolol, cyclosporine (Sandimmun^{TM}) and verapamil are 90, 80, 70, 50, 46, 26, 23 and 22%, respectively, according to the manufacturers' drug informa-

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Abbreviations used are: P-gp, P-glycoprotein; C_{max}, maximum concentration; AUC_{0-24hr}, area under the plasma concentration time-curve from time 0 to 24 hr; DDI, drug-drug interactions; CNS, central nervous system; SD, Sprague-Dawley; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; BCRP/bcrp, breast cancer resistance protein; MRP2/mrp2, multidrug resistance-associated protein 2; Hank's balanced salt solution, HBSS; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid.

tion sheets. Therefore, several researchers have suggested that the influence of P-gp on intestinal absorption of drugs is relatively limited.[3–7] However, other authors have found that P-gp substrates do show P-gp-dependent intestinal absorption.[8–11] Since P-gp substrates with low absorption would not have been successfully developed as oral drugs, it can not necessarily be assumed that P-gp has little effect on intestinal drug absorption, based on findings with clinically used drugs. In this study, we investigated the effect of P-gp on intestinal absorption of drugs by using FVB wild-type mice (mdr1a/1b$^{+/+}$), mdr1a/1b gene-deficient (mdr1a/1b$^{-/-}$) mice and Sprague-Dawley (SD) rats. Verapamil, which is administered orally in clinical use, and vinblastine, which is administered intravenously, were tested as typical substrates of P-gp. Moreover, we attempted to develop a biochemical classification procedure using Caco-2 cells, to predict which P-gp substrates are likely to show P-gp-dependent intestinal absorption.

Methods

Chemicals and animals: [^{3}H]Vinblastine (222 GBq/mmol) and [^{3}H]paclitaxel (236.8 GBq/mmol) were purchased from Moravek Biochemicals (Brea, CA). [^{3}H]Verapamil (3.15 TBq/mmol) and [^{3}H]mannitol (629 Gq/mmol) were purchased from PerkinElmer (Boston, MA). [^{14}C]Antipyrine (203.5 MBq/mmol) and [^{3}H]propranolol (1.07 TBq/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO) and Amersham (Buckinghamshire, UK), respectively. Fexofenadine was purchased from Toronto Research Chemicals (North York, On., Canada). All other chemicals and solvents were commercial products of analytical or HPLC grade. The animal study was performed according to the Guidelines for the Care and Use of Laboratory Animals at the Pharmaceutical Research Center, Mochida Pharmaceutical Co., Ltd., and approved by the Committee of Ethics of Animal Experimentation of the Pharmaceutical Research Center, Mochida Pharmaceutical Co., Ltd. Male FVB wild-type mice and mdr1a/1b$^{-/-}$ mice were purchased from Taconic Farms (Germantown, NY) and used at 7 to 14 weeks of age. Male SD rats, 6–7 weeks of age, were obtained from Japan SLC (Hamamatsu, Japan). The animals were individually housed at constant temperature (23 ± 2°C) and humidity (55 ± 15%), with a 12 h/12 h light/dark cycle, and were allowed free access to water and commercial diet, CE-2 (Clea Japan Inc., Tokyo, Japan).

Absorption ratio in intestine: Five 3-cm loops of segments of the small intestine, *i.e.*, duodenum, middle jejunum, distal jejunum, middle ileum and terminal ileum from the same FVB wild-type or mdr1a/1b$^{-/-}$ mice were made under anesthesia after binding the bile duct to stop the flow of bile into the intestine. [^{3}H]-Verapamil or [^{3}H]vinblastine (10 μg/100 μL/loop), appropriately diluted with non-labeled compounds and saline, was directly administered into the five intestinal loops of mice at the same time. At 30 min after injection of [^{3}H]verapamil or [^{3}H]vinblastine, mice were killed by exsanguination under anesthesia. Each isolated intestinal loop with its remaining contents was dissolved in 3 mL of Solvable™ (Packard, Meriden, CT), and then 300 μL of 100 mM EDTA, and 150 μL of 30% hydrogen peroxide was added. The bleached solution was mixed with a scintillation cocktail (Atomlight™; NEN, Boston, MA) to measure the radioactivity. The absorption ratio was calculated from the recovered radioactivity in the intestinal loop and contents,

$$\text{Absorption ratio} = 1 - (\text{Recovered radioactivity} / \text{Dosed radioactivity}) \times 100\ (\%)$$

