AAPS Pharmsci 1999; 1 (4) article 18 (http://www.pharmsci.org)

Comparative Studies to Determine the Selective Inhibitors for P-Glycoprotein and Cytochrome P4503A4

Submitted: November 2, 1999; Accepted: November 10, 1999; Published: December 22, 1999

Meguru Achira

Graduate School of Pharmaceutical Sciences, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

Kiyomi Ito

Graduate School of Pharmaceutical Sciences, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan (Present address: School of Pharmaceutical Sciences, Kitasato University, Shirokane, Minato-ku, Tokyo 108-8641,Japan)

Hiroshi Suzuki and Yuichi Sugiyama

Graduate School of Pharmaceutical Sciences, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan, and CREST, Japan Science and Technology Corporation.

ABSTRACT It has been suggested that cytochrome P450 3A4 (CYP3A4) and MDR1 P-glycoprotein (P-gp) act synergistically to limit the bioavailability of orally administered agents. In order to determine the relative role of these proteins, it is essential to identify a selective inhibitor for either P-gp or CYP3A4. In the present investigation, comparative studies were performed to examine the effect of inhibitors on the function of these proteins. The IC_{50} of P-gp function, determined by examining the inhibition of the transcellular transport of vinblastine across Caco-2 monolayers, was in the order PSC833 << ketoconazole, verapamil << N-(2(R)-hydroxy-1(S)-indanyl)-5-(2(S)-(1,1-dimethylethylaminocarbonyl)-4-(furo(2,3-b)pyridin-5-yl)methyl)piperazin-1-yl)-4(S)-hydroxy-2(R)-phenylmethylpentanamide (L-754,394). In contrast, the IC_{50} of CYP3A4 function, determined by examining the inhibition of the metabolism of midazolam by intestinal and liver microsomes, was in the order L-754,384 < ketoconazole << PSC 833 and verapamil. The ratio of IC_{50} for P-gp to that for CYP3A4 was more than 200 for L-754,394, 60 ~ 150 for ketoconazole, 1.5 for verapamil, and 0.05 for PSC 833. Collectively, it was demonstrated that PSC 833 and L-754,394 can be used as selective inhibitors of P-gp and CYP3A4, respectively.

INTRODUCTION

Based on the finding that intestinal epithelial cells contain significant amounts of metabolic enzymes (such as cytochrome P-450 3A4 (CYP3A4)), it has been suggested that the limited oral bioavailability of certain drugs can be ascribed to their first-pass elimination in the small intestine (1). The results of subsequent pharmacokinetic analysis of the data obtained in human subjects who were given substrates (such as cyclosporin A) i.v. and p.o. are consistent with this hypothesis (2,3,4). The direct demonstration of the validity of this hypothesis has been provided by examining the metabolic activity of midazolam, a CYP3A4 probe, during a survey of liver transplant recipients (5). Under anhepatic conditions, the fraction of midazolam metabolized during transcellular transport across the intestinal epithelial cells after intraduodenal administration was found to be as high as 0.43 (5).

In addition to CYP enzymes, MDR1 P-glycoprotein (P-gp), located on the brush border membrane of intestinal epithelium, may have a functional significance in limiting the oral bioavailability of its substrates (reviewed by Wacher et al. (6)). This hypothesis was proved directly by studies in mdr1a/1b knockout mice, in which reduced small intestinal excretion and enhanced oral bioavailability have been reported (7,8). Focusing on the fact that the substrates of CYP3A4 and P-gp are mutually overlapping (1), Benet and his collaborators proposed that these proteins act synergistically to present a barrier to absorption from the small intestine (1,6). The results of a series of experiments in which the simultaneous administration of inhibitors of CYP3A4/P-gp increased the oral bioavailability of substrates are consistent with this hypothesis (9,10,6). In addition, we have previously demonstrated the synergistic role of CYP3A4 and P-gp by simulation studies using a pharmacokinetic model in which the intracellular diffusion process has been considered (11).

