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# Interaction with P-glycoprotein and transport of erythromycin, midazolam and ketoconazole in Caco-2 cells

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**Abstract**

The effect of cytochrome P-450 3A (CYP3A) substrates (erythromycin, midazolam) and an inhibitor (ketoconazole) on P-glycoprotein-mediated transport was studied in Caco-2, the human colon adenocarcinoma cell line expressing various functions of differentiated intestinal epithelial cells. The involvement of P-glycoprotein in the transport of these drugs was also examined. The basal-to-apical transport of rhodamine 123, a P-glycoprotein substrate, was inhibited by erythromycin, midazolam and ketoconazole, as well as by P-glycoprotein inhibitors such as verapamil. The apical-to-basal transport of rhodamine 123 was increased by these drugs. The transepithelial transport of erythromycin and midazolam, but not of ketoconazole, was much greater from the basal to apical side than from the apical to basal side. The inhibitory effect of verapamil was observed on the basal to apical transport of erythromycin, but not on midazolam and ketoconazole transport. In conclusion, erythromycin, midazolam and ketoconazole could interact with P-glycoprotein-mediated transport, and P-glycoprotein could be, at least in part, involved in the transport of erythromycin, but not of midazolam and ketoconazole, in the intestinal epithelia. 

*Keywords:* P-glycoprotein; Cytochrome P-450 3A; Intestinal transport; Drug interaction; Caco-2 cell

## 1. Introduction

P-glycoprotein, a membrane glycoprotein of 170 kDa encoded by the multidrug resistance gene (*MDR1* in human), is responsible for conferring multidrug resistance by promoting the active efflux of chemotherapeutic agents out of cells (Gottesman and Pastan, 1993). The protein transports a variety of structurally and pharmacologically unrelated, hydrophobic drugs such as vinca alkaloids, anthracyclines, cyclosporin A, digoxin and glucocorticoids (Tanigawara et al., 1992; Ueda et al., 1992; Wacher et al., 1995; Tanaka et al., 1997). P-glycoprotein is expressed in normal tissues such as the brush-border membrane of renal proximal tubules, bile canalicular membrane of hepatocytes, intestinal epithelial cells, capillary endothelial cells of brain and testis, adrenal gland and placental trophoblasts as well as in multidrug-resistant tumor cells (Fojo et al., 1987; Thiebaut et al., 1987; Schinkel et al., 1994). This distribution of P-glycoprotein in various normal tissues indicates that the protein is involved in the excretion of xenobiotic and endogenous compounds into urine, bile and the intestinal lumen. In addition, the intestinal P-glycoprotein is thought to limit the absorption of drugs after oral administration (Hsing et al., 1992; Hunter et al., 1993; Terao et al., 1996; Sparreboom et al., 1997).

Cytochrome P450 (CYP) 3A is the enzyme responsible for the phase I metabolism of a variety of clinically used drugs. CYP3A is present primarily in hepatocytes and in the epithelial cells of the small intestine (McKinnon et al., 1995), where P-glycoprotein is also expressed. Recently, it has been suggested that there is overlap in the substrate specificity of CYP3A and P-glycoprotein (Wacher et al., 1995). However, further studies are needed to clarify the overlap and relative contribution of these functional proteins to protection of the body from unfavorable compounds including drugs. In this study, the effect of CYP3A substrates (erythromycin, midazolam) and an inhibitor (ketoconazole) on P-glycoprotein-mediated transport and the involvement of P-glycoprotein in the transport of these drugs were evaluated in Caco-2 cells. Caco-2 is a cell line derived from the human colon adenocarcinoma and is

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often used as a model for studying various functions of differentiated intestinal cells including that of P-glycoprotein (Hunter et al., 1993; Wils et al., 1994; Yee, 1997).

## 2. Materials and methods

### 2.1. Materials

The following reagents were used: rhodamine 123 (Kanto Chemical, Tokyo, Japan), verapamil hydrochloride and quinidine sulfate dihydrate (Wako, Osaka, Japan), erythromycin (Merck, Darmstadt, Germany), midazolam (Nippon Roche, Tokyo, Japan), ketoconazole (Janssen Pharmaceutica, Beerse, Belgium), cell culture medium and reagents (Gibco Laboratories, Life Technologies, Grand Island, NY, USA). All other chemicals used were of the highest purity available.