Extraction of RNA, reverse transcription, and assay of mRNA expression by real-time PCR: Mdr1a/1b$^{+/+}$ and mdr1a/1b$^{-/-}$ mice were killed, and tissues were collected and rapidly frozen until required. Duodenum, jejunum, ileum, colon, rectum and liver were identified and cleaned several times in ice-cold phosphate-buffered saline (PBS), then the mucosal cell layers were scraped off on ice and rapidly frozen. The duodenum was identified as proximal to the ligament of Treitz (first 2 cm), the jejunum as the upper part (upper 2/5) of the small intestine distal to the ligament of Treitz and the ileum as the lower part (lower 2/5) of the small intestine ending before the cecum. Between the duodenum and jejunum, 5 cm of tissue was removed as a safety margin, as well as 10 cm between the jejunum and ileum. The total colon was used for mucosa isolation. Total RNA was extracted from 30 mg of mucosal tissue using Sepasol RNA I™ reagent (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's instructions. First-strand cDNA was synthesized using ReverTra Ace™ (Toyobo, Osaka, Japan) with an oligo(dT) primer. Real-time PCR was performed as previously described[12] according to the recommendations of Applied Biosystems (http://home.appliedbiosystems.com). Taqman-Primers for mdr1a, mdr1b, breast cancer resistance protein (bcrp), multidrug resistance-associated protein 2 (mrp2), and cyp3a11 genes were purchased from Applied Biosystems (Tokyo, Japan). The passive reference dye (ROX) was included in the Taqman buffer supplied by the manufacturer. Twenty microliters of cDNA obtained from the RT reaction was diluted to 100 μL with RNAse-free water. PCR was conducted in a 25 μL reaction volume, using 5 μL of diluted cDNA as a template, with sense and antisense primers (25 μM each) and the labeled probe (5 μM). The Taqman Universal PCR Master Mix™ (Applied Biosystems) was added to the final volume. Prior quantification experiments had determined the optimal

concentrations of primers and probes, and the optimum temperature settings to maximize fluorescence signals and PCR products. Reactions were run in 96-well optical reaction plates using a Prism 9600 cycler (Applied Biosystems). Conditions were as follows: initial 95°C (10 min) and then 40 cycles of 95°C (15 s) and 60°C (1 min) with auto ramp time. For analysing the data, the threshold was set to 0.06, as this value had been determined to be in the linear range of the amplification curves for all mRNAs in all experimental runs. All reactions were run in triplicate. The abundance of the target mRNAs was calculated relative to a reference mRNA (glyceraldehyde 3-phosphate dehydrogenase, GAPDH). Since standard curves made for all primer pairs with jejunum RNA had revealed an efficiency value close to 2 (fold-increase in input mRNA required to decrease the cycle number by 1), relative expression ratios were calculated as $R = 2^{(Ct(GAPDH)-Ct(test))}$, where Ct is the cycle number at the threshold and test indicates the tested mRNA. GAPDH mRNA was measured in all samples and the values obtained were used to normalize data for test mRNAs.