Because, in general, inhibitors for CYP3A4 inhibit P-gp function (6), the relative role of CYP3A4 and P-gp in reducing the oral bioavailability still needs to be clarified. The purpose of the present study is to identify a selective inhibitor of either CYP3A4 or P-gp. Comparative studies were performed to examine the IC_{50} of four kinds of CYP3A4/P-gp inhibitors on

Corresponding Author: Dr. Yuichi Sugiyama, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan; telephone: +81-3-5841-4770; facsimile: +81-3-5800-6949; e-mail: sugiyama@seizai.f.u-tokyo.ac.jp

these two proteins. P-gp and CYP3A4 activity was monitored by measuring the transcellular transport of vinblastine across Caco-2 monolayers, and the metabolic conversion of midazolam to -OH midazolam in human liver and small intestinal microsomes (12,13).

MATERIALS AND METHODS

Materials

PSC 833, N-(2(R)-hydroxy-1(S)-indanyl)-5-(2(S)-(1,1-dimethylethyl-aminocarbonyl)-4-(furo(2,3-b)pyridin-5-yl)methyl)piperazin-1-yl)-4(S)-hydroxy-2(R)-phenylmethylpentanamide (L-754,394), midazolam andα-hydroxy midazolam were provided by Novartis Pharma AG (Basel, Switzerland), Merck Research Laboratories (West Point, PA) and Hoffmann La Roche (Basel, Switzerland), respectively. (^{3}H) Labeled and unlabeled vinblastine were purchased from Amersham International (Buckinghamshire, UK) and Sigma Chemical (St. Louis, MO), respectively. Human intestinal and liver microsomes were purchased from Human Cell Culture Center. All other chemicals were commercial products of analytical grade.

Transcellular Transport Studies

Transcellular transport studies across the Caco-2 monolayer were performed according to the method described by Hunter et al. (14). Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose, 5% fetal bovine serum (FBS), 0.1 mM non-essential amino acids, 4 mM L-glutamine and antibiotics/antifungal agents in an atmosphere of 95% O_2-;5% CO_2. Monolayers of Caco-2 cells, formed by incubating the confluent cells for an additional 20 days, were used for the transcellular transport studies. Each Caco-2 monolayer was washed twice with phosphate buffered saline (PBS) prior to the preincubation for 30 minutes in DMEM containing no FBS. Transcellular transport experiments were initiated by adding (^{3}H)vinblastine (100 nM) to the apical or basal side. At 1, 2, and 4 hours, aliquots of medium in the receiver side were removed to determine the radioactivity in a liquid scintillation counter (Model 2500TR, Packard, CA). Permeability of vinblastine across the monolayer (P_{app}) was calculated from the following equation:

$P_{app} = dA/dt/S/C_0$,

where S, C_0, and A represent the surface area of the Caco-2 monolayer, initial concentration of vinblastine in the donor side, and amount of vinblastine transferred to the receiver side, respectively.

Metabolic Studies

The metabolism of midazolam was examined according to the method described previously (12,13). 100 mM potassium phosphate buffer (pH 7.4) containing human microsomes (0.2 mg protein/ml), 9.63 mM glucose-6-phosphate, 0.356 mM NADP, 0.1 U/ml glucose-6-phosphate dehydrogenase and 0.25 mM EDTA, with or without inhibitors, were preincubated at 37^{o}C for 5 minutes. The reaction was initiated by the addition of midazolam (2 μM). After 20 minutes incubation at 37^{o}C, the same volume of methanol/acetonitrile = 1/1 (v/v) containing etizolam (an internal standard) was added to the incubation mixture to terminate the reaction. After centrifugation, the supernatant was evaporated to dryness. 10 mM PBS (pH 7.4)/methanol/acetonitrile = 10/5/7 (v/v/v) was added to the residue prior to the injection onto the high-performance liquid chromatography (HPLC) column. Our preliminary experiments indicated that the metabolic reaction takes place over a linear range in terms of incubation time and protein concentration under the present experimental conditions.