### 2.2. Cell culture

Caco-2 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% nonessential amino acids, 2 mM L-glutamine, 100 units/ml penicillin G and 100 μg/ml streptomycin in an atmosphere of 5% $CO_2$–95% air at 37°C, and were subcultured every 4 days using 0.02% EDTA and 0.05% trypsin. The cells were used between passages 57 and 77.

### 2.3. Transport study

Transport measurements were performed in a Transwell chamber (Costar, Cambridge, MA, USA) (Takano et al., 1994). To prepare cell monolayers, Caco-2 cell monolayers were seeded at a cell density of $3 \times 10^5$ cells/filter on a polycarbonate filter (pore size 3.0 μm) in Transwell cell chambers (4.71 $cm^2$ surface area). The volume of medium inside and outside the chambers was 1.5 and 2.6 ml, respectively. Cells were used at 16–21 days after seeding. Transport was measured at 37°C in Hanks' balanced salt solution containing 25 mM HEPES and 25 mM glucose (HBSS, pH 7.4). After removal of the culture medium, cells were washed with HBSS and allowed to preincubate for 10 min. Transepithelial transport was initiated by adding HBSS including rhodamine 123, erythromycin, midazolam or ketoconazole to the basal or to the apical side of the monolayers. After incubation for the specified period of time, the medium on the other side was collected, and the concentration of each drug was determined as described below.

### 2.4. Analytical methods

The concentration of rhodamine 123 was determined fluorimetrically (excitation, 501 nm; emission, 524 nm) with a Hitachi fluorescence spectrophotometer F-3000 (Tokyo, Japan). Other compounds were assayed by using a high-performance liquid chromatography (PU-980, Jasco, Tokyo, Japan) equipped with an electrochemical detector (840-EC, Jasco) for erythromycin and ketoconazole (oxidation at applied voltage of 1.0–1.2 V), or a UV spectrophotometric detector (UV-970, Jasco) for midazolam (wavelength, 229 nm). The conditions were as follows: column, $4.6 \times 150$ mm CAPCELLPACK $C_{18}$ SG120 (Shiseido, Tokyo, Japan) for erythromycin and ketoconazole, and $4.6 \times 100$ mm TSK gel ODS-80TM (Tosoh, Tokyo, Japan) for midazolam; mobile phase, 0.1 M phosphate buffer (pH 8.0)–acetonitrile–methanol (v/v) = 48:47:5 for erythromycin and 50:45:5 for ketoconazole, and methanol–water–0.1 M phosphate buffer (pH 7.4)–acetonitrile (v/v) = 63:26:10:1 for midazolam; flow rate 1.0 ml/min; temperature, room temperature. Protein was determined by the method of Bradford (1976) with bovine γ-globulin as the standard.

## 3. Results

### 3.1. Transepithelial transport of rhodamine 123 in Caco-2 cells

To confirm the existence of P-glycoprotein-mediated transport in Caco-2 cells, the transport of rhodamine 123, a substrate for P-glycoprotein, was examined. As shown in Fig. 1, the transepithelial transport of rhodamine 123 was more than 30-fold higher from the basal to apical side than from the apical to basal side (at 120 min, 131.7 ± 5.2,

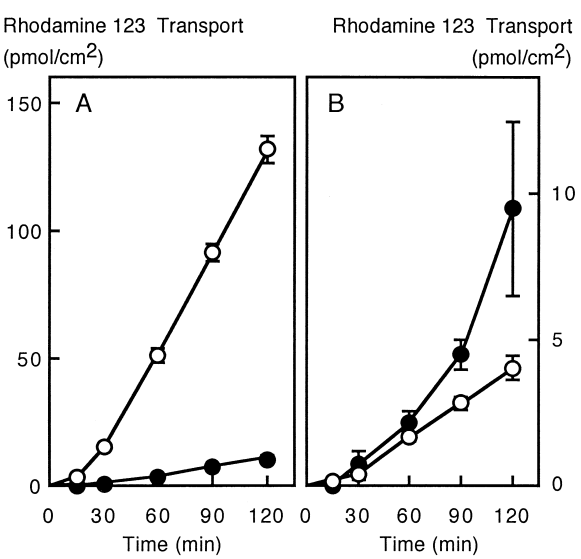


Fig. 1. Transepithelial transport of rhodamine 123 and effect of verapamil on the transport in Caco-2 cell monolayers. Rhodamine 123 (5 μM) was added to the basal side (A) or to the apical side (B) of monolayers in the absence (○) or presence (●) of 100 μM verapamil. After incubation for 15, 30, 60, 90 and 120 min, the medium on the other side was collected and assayed. Verapamil was applied to both the basal and apical sides. Each point is the mean ± S.E. of three experiments.