Cell culture and transport experiments Caco-2: Cells were obtained from American Type Culture Collection (Rockville, MD). Polycarbonate membrane Transwell™ clusters, 11.2 mm in diameter and 3.0 μm pore size, were purchased from Corning (Acton, MA). The cultivation of Caco-2 cells was performed as described previously.[13] All cells used in this study were between passages 17 and 26. Caco-2 cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were seeded on a 12-well Transwell at a density of 60000 cells/cm^2. Caco-2 cells were cultured for 21 days with Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) with 10% fetal bovine serum. Culture medium was replaced three times a week. The cells were used for transport experiments as described previously.[13,14] Monolayer integrity was assessed by measuring TEERs using a Millicell electrical resistance system (Millipore Corp., Bedford, MA) and the average TEER value was $738 \pm 94\ \Omega \cdot cm^2$. Transport solutions of non-labeled compounds were prepared by adding 1 mM DMSO stock solutions of test compounds to transport buffer to give the final concentration at 5 μM. For the preparation of [^{14}C]antipyrine transport solution, [^{14}C]-antipyrine solution (1.82 mM as antipyrine) was diluted with transport buffer to give the final concentration of 5 μM. For other labeled compounds, to the transport buffer was added a non-labeled 1 mM DMSO stock solution of each compound together with radiolabeled compound to give the final concentration of 5 μM. The cells grown on a polycarbonate membrane were washed twice with Hank's balanced salt solution (HBSS; 0.952 mM $CaCl_2$, 5.36 mM KCl, 0.441 mM KH_2PO_4, 0.812 mM $MgSO_4$, 136.7 mM NaCl, 0.385 mM Na_2HPO_4, 30 mM D-glucose and 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) for pH 7.4 or 10 mM 2-(N-morpholino)ethanesulfonic acid (MES) for pH 6.0; osmolarity 315 mOsm kg^{-1}). To initiate transport for the apical-to-basolateral study, 1.5 mL of HBSS (pH 7.4, 37°C) was introduced on the basolateral side (receiver side) and 0.5 mL of the test solution (pH 6.0, 37°C) containing radio-labeled or non radio-labeled compound was loaded on the apical side (donor side) of a cell insert. At 15, 30, 45 and 60 min after the loading, 0.5 mL aliquots of the solution were removed from the receiver side and replaced with an equal volume of fresh HBSS. In the case of efflux evaluation (basolateral-to-apical study), to initiate transport, 1.5 mL of the test solution (pH 7.4, 37°C) containing radio-labeled or non radio-labeled substrate was loaded on the basolateral side as the donor side and 0.5 mL of MES (pH 6.0, 37°C) was introduced on the apical side as the receiver side of a cell insert. At designated times after loading, 0.1 mL aliquots of the solution were removed from the receiver side and replaced with an equal volume of fresh MES. The apparent permeability coefficients ($P_{app,influx}$ and $P_{app,efflux}$) were calculated using the following equation:

$$P_{app} = (dQ/dt)/(C_0 \times A) \quad (cm/sec)$$

where dQ/dt is the permeability rate, C_0 is the initial concentration in the donor compartment, and A is the surface area of the membrane. The efflux rate ($dQ/dt \times 1/A$) was calculated by plotting the amount transported per unit area as a function of time and determining the slope of the line using linear regression. Each result represents the mean of two experiments using the same culture of Caco-2 cells.

Pharmacokinetic study: Animals was deprived of food for 12 hrs before experiments. [^{3}H]Verapamil or [^{3}H]vinblastine was dissolved at a concentration of 1 mg/10 mL with non-labeled compound and physiological saline, and orally administered to mice at a single dose of 1 mg/kg. In the rat study, only [^{3}H]vinblastine was administered. Blood samples were withdrawn from the orbital sinus of mice with a heparinized capillary or from the jugular vein of rats with a heparinized syringe at designated times, under anesthesia induced with diethyl ether. Blood were centrifuged (1700 × g) for 15 min at 4°C to obtain plasma. The plasma sample was mixed with a scintillation cocktail (Aquasol™; Packard, Meriden, CT) to measure the radioactivity. The maximum plasma concentration (C_{max}) and time to C_{max} (T_{max}) were determined directly from the observed data. The area under the plasma concentration-time curve from time 0 to 24 hr (AUC_{0-24hr}) was estimated by the linear trapezoidal method. Each value is the mean ± S.D. of three animals. Statistical analysis for C_{max} and AUC_{0-24hr} was performed with Student's two-tailed *t*

Table 1. Absorption of [^{3}H]verapamil and [^{3}H]vinblastine from mouse intestine loops

Segment	Absorbed at 30 min (% of dose)			
	[^{3}H]Verapamil		[^{3}H]Vinblastine	
	Wild-type	mdr1a/1b$^{-/-}$	Wild-type	mdr1a/1b$^{-/-}$
Duodenum	50.9±31.8	58.4±10.4	44.6±27.9	32.5±14.4
Middle Jejunum	47.1±26.4	56.5±6.42	5.51±4.30	21.7±11.6
Distal Jejunum	38.0±18.0	36.3±22.6	0.01±4.75	28.7±15.1
Middle Ileum	44.7±33.6	53.8±21.9	23.0±4.03	40.3±18.9
Terminal Ileum	48.7±14.2	40.9±20.5	43.9±10.2	42.5±16.4

Each value represents the mean±S.D. of three or four animals.

test. The difference between means was considered to be significant when the P-value was less than 0.05.