The amount of midazolam and α-hydroxy midazolam was determined by HPLC using a reversed-phase column (4.6 mm x 250 mm TSK gel ODS-80Ts, Tosoh, Tokyo, Japan). The mobile phase consists of 10 mM PBS (pH 7.4)/methanol/acetonitrile = 10/5/7 (v/v/v) and the flow rate was 1 ml/minute. Both midazolam and α-OH midazolam were detected by UV absorption at a wavelength of 220 nm.

RESULTS

Transcellular Transport

Figure 1 represents the transcellular transport of vinblastine across Caco-2 monolayers. The transport of vinblastine was linear up to 4 hours. P_{app} for the basal-to-apical compartment (2.86 x $10^{-;5}$ cm/second) was 22-fold higher than that in the opposite direction (1.28 x $10^{-;6}$ cm/second) (Figure 1). This basal-to-apical preferential flux was almost completely abolished in the presence of 100 μM verapamil (Figure 1). Indeed, the P_{app} for apical-to-basal flux

(7.52×10^{-6} cm/second) was comparable to that in the opposite direction (9.66×10^{-6} cm/second).

The concentration-dependent effect of inhibitors on vinblastine flux across the monolayers was determined. As shown in Figure 2, PSC 833 was the most potent inhibitor of P-gp function, with an IC_{50} of 0.1 μM (Table 1). Although ketoconazole and verapamil significantly reduced P-gp function with an IC_{50} of 3 and 30 μM, respectively, the effect of L-754,394 was not significant at a concentration of 1 μM (Figure 2, Table 1).

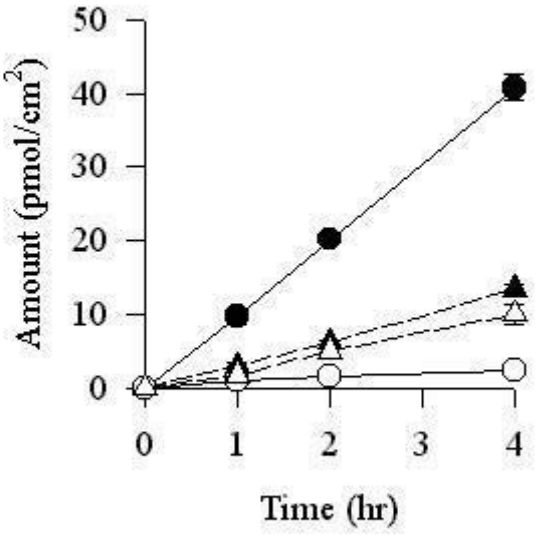

Figure 1. Transcellular transport of vinblastine across Caco-2 monolayers. Transcellular transport of (^{3}H)vinblastine (100 μM) across Caco-2 monolayers was examined in the presence and absence of 100 μM verapamil. Each point and vertical bar represent the mean + S.E. of 4 independent experiments. Open and closed symbols represent the apical-to-basal and basal-to-apical flux, respectively. Triangles and circles represent the flux in the presence and absence of verapamil, respectively.

