Carrier-Mediated Transport of Macrolide Antimicrobial Agents Across Caco-2 Cell Monolayers

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INTRODUCTION

Erythromycin (EM) is known to be poorly absorbed from the gastrointestinal tract after oral administration due to extensive hydrolysis in the stomach under acidic conditions (1). Clarithromycin (CAM) was synthesized in order to overcome this disadvantage of EM, as a new 14-membered macrolide antibiotic in which the hydroxy group of EM is methylated at the C6 position of the lactone ring (2). CAM is very stable in acidic solution (3), well absorbed after oral administration and is distributed to various tissues in animals at significantly higher levels than EM. Moreover, CAM has excellent clinical efficacy and is now in widespread clinical use (4).

Recent studies of drug absorption from the gastrointestinal tract have suggested P-glycoprotein (P-gp) acts as an intestinal excretion system, and that it might reduce the oral absorption of various hydrophobic compounds (5). In addition, some substrates of P-gp are substrates of P-450 (CYP3A4) simultaneously (6), and first-pass metabolism in intestinal cells also limits their bioavailability. Since EM and CAM are substrates of CYP3A4 (7,8), and EM and CAM also inhibit P-gp in liver and kidney, it is presumed these systems are involved in the process of absorption from the GI tract of both drugs affecting their oral bioavailability.

The use of the transformed human intestinal cell line, Caco-2, as a model of small intestinal cell absorption has been proposed by several investigators. Although Caco-2 cells express P-gp on their apical (brush-border) membrane (9), they appear to express insignificant levels of drug metabolizing enzymes of the cytochrome P450 class, which are found at high levels in the human intestine (10). This functional feature of Caco-2 cells might permit evaluation of the effect of P-gp on the intestinal absorption of CAM and EM, without any effects by CYP3A4 metabolism.

In this study, we focused on the mechanisms of membrane transport of EM and CAM. The effects of drug concentration and P-gp inhibitors on the apical-to-basal and basal-to-apical transport of these drugs were investigated in Caco-2 cell monolayers.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle medium (DMEM), non-essential amino acids (NEAA), fetal bovine serum (FBS), L-glutamine (200 mM), trypsin (0.25%)-EDTA (1 mM), and antibiotic-antimycotic mixture (10000 U/ml penicillin G, 10000 μ g/ml streptomycin sulfate and 25 μg/ml amphotericin B in 0.85% saline) were purchased from Life Technologies, Inc., Rockville, MD, USA. [^{14}C]-CAM and [^{14}C]-EM were purchased from Daiichi Pure Chemicals Co.,Ltd. and American Radiolabeled Chemicals, Inc., respectively. All other reagents used were of the highest purity.

Preparation of Caco-2 Monolayer

Caco-2 cells (American Type Culture Collection, Rockville, MD) were seeded on polycarbonate filters (0.3 μm pores, 4.71 cm^2 growth area) inside Transwell cell culture chambers (Coaster, Cambrige, MA) at a density of 2×10^5 cells/filter. The cells were grown in DMEM supplemented with 10% FBS, 1% L-glutamine, 1% NEAA, and 5% antibiotic-amimycotic solution at 37°C in a humidified air-5% CO_2 atmosphere (11). The culture medium (1.5 ml in the insert and 2.6 ml in the well) was first replaced after 72 hour and every 48 hour thereafter. The transport studies were conducted with the monolayers between 15 to 20 days in culture. Cell passage number were between 40 to 48.

Transport Studies

The transport experiments were performed in Hank's balanced salts solution (HBSS) using Transwell cell culture chambers. Initially, the Caco-2 cell monolayers were pre-incubated for 20 min at 37°C in HBSS. Then, new prewarmed HBSS containing a radioactive compound, [^{14}C]-CAM or [^{14}C]-EM, was applied to either the apical (1.5 ml) or the basal (2.6 ml) side of Caco-2 cell monolayers and drug-free HBSS was applied to the opposite side. In the inhibition study, verapamil was added to either the apical or the basal side at a concentration of 0.5 mM (12). Aliquots of samples (0.1 ml) were taken from the apical or the basal side every 10 minutes for 1 hour and were replaced with equal volumes of drug-free HBSS. The apical-to-basal or basal-to-apical permeability of each drug was calculated from its flux rate estimated as the rate of increase in the basal or apical concentration.