Analytical method: Radioactivity was determined with a liquid scintillation counter (LS6000TA, Beckman, Fullerton, CA). Non-labeled samples were analyzed with a NanoSpace™ HPLC system (Shiseido, Tokyo, Japan) coupled to a TSQ Quantum™ triple quadrupole mass spectrometer (Thermo Electron Corp., San Jose, CA) *via* an electron spray ionization interface under the conditions described below. Colchicine was separated with a Capcellpak™ DD column (150×2.0 mm i.d., Shiseido, Tokyo, Japan) using isocratic elution at 0.2 mL/min with 55% 0.1%-formic acid/45% methanol. Positive ion selected reaction monitoring was done using the transition 400.0 to 358.0. Fexofenadine was separated with a Capcellpak™ DD column using isocratic elution with 46% 0.1%-formic acid/54% methanol, with monitoring of the 502.2 to 466.1 transition. Cyclosporine was separated with a Capcellpak™ MGII column (150×2.0 mm i.d., Shiseido, Tokyo, Japan) using isocratic elution with 14.5% 0.1%-formic acid/85.5% methanol; the 1219.9 to 1202.9 transition was monitored. Digoxin was separated with a Capcellpak™ MGII column using isocratic elution with 55% 20 mM ammonium acetate/45% acetonitrile. The transition 798.3 to 651.1 was monitored. Quinidine was separated with a Capcellpak™ MGII column using isocratic elution with 55% 0.1%-formic acid/45% methanol, with monitoring of the 325.0 to 307.2 transition.

Results and Discussion

Nakayama *et al*. reported the absorption profile of vinblastine from rat intestinal loops.[15] They found that vinblastine was absorbed in the duodenum and ileum, but not at all in jejunum. Therefore, we first examined the absorption ratio in various intestinal segments of mice. **Table 1** shows the absorption ratio at 30 min after a single administration of [^{3}H]verapamil or [^{3}H]-vinblastine into five intestinal loops of male mice. The absorption ratio of [^{3}H]verapamil ranged from 38.0 to 50.9% in mdr1a/1b$^{+/+}$ mice. There was no remarkable variation among segments of the intestine, or between wild-type and mdr1a/1b$^{-/-}$ mice. In the case of [^{3}H]-vinblastine, the values of the absorption ratio in the middle and distal jejunum of wild-type mice were very low, 5.51 and 0.01%, respectively, whereas those elsewhere were high, 44.6, 23.0 and 43.9% in duodenum, middle ileum and terminal ileum, respectively. On the other hand, the absorption ratio of [^{3}H]vinblastine for mdr1a/1b$^{-/-}$ mice showed no remarkable variation among segments of the intestine, ranging from 21.7 to 42.5%. These results are similar to the pattern seen in rats by Nakayama *et al*..[15]

To examine the reason for the segment-dependent absorption of vinblastine in mouse intestine, real-time PCR was used to assess the relative abundance of mRNAs of various efflux transporters and cyp3a11 in the epithelial layer of small intestine and liver (**Fig. 1**). There were clear differences in the levels of expression of mdr1a mRNA between intestinal segments in wild-type mice, *i.e.* colon > ileum > rectum > jejunum > duodenum, and this is in accordance with human data reported by various researchers.[16–18] However, the differences of mdr1a mRNA expression could not account for the variation of the absorption ratio of vinblastine in different intestinal regions. A possible explanation might be segment-related differences in the permeability of vinblastine, as discussed below. No significant differences in the expression of multidrug resistance-associated protein 2 (mrp2), breast cancer resistance protein (bcrp) and cyp3a11 mRNAs were observed between mdr1a/1b$^{+/+}$ and mdr1a/1b$^{-/-}$ mice. Thus, mdr1a/1b$^{-/-}$ mice did not functionally compensate for mdr1a/1b gene-deficiency with enhanced synthesis of mRNAs for mrp2, bcrp and cyp3a11.