4.0 ± 0.4 pmol/cm$^2$, respectively). In addition, verapamil, an inhibitor of P-glycoprotein, decreased the basal-to-apical transport of rhodamine 123 (Fig. 1A) and increased the apical-to-basal transport of rhodamine 123 (Fig. 1B). Quinidine, another P-glycoprotein inhibitor, also decreased the basal-to-apical transport of rhodamine 123 and increased its apical-to-basal transport (data not shown). Thus, the expression of functional P-glycoprotein in Caco-2 cells was confirmed.

### *3.2. Effect of CYP3A substrate and inhibitor on transepithelial transport of rhodamine 123*

The effects of erythromycin, midazolam and ketoconazole on P-glycoprotein-mediated transport were examined in Caco-2 cells. As shown in Fig. 2, the basal-to-apical transport of rhodamine 123 was inhibited by all drugs examined. In addition, the apical-to-basal transport of rhodamine 123 was increased by these drugs (data not shown). These findings suggest that erythromycin, midazolam and ketoconazole interact with P-glycoprotein and inhibit its transport activity.

### *3.3. Transepithelial transport of CYP3A substrate and inhibitor*

The transport of erythromycin, midazolam and ketoconazole was examined in Caco-2 cells. The transepithelial transport of erythromycin was much greater from the basal to apical side than from the apical to basal side (Fig. 3A). The basal-to-apical transport of erythromycin was inhibited by verapamil (Fig. 3B). The concentration dependence of erythromycin transport across Caco-2 cell monolayers was examined (Fig. 4). The basal-to-apical transport of erythromycin was saturable when its concentration was

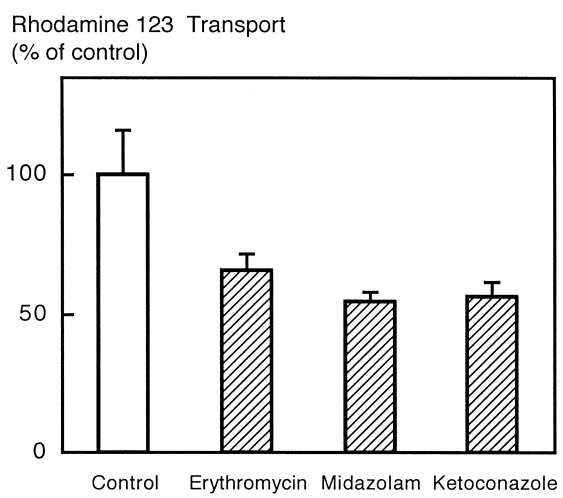


Fig. 2. Effect of erythromycin, midazolam and ketoconazole on basal-to-apical transport of rhodamine 123 in Caco-2 cell monolayers. Rhodamine 123 (5 μM) was added to the basal side of monolayers, and the transepithelial transport of rhodamine 123 from the basal to apical side was measured at 120 min in the absence (open column) or presence (hatched column) of 100 μM erythromycin, midazolam and ketoconazole. These compounds were applied to both the basal and apical sides. Rhodamine 123 transport (control) was 179.6 ± 28.5 pmol/cm$^2$ per 2 h. Each column is the mean ± S.E. of three experiments.

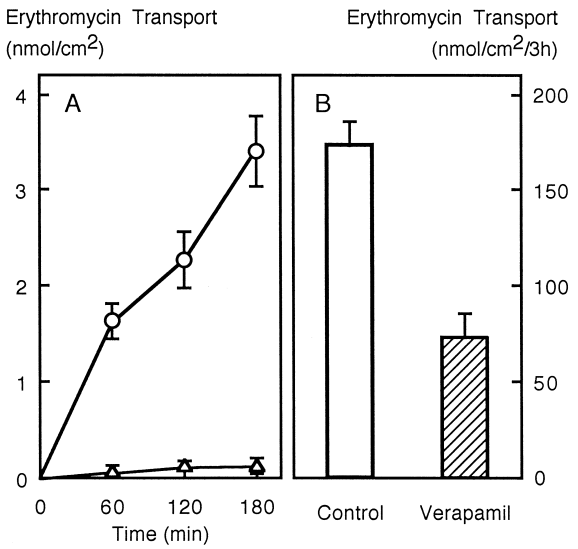


Fig. 3. Transepithelial transport (A) and effect of verapamil on basal-to-apical transport (B) of erythromycin in Caco-2 cell monolayers. (A) Erythromycin (50 μM) was added to the basal side (○) or to the apical side (△) of monolayers. After incubation for 60, 120 and 180 min, the medium on the other side was collected and assayed. (B) Erythromycin (10 μM) was added to the basal side of monolayers, and the transepithelial transport of erythromycin from the basal to apical side was measured at 180 min in the absence (open column) or presence (hatched column) of 100 μM verapamil. Verapamil was applied to both the basal and apical sides. Each point or column is the mean ± S.E. of three experiments.