Drug Accumulation in Caco-2 Cells

After the transport experiment, the Caco-2 monolayer was rinsed with ice-cold saline solution, blotted gently to remove residual water, and then scraped off to be dissolved overnight with 1N NaOH. The accumulated amount of drugs in Caco-2 cells was calculated from the radioactivity in the dissolved tissue sample and expressed as nmol/mg protein. The amount of total protein in each sample was determined by the method of Lowry *et al.* (13).

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Analytical Methods

The radioactivity of the samples was determined by liquid scintillation counter (LS6000TA, Beckman Instruments, Inc., CA).

Kinetic Analysis of Drug Transport Across Cell Membranes

Kinetic parameters for drug transport across Caco-2 cell membranes were estimated according to the following equation, based on the drug concentration in Caco-2 cells.

$$J_{ca} \text{ or } J_{cb} = J_{max} \cdot C_{cell}/(Km + C_{cell}) + P_{dif} \cdot C_{cell} \quad (1)$$

where J_{ca} and J_{cb} are the cell-to-apical and cell-to-basal rate of flux of drugs (n mol/cm^2/min), respectively, C_{cell} is the drug concentration in the Caco-2 cell (n mol/mg protein), Km is the Michaelis constant (n mol/mg protein), J_{max} is the maximum flux rate (n mol/cm^2/min), and P_{dif} is the nonspecific efflux clearance (mg protein/cm^2/min). Assuming the paracellular transport of both drugs is negligible due to their large molecular weight (733.94 and 747.96 for EM and CAM, respectively), the transmural flux rate is identical to the flux rate across the cell membrane, i.e.,

$$J_{ab} = J_{cb} \text{ and } J_{ba} = J_{cb} \quad (2)$$

where J_{ab} and J_{ba} are the apical-to-basal and basal-to-apical flux rates, respectively. Then, Eq. (1) was fitted to the data sets (transmural flux rate and concentration in Caco-2 cells) by an iterative nonlinear least-squares method using a MULTI program (14) to obtain kinetic parameters for the cell-to-apical or cell-to-basal flux of each drug.

RESULTS

Figure 1 shows the time course of apical-to-basal (a → b) and basal-to-apical (b → a) transport of CAM and EM across Caco-2 monolayers. The initial concentration of drugs applied was 0.5 mM. The a → b and b → a transport rates of CAM were both faster than those of EM. In the case of CAM, a → b flux was greater than b → a flux at this concentration. On the other hand, b → a transport of EM was much faster than a → b transport, suggesting the involvement of efflux systems in its transport from the basal to apical direction.

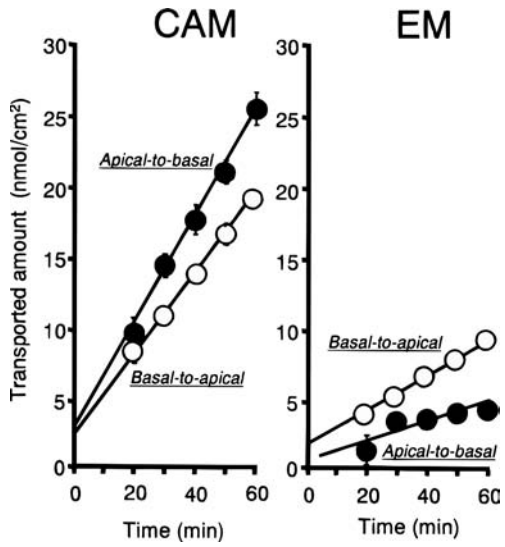

Fig. 1. Transport of CAM and EM across Caco-2 monolayers. CAM or EM was added to apical or basal side of Caco-2 monolayers at an initial concentration of 0.5 mM. Each point represents the mean ± S.E. of at least three experiments.

The effects of verapamil, a typical inhibitor of P-gp efflux pump, on a → b and b → a permeability of CAM and EM are shown in Fig. 2. In the case of EM, verapamil markedly increased a → b permeability and reduced b → a permeability, regardless of the side to which it was added. Verapamil also significantly increased the a → b permeability of CAM, but its effect on b → a permeability was not clear. When cyclosporin A was used as the inhibitor instead of verapamil, almost the same results were obtained (data not shown). The b → a permeability of CAM increased significantly only when verapamil was added to the basal side.