It is important to know what kinds of substrates are likely to show P-gp-dependent intestinal absorption. Although several researchers have concluded that the effect of P-gp on intestinal absorption of drugs can not be predicted from *in vitro* data,[5,19] we investigated the feasibility of using a simple assay with Caco-2 cells to classify P-gp substrates. **Figure 2** shows the intestinal permeability of several P-gp substrates and two standard compounds, mannitol and antipyrine. Horizontal and vertical axes in **Fig. 2** represented influx permeability (apical-to-basolateral: $P_{app,influx}$) and the ratio of efflux to influx permeability (basolateral-to-apical/apical-to-basolateral: $P_{app,efflux}/P_{app,influx}$) of compounds, respectively. For all the substrates examined, except vinblastine, colchicines and fexofenadine, influx permeability was higher than 1×10^{-6} cm/sec. For P-gp substrates except vinblastine, the values of the B-to-A/A-to-B ratio were relatively low, *i.e.*, below 8. On the other hand, the efflux/influx permeability ratio of vinblastine was quite high, at 12.1. Thus, there seem to

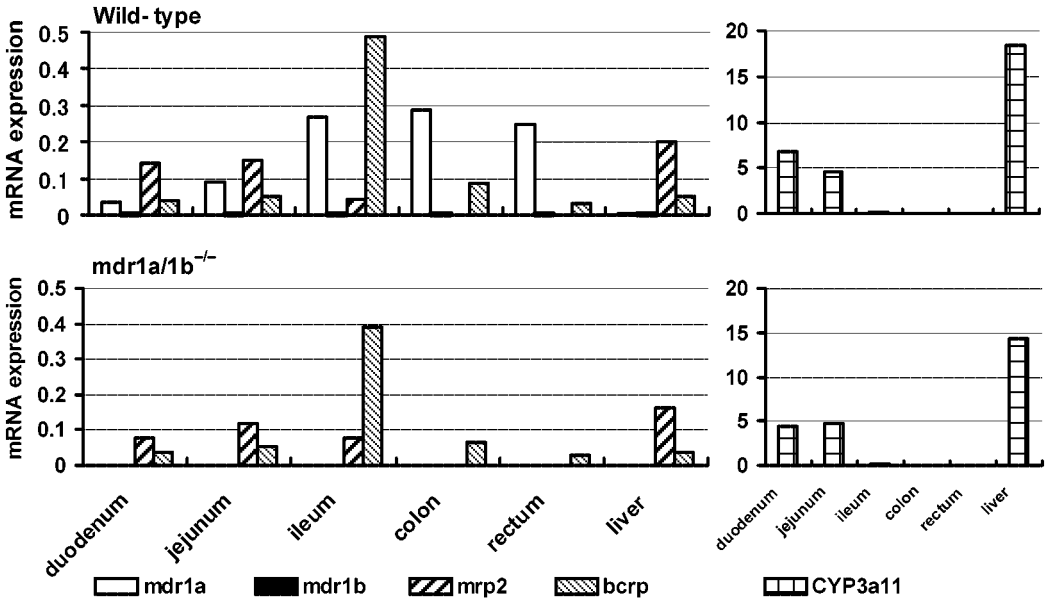

Fig. 1. Gene expression in different intestinal segments normalized to GAPDH mRNA espression. Data represent means of triplicate experiments.