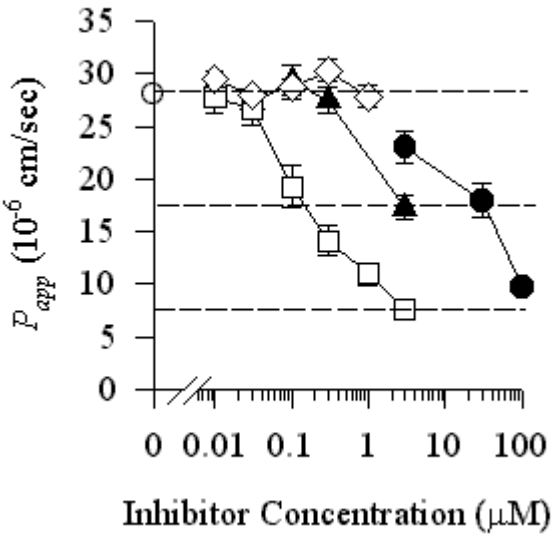

Figure 2. Effect of inhibitors on the transcellular transport of vinblastine across Caco-2 monolayers. The permeability of (^{3}H)vinblastine (100 μM) across Caco-2 monolayers was determined in the presence and absence of the indicated concentrations of inhibitors. The permeability in the absence of inhibitors was 28×10^{-6} cm/second. Each point and vertical bar represent the mean + S.E. of 4 independent experiments. Key; ●+ verapamil; ▲+ ketoconazole; □+ PSC 833; ◇+ L-754,394.

Metabolic Studies

The effect of inhibitors on the metabolic conversion of midazolam (2 μM) by human liver and intestinal microsomes is shown in Figure 3. The metabolic activity of human jejunum microsomes (Lot #HJ005 and #HJ006) was 116 + 3 and 39.3 + 1.9 pmol/minute/mg microsomal protein, respectively, whereas that of human liver microsomes (Lot #HH459) was 233 + 9 pmol/minute/mg microsomal protein. The difference in the absolute value of the metabolic activity may be attributed to the difference in CYP3A4 content. L-754,394 was the most potent inhibitor, with an IC_{50} value of 0.005 and 0.05 μM for jejunum and liver microsomes, respectively (Table 1). Ketoconazole was also a potent inhibitor, with an IC_{50} of 0.02 μM for both jejunum and liver microsomes (Figure 3 and Table 1). Although PSC 833 and verapamil reduced the metabolic conversion of midazolam, their IC_{50} values (3 and 20 μM, respectively) were much higher than those for L-754,394 and ketoconazole (Table 1).