The permeability coefficients for both a → b and b → a directions were determined at six different concentration of CAM and EM (Fig. 3). For both drugs, b → a permeability decreased with increasing drug concentration on the donor (basal) side. At low concentrations, the b → a permeability of CAM was significantly higher than a → b permeability, suggesting that efflux systems, probably P-gp, participated in the transport of not only EM but also CAM. The a → b permeability of EM increased slightly with increasing concentrations of EM, whereas that of CAM peaked at 0.05 mM concentration, sharply decreased, and then became constant in the range of higher concentrations.

Figure 4 shows the relationship between drug concentration added to the apical or basal side and the steady-state accumulation of drugs into Caco-2 cells, which was standardized to the initially applied drug concentration and represented as n mol/mg protein/mM. The cellular accumulation of CAM was markedly higher than that of EM regardless of the side of drug application, and increased with increasing drug concentration in the range of 0.01 to 0.25 mM. EM exhibited nearly constant accumulation at all concentrations tested. Cellular accumulation of CAM from the apical side was about twice that from the basal side. In contrast, EM exhibited higher accumulation from basal solution.

The a → b and b → a transport rates of both drugs were plotted against their accumulated amount Caco-2 cells (Fig. 5). This figure enables characterization of the transport of drugs across apical and basal membranes of Caco-2 cells, individually. For both drugs, cell-to-apical transport exhibited clear saturation against cellular concentration, indicating the involvment of a carrier-mediated process. A saturable process was also detected in the cell-to-basal transport of CAM across the basal membrane, but that of EM was non-saturable. Kinetic analysis based on the Michaelis-Menten equation revealed that both drugs possess Km and Jmax values similar to those of the efflux system at the apical membrane (Table 1). The affinity of CAM for the saturable process in cell-to-basal transport was lower (thus the higher value of Km) than that for the process in cell-to-apical transport.

DISCUSSION

Concerning transport of EM across Caco-2 monolayers, all results presented here clearly indicate EM is effluxed by P-gp from the inside of the cell to the apical solution. The effect

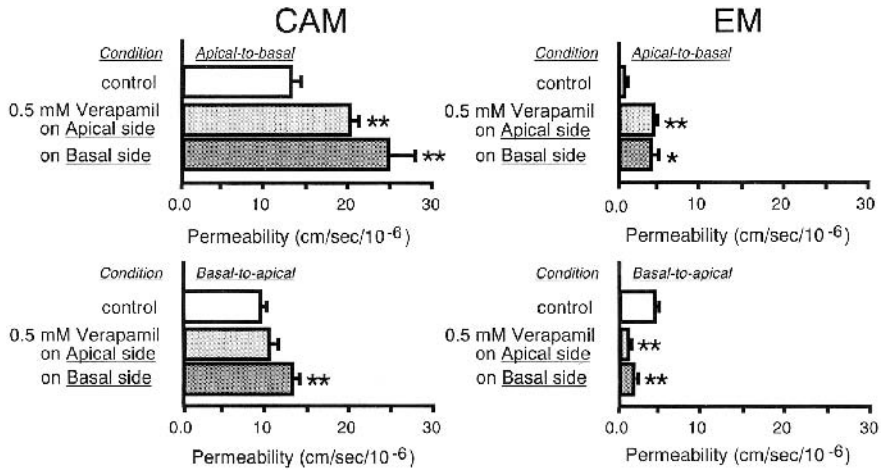

Fig. 2. Effect of P-glycoprotein inhibitor (verapamil) on the permeability of CAM and EM to Caco-2 monolayers. CAM or EM was added to apical or basal side of Caco-2 monolayers at an initial concentration of 0.5 mM with or without verapamil. Initial concentration of verapamil applied was 0.5 mM. Each bar represents the mean ± S.E. of at least three experiments. Significant differences are *: $P < 0.05$ and **: $P < 0.01$.

of verapamil on the transport of EM (Fig. 2) was characterized by a typical pattern of P-gp-mediated transport (15). The saturable fraction detected in the cell-to-apical transport of EM (Fig. 5) appeared to represent the fraction of active efflux by P-gp at the brush-border membrane. The partition coefficient of EM measured in an n-octanol/transport medium (pH 7.4) system was 12.7, and thus logD was 1.1. EM thus appeared to exhibit fairly high permeability through the intestinal membrane via a simple diffusion mechanism. In fact, as shown in Fig. 2, the a → b permeability of EM after inhibition of P-gp is high enough (about 4.2 cm/sec x 10^{-6}) to assume good oral absorption in humans (16). The very low permeability of EM in the absorptive (a → b) direction in Caco-2 monolayers was thus largely due to active efflux by P-gp, which might account in part for the low bioavailability of EM after oral administration.