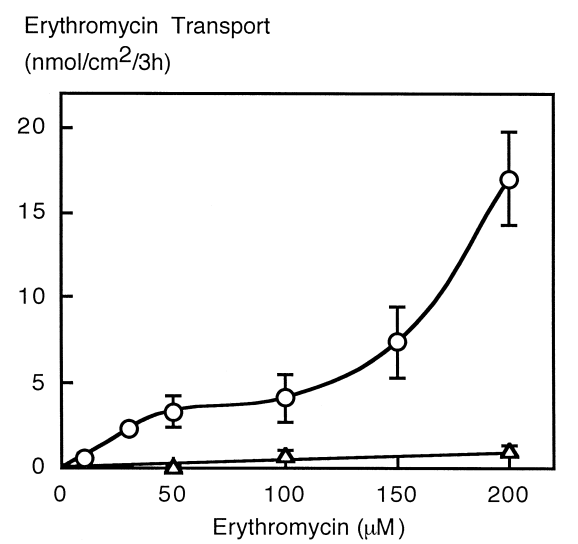


Fig. 4. Concentration dependence of the transepithelial transport of erythromycin in Caco-2 cell monolayers. Erythromycin of the various concentrations (10, 30, 50, 100, 150 and 200 μM) was added to the basal side (○) or to the apical side (△) of monolayers. After incubation for 180 min, the medium on the other side was collected and assayed. Each point is the mean ± S.E. of three experiments.

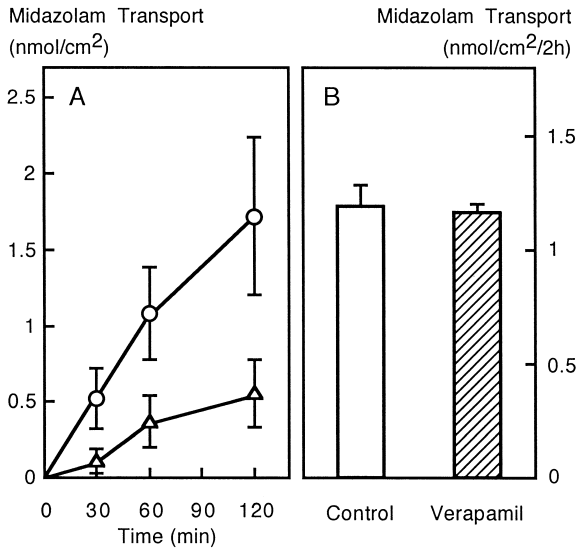


Fig. 5. Transepithelial transport (A) and effect of verapamil on basal-to-apical transport (B) of midazolam in Caco-2 cell monolayers. (A) Midazolam (10 μM) was added to the basal side (○) or to the apical side (△) of monolayers. After incubation for 30, 60 and 120 min, the medium on the other side was collected and assayed. (B) midazolam (10 μM) was added to the basal side of monolayers, and the transepithelial transport of midazolam from the basal to apical side was measured at 120 min in the absence (open column) or presence (hatched column) of 100 μM verapamil. Verapamil was applied to both the basal and apical sides. Each point or column is the mean ± S.E. of three experiments.