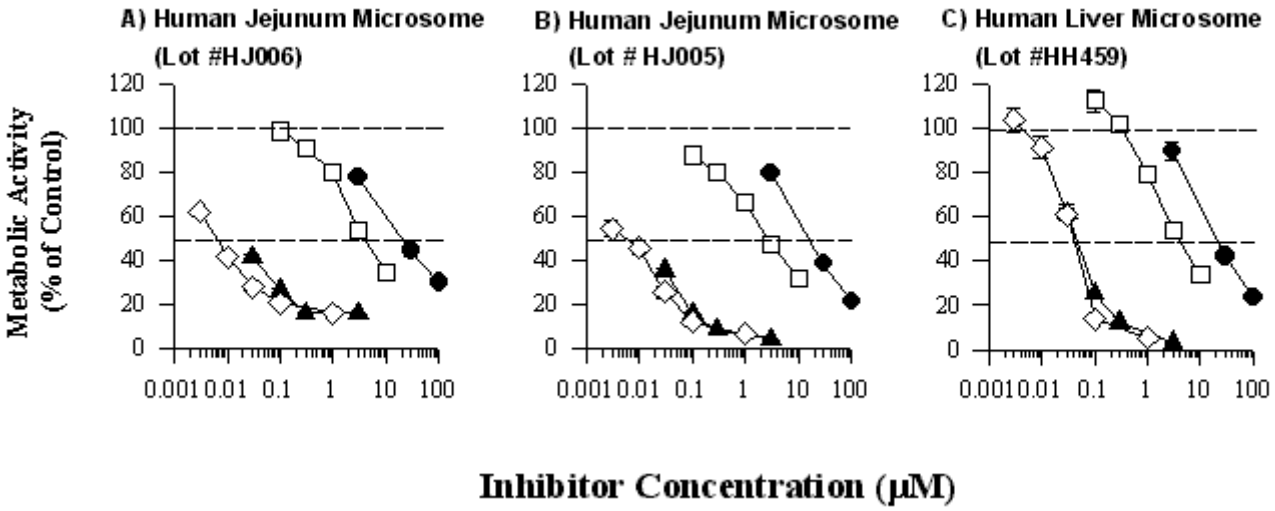

Figure 3. Effect of inhibitors on α-hydroxylation of midazolam. The α-Hydroxylation of midazolam (2 μM) by human jejunum (Lot #HJ005 and HJ006) and liver microsome (Lot #HH459) was determined in the presence of the indicated concentrations of inhibitors. Each point and vertical bar represents the mean + S.E. of four independent experiments. Key; ● + verapamil; ▲+ ketoconazole; □+ PSC 833; ◇+ L-754,394.

Table 1 IC_{50} values for the inhibition of P-gp and CYP3A4 activity. Results shown in Figs. 2 and 3 are summarized. Ratio of IC_{50} for P-gp to that for CYP3A4 is also indicated.

	IC_{50} (μM)			
	Verapamil	Ketoconazole	PSC-833	L-754,394
P-glycoprotein				
Caco-2 cell monolayer	30	3	0.15	>> 1
CYP3A4				
Human Jejunum Microsomes (lot HJ006)	20	0.02	3	0.005
Human Jejunum Microsomes (lot HJ005)	20	0.02	3	0.006
Human Liver Microsomes (lot HH459)	20	0.05	3	0.05
P-gp/CYP3A4 Ratio	1.5	60-150	0.05	>> 20-200

\The ratio of IC_{50} for P-gp to that for intestinal CYP3A4 is also summarized in Table 1. This ratio was more than 200 for L-754,394, and 60-;150 for ketoconazole (Table 1). Although verapamil inhibited both P-gp and CYP3A4 function, exhibiting comparable IC_{50} values (ratio = 1.5), PSC 833 was selective for P-gp (ratio = 0.05) (Table 1).

DISCUSSION

In the present study, we determined the selectivity of the effect of verapamil, ketoconazole, PSC 833, and L-754,394 on the function of P-gp and CYP3A4, because the substrates/inhibitors for these proteins mutually overlap. It has been demonstrated that verapamil and PSC 833 are substrates for both CYP3A4 (15,16) and P-gp (17,18), whereas ketoconazole, a CYP3A4 substrate, is not significantly transported via P-gp (19). There are no published reports showing that L-754,394 is transported via P-gp. The results obtained showed that the potency of inhibition of P-gp is in the order PSC 833 >> ketoconazole, verapamil >> L-754,394, whereas that of CYP3A4 is in the order L754,394 > ketoconazole >> PSC 833 and verapamil (Table 1). The ratio of the IC_{50} for P-gp to that for CYP3A4 was more than 200 for L-754,394, 60-;150 for ketoconazole, 1.5 for verapamil, and 0.05 for PSC 833 (Table 1). Collectively, it was shown that PSC 833 and L-754,394 can be used as selective inhibitors of P-gp and CYP3A4, respectively. However, because the selectivity of PSC 833 toward P-gp is marginal (20-fold higher than that for CYP3A4), and because the IC_{50} value for P-gp may depend on the experimental conditions (as discussed below), we have to be cautious when interpreting the effect of this inhibitor in any in vivo experiments.

The IC_{50} values determined in the present study should be compared with those reported previously. The IC_{50} value of PSC 833 (0.15 μM, Table 1.) was comparable with that previously reported from this laboratory (~0.1 μM) (20). We have examined the effect of PSC 833 on the accumulation of

(^{3}H)vinblastine and (^{3}H)daunomycin by HCT15 cells that overexpress P-gp and found that the accumulation of both antitumor drugs was enhanced by PSC 833 with an IC_{50} of 0.1 μM (20).