In contrast, the mechanisms of transport of CAM in Caco-2 monolayers appear to be more complicated. In Fig. 5, a saturable fraction was detected in cell-to-apical transport of CAM with kinetic parameters (Km, Jmax) similar to those of EM. This finding indicates the efflux of CAM across the brush-border membrane is also promoted by the P-gp active efflux system. This result is consistent with the report that CAM inhibited the P-gp-mediated tubular secretion of digoxin (17).

However, even in the presence of P-gp, CAM exhibited high permeability to Caco-2 monolayers in both directions (13.2 cm/sec × 10^{-6} for a → b and 9.5 cm/sec × 10^{-6} for b → a), indicating good oral absorption in humans. This might have been due to CAM having a higher partition coefficient (logD

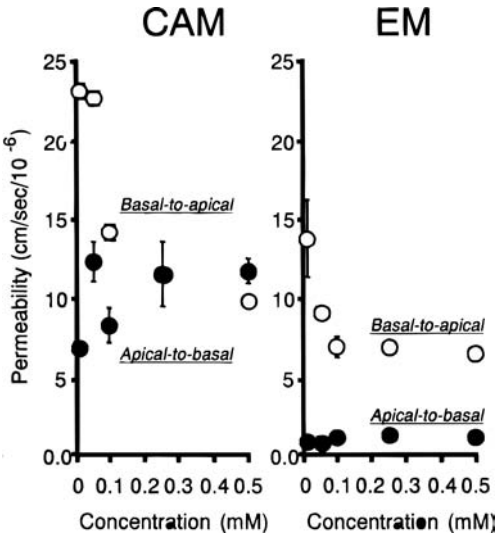

Fig. 3. Concentration-dependence of CAM and EM permeability across Caco-2 monolayers. CAM or EM was added to apical or basal side of Caco-2 monolayers at various concentrations. Initial concentration of verapamil applied was 0.5 mM. Each point represents the mean ± S.E. of at least three experiments.

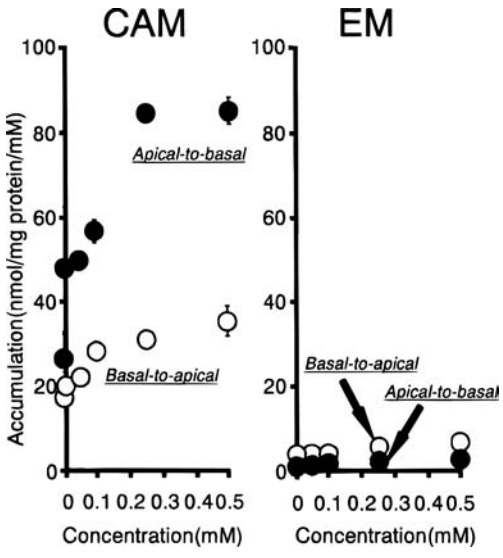

Fig. 4. Concentration-dependence of CAM and EM accumulation in Caco-2 cells. Steady-state accumulation of CAM and EM into Caco-2 cells was measured after finishing the transport experiments. Cellular accumulation was standardized to the initially applied drug concentration and represented as n mol/mg protein/mM. Each point represents the mean ± S.E. of at least three experiments.