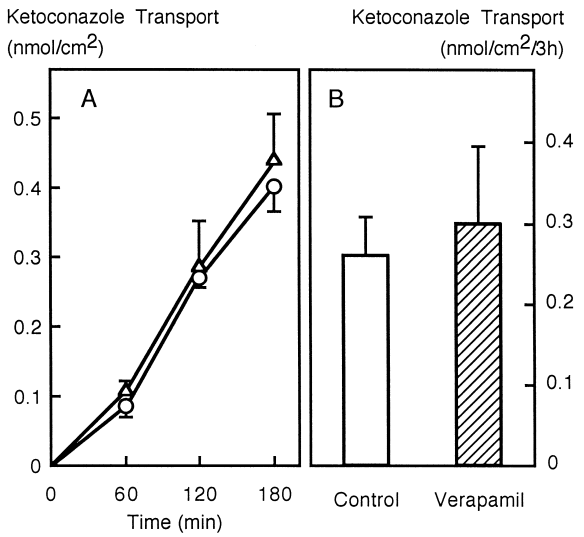


Fig. 7. Transepithelial transport (A) and effect of verapamil on basal-to-apical transport (B) of ketoconazole in Caco-2 cell monolayers. (A) Ketoconazole (10 μM) was added to the basal side (○) or to the apical side (△) of monolayers. After incubation for 60, 120 and 180 min, the medium on the other side was collected and assayed. (B) Ketoconazole (10 μM) was added to the basal side of monolayers, and the transepithelial transport of ketoconazole from the basal to apical side was measured at 180 min in the absence (open column) or presence (hatched column) of 100 μM verapamil. Verapamil was applied to both the basal and apical sides. Each point or column is the mean ± S.E. of three experiments.

less than 100 μM, but transport was increased again at higher concentrations. In contrast, the apical-to-basal transport was much smaller than the basal-to-apical transport at all concentrations examined, and the transport was increased linearly with erythromycin concentration. We then examined whether the transport of midazolam across Caco-2 cell monolayers is a vectorial process or not. The transport of midazolam from the basal to apical side at the concentration of 10 μM was also greater than that from the apical to basal side (Fig. 5A). In contrast to erythromycin, however, verapamil did not affect the basal-to-apical transport of midazolam (Fig. 5B). In addition, when the concentration of midazolam was raised, the ratio of the basal-to-apical transport to the apical-to-basal transport of midazolam approached to unity (Fig. 6). In the case of ketoconazole, there was no difference between transepithelial transport from the basal to apical side and from the apical to basal side (Fig. 7A), and the basal-to-apical transport was not affected by verapamil (Fig. 7B).

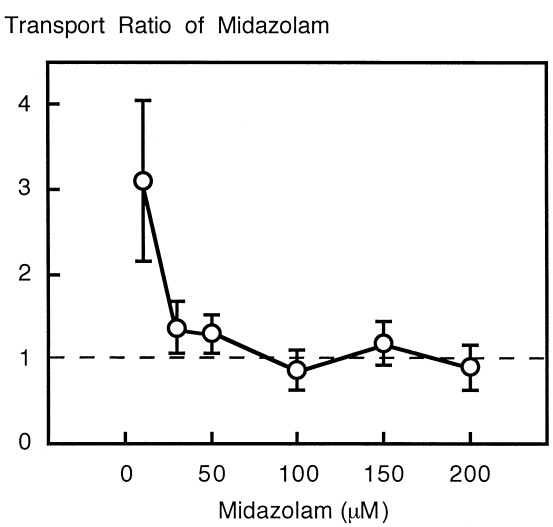


Fig. 6. Transepithelial transport of midazolam in Caco-2 cell monolayers. Midazolam at various concentrations (10, 30, 50, 100, 150 and 200 μM) was added to the basal side or to the apical side of monolayers. After incubation for 120 min, the medium on the other side was collected and assayed. Each value is expressed as the ratio of basal-to-apical transport to apical-to-basal transport at each concentration. Each point is the mean ± S.E. of three experiments.

## 4. Discussion

In recent years, some reports have suggested the involvement of intestinal CYP3A in decreasing the bioavailability of orally administered drugs. In addition, the substrate specificity of CYP3A and P-glycoprotein is suggested to overlap. P-glycoprotein is expressed in the apical membrane of mucosal cells in the intestine and thus this protein may also play an important role in the absorption of orally administered drugs. Lown et al. (1997) reported

that the interpatient variation in the oral bioavailability of cyclosporin A depended on variation in the expression of intestinal P-glycoprotein as well as on the activity of hepatic CYP3A. Thus, P-glycoprotein in the intestine would be an important determinant of drug absorption. In the present study, we examined whether erythromycin, midazolam (CYP3A substrates) and ketoconazole (CYP3A inhibitor) interact with P-glycoprotein-mediated transport, using the human intestinal cell line Caco-2.