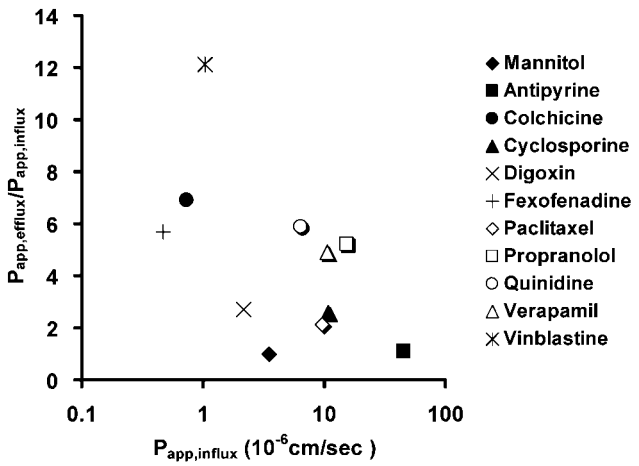

Fig. 2. Permeability for the transcellular transport of P-gp substrates across Caco-2 monolayer cells. Each point represents the mean of two experiments using the same cultivation of Caco-2 cells.

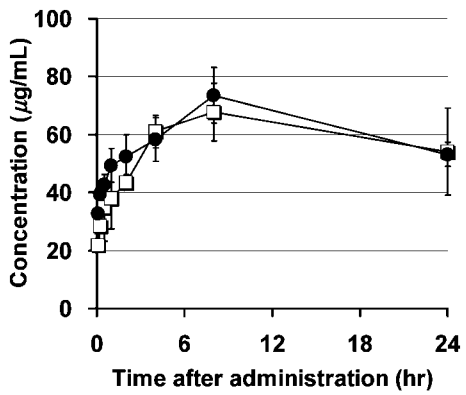

Fig. 3. Plasma concentrations of radioactivity after a single oral administration of [^{3}H]verapamil at a dose of 1 mg/kg to male wild-type (closed circles) and mdr1a/1b$^{-/-}$ (open squares) mice. Each point represents the mean ± S.D. of three animals.

be at least two classes of P-gp substrates. The intestinal absorption profile of some substrates, such as verapamil, was not affected by P-gp, whereas that of others such as vinblastine was affected by P-gp. The vinblastine-type substrates have high affinity for P-gp, as judged from the value of the efflux/influx ratio in Caco-2 cells. Moreover, they may be easily captured by P-gp in intestinal epithelial cells before transport to the basolateral side, since they show low permeability. Consequently, the absorption profile of vinblastine-type substrates is quite poor, and such P-gp substrates with low absorption and high P-gp affinity would be unfavorable for development as oral drugs. Therefore, we suggest that our Caco-2 assay represents a useful screening method.

After oral administration of [^{3}H]verapamil (1 mg/kg), the plasma concentration of total radioactivity reached C_{max} of 73.5 ± 9.6 μg/mL at 8 hr in mdr1a/1b$^{+/+}$ mice and 67.8 ± 9.6 μg/mL at 8 hr in mdr1a/1b$^{-/-}$ mice. The $AUC_{0\text{-}24hr}$, an index of absorbability, were not significantly different between the strains, *i.e.*, 1479 ± 105 and 1409 ± 92 μg·hr/mL for mdr1a/1b$^{+/+}$ and mdr1a/1b$^{-/-}$ mice, respectively (**Fig. 3**, **Table 2**). In the case of [^{3}H]vinblastine, the C_{max} values in mdr1a/1b$^{+/+}$ and mdr1a/1b$^{-/-}$ mice were 117 ± 27 and 170 ± 32 ng/mL at 8 hr after oral administration, respectively.

Table 2. Pharmacokinetic parameters of [^{3}H]verapamil and [^{3}H]vinblastine after a single oral administration at a dose of 1 mg/kg to male wild-type and mdr1a/1b$^{-/-}$ mice

Substrate	Strain	C_{max} (μg/mL for verapamil) (ng/mL for vinblastrine)	T_{max} (hr)	AUC_{0-24hr} (μg·hr/mL for verapamil) (ng·hr/mL for vinblastrine)
[^{3}H]Verapamil	Wild-type	73.5±9.6	8	1479±105
	mdr1a/1b$^{-/-}$	67.8±9.6	8	1409±92
[^{3}H]Vinblastine	Wild-type	117±27	8	2423±502
	mdr1a/1b$^{-/-}$	170±32*	8	3714±318*

*Each value of C_{max} and AUC_{0-24hr} represents the mean±S.D. of three animals.