By examining the inhibitory effect of verapamil on the transcellular transport of morpholine-urea-phenylalanine-homophenylalanine-vinylsulfone-phenyl (K-02), a peptidomimetic cysteine protease inhibitor, across MDR1 cDNA-transfected MDCK and Caco-2 monolayers, its IC_{50} values were determined as 236 + 63 μM and 339 + 19 μM, respectively (21). These values are much higher than those determined in the present study; the IC_{50} value for verapamil being found to be ~30 μM (Table 1). This discrepancy may be accounted for by the fact that the previous authors had added the inhibitors to the basal side only (21), whereas in the present study we added inhibitors to both basal and apical sides. If we liken the action of P-gp to that of a vacuum cleaner, the ligand molecules in the outer leaflet of the lipid bilayer of the plasma membrane are more efficiently extruded into the medium than those in the inner leaflet (22). In the same manner, it is possible that the inhibitors can efficiently reduce the function of P-gp after addition so that they can easily access the outer leaflet of the plasma membrane. Because we also added the inhibitors to the apical side, it is possible that the inhibitory effect was much more potent (in the present study, inhibitor molecules can access P-gp directly from the outer leaflet) than the situation where the inhibitors were added to the basal side only (in the previous studies, inhibitor molecules could access P-gp from the inner leaflet (21)).

In the same manner, the IC_{50} of ketoconazole on the transcellular transport of K-02 across the MDR1 cDNA-transfected MDCK monolayers was determined as 119 ± 10 μM (10), which was much higher than that obtained in the present study (~3 μM, Table 1). This discrepancy can also be accounted for by the hypothesis described previously. The IC_{50} value of ketoconazole determined in the present study is similar to that reported by examining its P-gp-modulating activity in tumor cells (KB-V1 cells) overexpressing this extrusion pump (23). KB-V1 cells were 281- and 256-fold resistant to vinblastine and daunorubicin, respectively, compared with the parental cell line (KB-3-1) (23). Ketoconazole reduced this resistance in a concentration-dependent manner; 2 μM was sufficient to reduce the resistance to antitumor drugs to 27- and 91-fold, respectively (23).

Concerning the effect of inhibitors on metabolic enzymes, it has been reported that verapamil and ketoconazole reduce the function of CYP3A4 with K_i values of 41 and 0.015 μM, respectively (15,24,13); these values are consistent with those determined in the present study (20 and 0.02 μM, respectively, Table 1). L-754,394 has been reported to reduce CYP3A4 activity in a concentration- and time-dependent manner (25); unstable metabolite formed with the aid of CYP3A4 covalently binds to this enzyme. Chiba et al. (25) analyzed the characteristics of the inhibitory effect of L-754,394 on testosterone 6 ß-hydroxylation activity using the following equation:

$$k = k_{inact} \times I_0/(K_i + I_0),$$

where k, k_{inact}, K_i, and I_0 represent the initial rate constant for inactivation, maximum rate constant for inactivation, dissociation constant for the enzyme-inactivator complex, and the initial inhibitor concentration, respectively (25). They reported the values of K_i and k_{inact} as being 7.5 μM and 1.62 minutes$^{-;1}$, respectively (25). Substitution of the IC_{50} value of L-754,394 determined in the present study (0.05 μM, Table 1) in this equation yields a k value of 0.0107 minutes$^{-;1}$. These results appear to be reasonable, because, by substituting this k value and incubation time, it was calculated that approximately 25% of the CYP3A4 enzyme should be reduced under the present experimental conditions.

The fact that L-754,394 did not significantly inhibit P-gp function up to 1 μM (Figure 2) may also be related to its inhibition characteristics. The affinity of L-754,394 itself for CYP3A4 has also been demonstrated to be much lower compared with that of its reactive metabolite (25). The apparent selective inhibition of CYP3A4 by L-754,394 can be attributed to its reactive metabolite (25).