Erythromycin is frequently used in the treatment of infections caused by *Legionella pneumophila*, *Mycoplasma pneumoniae* and other bacteria. Coadministration of erythromycin with cyclosporin A, a substrate for both CYP3A and P-glycoprotein, increases the blood cyclosporin A concentration, possibly by the inhibition of the hepatic metabolism of cyclosporin A by erythromycin (Ptachcinski et al., 1985; Kohan, 1986). Recent evidence suggests that the increase in the absorption of cyclosporin A by erythromycin is also involved in the change in blood cyclosporin A concentration (Campana et al., 1996). The present results showed that erythromycin inhibits P-glycoprotein-mediated transport. Therefore, erythromycin affects the absorption of cyclosporin A, probably by decreasing the P-glycoprotein-mediated excretion of the drug from the cells back into the intestinal lumen. We also showed that the transport of erythromycin from the basal to apical side in Caco-2 cells is greater than that from the apical to basal side as reported by Yee (1997), and that the basal-to-apical transport is decreased by the addition of verapamil, a P-glycoprotein inhibitor. Erythromycin was transported from the basal-to-apical side in a saturable manner at concentrations lower than 100 μM, but the transport was increased again at higher concentrations. Thus, in contrast to other transcellular transport systems such as *p*-aminohippurate transport in OK cells (Hori et al., 1993), the basal-to-apical transport of erythromycin could not be explained by simple Michaelis–Menten kinetics. Therefore, we did not estimate the kinetic parameters for erythromycin transport. Rather, it may be more important that a similar pattern was observed for the transport of rhodamine 123 in Caco-2 cells in the present study (data not shown) and for cepharanthin, a substrate for P-glycoprotein, in P-glycoprotein-overexpressing LLC-$PK_1$ cells (Hirai et al., 1995). Recently, Schuetz et al. (1998), using derivative LLC-$PK_1$ cells expressing P-glycoprotein and *mdr1a* (−/−) mice, suggested that P-glycoprotein affects the transport of erythromycin. Taken together, erythromycin appears to be a substrate for P-glycoprotein. Thus, the poor absorption of erythromycin with 30–40% bioavailability (Somogyi et al., 1995) might be, at least in part, due to the P-glycoprotein-mediated excretion of the drug into the intestinal lumen.

Azole antifungal agents such as ketoconazole and itraconazole have been also reported to interact with cyclosporin A and to increase blood cyclosporin A concentrations (Campana et al., 1996). In addition, Floren et al. (1997) reported the marked increase in the bioavailability of another immunosuppressive agent tacrolimus, a substrate for both P-glycoprotein and CYP3A, after the coadministration of ketoconazole. The present results showed that ketoconazole inhibited the P-glycoprotein-mediated transport of rhodamine 123 in Caco-2 cells, as reported in multidrug-resistant KB-V1 cells (Siegsmund et al., 1994). Therefore, the inhibition of drug excretion into the intestinal lumen via P-glycoprotein might play an important role in the increased absorption of cyclosporin A and tacrolimus. However, the directional transport of ketoconazole was not observed in Caco-2 cells. In addition, there was no effect of verapamil on the transport of ketoconazole. These results suggest that ketoconazole could inhibit P-glycoprotein-mediated transport, but the drug itself may not be transported by P-glycoprotein.

Midazolam, a 1,4-benzodiazepine, is widely used as a short-acting hypnotic agent and for the induction of anesthesia. In the present study, midazolam inhibited P-glycoprotein-mediated transport in Caco-2 cells. This finding could indicate that midazolam affects the intestinal absorption of P-glycoprotein substrates, such as anticancer drugs, immunosuppressive agents and digoxin. There are several reports indicating that the bioavailability of orally administered midazolam is increased by the coadministration of drugs such as erythromycin, ketoconazole and itraconazole (Olkkola et al., 1993, 1994). These increases in the absorption of midazolam can be explained mainly by the inhibition of CYP3A metabolism in the intestine and in the liver, but not by the interaction with the intestinal P-glycoprotein, because our results suggest that midazolam is not a substrate for P-glycoprotein. However, the secretory efflux of midazolam across Caco-2 cells was observed at low concentrations, which may indicate the involvement of a high-affinity and low-capacity transport system. Therefore, transporter(s) other than P-glycoprotein might be involved in midazolam transport in the intestine and in the drug interactions described above.

In conclusion, erythromycin, midazolam and ketoconazole affect P-glycoprotein-mediated transport in Caco-2 cells. In addition, the transport of erythromycin, but not the transport of midazolam and ketoconazole, is, at least in part, mediated by P-glycoprotein. Since the cells were not grown under selective media and the conclusions were contingent upon the functional expression of P-glycoprotein in these cells, it will be important to demonstrate that P-glycoprotein is indeed expressed in these Caco-2 cells using Western blot analysis.

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