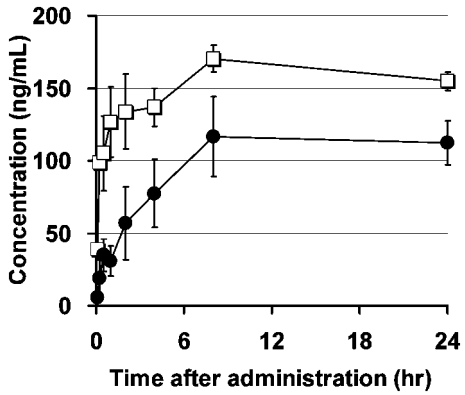

Fig. 4. Plasma concentrations of radioactivity after a single oral administration of [^{3}H]vinblastine at a dose of 1 mg/kg to male wild-type (closed circles) and mdr1a/1b$^{-/-}$ (open squares) mice. Each point represents the mean±S.D. of three animals.

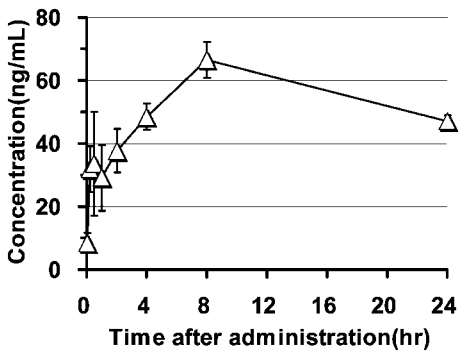

Fig. 5. Plasma concentrations of radioactivity after a single oral administration of [^{3}H]vinblastine at a dose of 1 mg/kg to male rats. Each point represents the mean±S.D. of three animals.

The AUC_{0-24hr} of [^{3}H]vinblastine was 3714±318 ng·hr/mL for mdr1a/1b$^{-/-}$ mice, 1.5 times greater than that for wild-type mice, as shown in **Fig. 4** and **Table 2**. Thus, the pharmacokinetic profile of vinblastine was affected by P-gp, while that of verapamil was not. This difference of pharmacokinetic profiles between verapamil and vinblastine is considered to be due to the difference of intestinal absorption characteristics between them. Therefore, pharmacokinetic profiling using mdr1a/1b$^{+/+}$ and mdr1a/1b$^{-/-}$ mice represents another possible screening method to confirm whether a compound is a vinblastine-type substrate or not.

We also examined the plasma concentration of radioactivity after oral administration of [^{3}H]-vinblastine to male rats. The PK profile showed puzzling twin peaks of plasma concentration, *i.e.*, the first C_{max} was 33.5 ng/mL at 0.5 hr, and this was followed by a higher peak of 66.5 ng/mL at 8 hr after administration, as shown in **Fig. 5**. We have encountered a series of P-gp substrates, which exhibit vinblastine-type low permeability of less than 1×10^{-6} cm/sec, and high affinity for P-gp (ratio of more than 10 in our Caco-2 assay). Despite the apparently desirable physicochemical parameters (solubility, metabolic stability and permeability through artificial membranes) of these compounds, they showed poor bioavailability and vinblastine-like twin peaks in the PK profiles after oral administration to rats. Their bioavailability increased slightly with increasing dose, and the twin peaks disappeared upon oral co-administration of these compounds with verapamil or cyclosporine as P-gp substrates/inhibitors (data not shown).

In conclusion, there appear to be at least two types of P-gp substrates in terms of intestinal absorption characteristics, *i.e.*, verapamil-type and vinblastine-type, and these could be differentiated with our Caco-2 system. Vinblastine-type substrates may be absorbed in the duodenum and ileum, but not the jejunum, and show twin C_{max} peaks and quite low bioavailability after oral dosing in rats. In general, hepatic metabolism of drugs by CYPs and enzymatic conjugation of drugs are called Phase I and Phase II elimination processes, respectively. Recently, primary active excretion into bile (positive removal of xenobiotics from the body) has been designated as Phase III elimination.[20,21] We suggest that intestinal carrier-mediated efflux may be regarded as a Phase 0 clearance system for removal of xenobiotics from the body.

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