We have no satisfactory explanation to account for the discrepancy in the IC_{50} values between the intestinal and liver microsomes (0.005 and 0.05 μM, respectively, Table 1), because the CYP3A4 cDNA sequence, along with its metabolic activity, has been demonstrated to be equivalent between these two tissues; Thummel et al. (12) have reported that the K_m values of midazolam are 3.58 and 2.74 μM in

human intestinal and liver microsomes, respectively. In addition, we had also found the comparable IC_{50} values for other inhibitors (verapamil, ketoconazole, and PSC 833; Table 1), which may competitively inhibit the function of CYP3A4, between intestine and liver. It is possible that the mode of inhibition of L-754,394 (25) may be related to this difference.

In conclusion, the results of the present study indicate that PSC 833 and L-754394 can be used as selective inhibitors of P-gp and CYP3A4, respectively. By using these selective inhibitors, it should be possible to determine the relative role of P-gp and CYP3A4 in reducing the oral bioavailability of certain drugs.

REFERENCES

1. Wacher VJ, Wu C-Y, Benet LZ. Overlapping substrate specificities and tissue distribution of cytochrome P450 3A and P-glycoprotein: implications for drug delivery and activity in cancer chemotherapy. Mol Carcinog. 1995;13:129-134.

2. Kolars JC, Awni WM, Merion RM, Watkins PB. First-pass metabolism of cyclosporine by the gut. Lancet. 1991;338:1488-1490.

3. Hebert MF, Roberts JP, Prueksaritanont T, Benet LZ. Bioavailability of cyclosporine with concomitant rifampin administration is markedly less than predicted by hepatic enzyme induction. Clin Pharmacol Ther. 1992;52:453-457.

4. Wu CY, Benet LZ, Hebert MF, Gupta SK, Rowland M, Gomez DY, Wacher VJ. Differentiation of absorption and first-pass gut and hepatic metabolism in humans: studies with cyclosporine. Clin Pharmacol Ther. 1995;58:492-497.

5. Paine MF, Shen DD, Kunze KL, Perkins JD, Marsh CL, McVicar JP, Barr DM, Gillies BS, Thummel KE. First-pass metabolism of midazolam by the human intestine. Clin Pharmacol Ther. 1996;60:14-24.

6. Wacher VJ, Silverman JA, Zhang Y, Benet LZ. Role of P-glycoprotein and cytochrome P450 3A in limiting oral absorption of peptides and peptidomimetics. J Pharm Sci. 1998;87:1322-1330.

7. Sparrenboom A, van Asperen J, Mayer U, Schinkel AH, Smit JW, Meijer DKF, Borst P, Nooijen WJ, Beijnen, JH, van Tellingen O. Limited oral bioavailability and active epithelial excretion of paclitaxel (Taxol) caused by P-glycoprotein in the intestine. Proc Natl Acad Sci U S A. 1997;94:2031-2035.

8. Schinkel AH, Mayer U, Wagenaar E, Mol CAAM, van Deemter L, Smit JJM, van der Valk MA, Voordouw AC, Spits H, van Tellingen O, Zijlmans JMJM, Fibbe WE, Borst P. Normal viability and altered pharmacokinetis in mice lacking mdr1-type (drug-transporting) P-glycoproteins. Proc Natl Acad Sci U S A. 1999;94:4028-4033.

9. Floren LC, Bekersky I, Benet LZ, Mekki Q, Dressler D, Lee JW, Roberts JP, Hebert MF. Tacrolimus oral bioavailability doubles with coadministration of ketoconazole. Clin Pharmacol Ther. 1997;62:41-49.

10. Zhang Y, Hsieh Y, Izumi T, Lin ET, Benet LZ. Effects of ketoconazole on the intestinal metabolism, transport and oral bioavailability of K02, a novel vinylsulfone peptidomimetic cysteine protease inhibitor and a P450 3A, P-glycoprotein dual substrate, in male Sprague-Dawley rats. J Pharmacol Exp Ther. 1998;287:246-252.

11. Ito H, Kusuhara H, Sugiyama Y. Effects of intestinal CYP3A4 and P-glycoprotein on oral drug absorption: theoretical approach. Pharm Res. 1999;16:225-231.