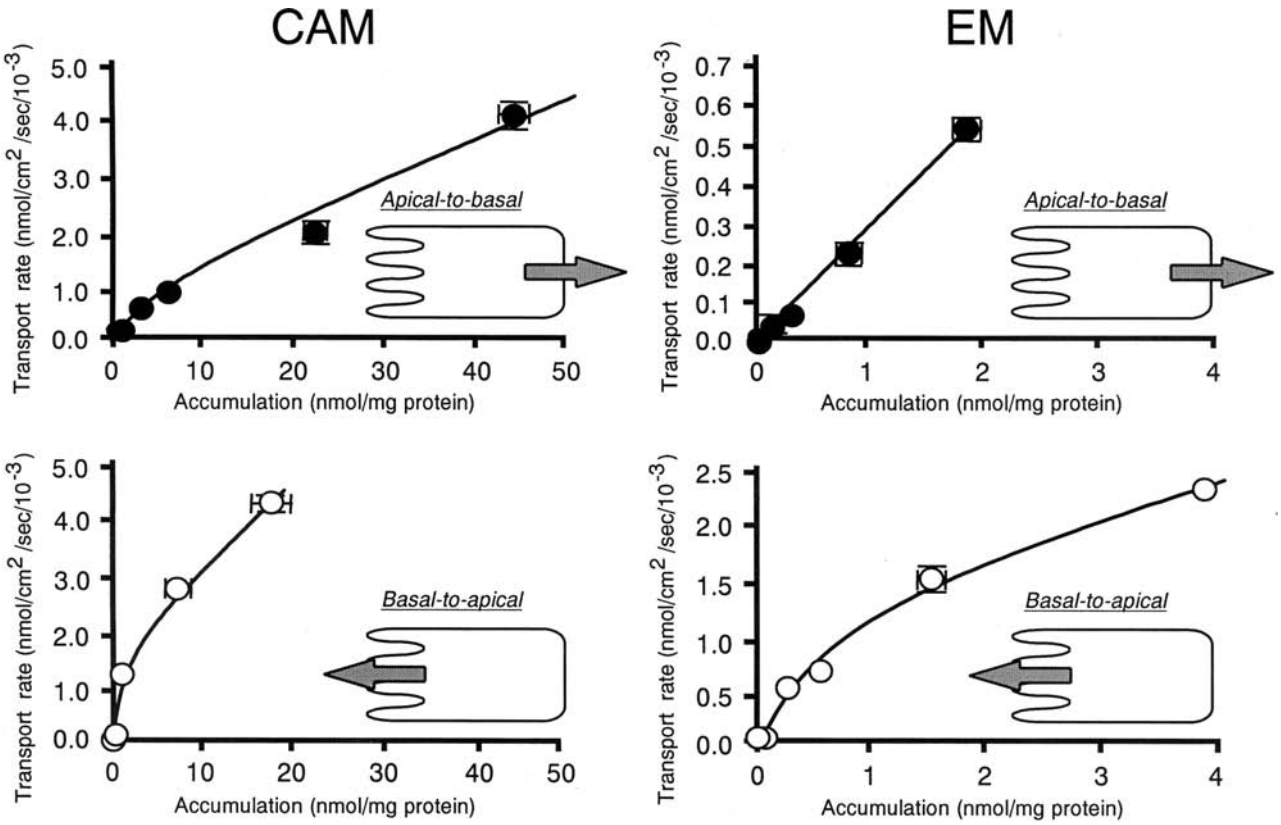

Fig. 5. Accumulation-dependence of CAM and EM transport across Caco-2 monolayers. Transport rates of CAM and EM were plotted against their accumulated amount in Caco-2 cells. The line in each figure demonstrates the theoretical correlation obtained by the nonlinear least-squares fitting based on the Michaelis–Menten equation. Each point represents the mean ± S.E. of at least three experiments.

= 1.8) than EM. When CAM was applied to the apical side of the monolayer, its influx into cells across the brush-border membrane should have been faster than the capacity of active efflux by P-gp, resulting in high permeability in the a → b direction. The finding of very high cellular accumulation of CAM compared with that of EM (Fig. 4) also indicates a high rate of influx of CAM into cells from both apical and basal solutions. In the case of EM, the rate of influx across the brush-border membrane was low and a large portion of the influxed EM could be effluxed again promptly by P-gp. This resulted in the low permeability of EM in the a → b direction, although the affinity of EM for P-gp is almost the same as with that of CAM.

Table 1. Kinetic Parameters of CAM and EM Transport Based on the Cellular Concentration of Drugs

	Apical to Basal		Basal to Apical	
	CAM	EM	CAM	EM
Km	5.23	–	1.26	0.645
J_{max}	1.03	–	1.75	1.40
P_{dif}	0.07	0.27	0.15	0.30

Note. Parameters were determined by Damping Gauss-Newton analysis according to equation (1) in the text. Parameters were expressed as follows: Km (nmol/mg protein), J_{max} (nmol/cm^2/sec/10^{-3}), P_{dif} (mg protein/cm^2/sec/10^{-3}).

In addition to the high partition of CAM to the cellular membrane, some of our findings suggest the possibility that transporters other than P-gp participate in the transport of CAM across Caco-2 monolayers. In particular, the concentration-dependent pattern of a → b permeability of CAM (Fig. 3), which peaked at 0.05 mM, appeared to be similar to that demonstrated for azasetron absorption from rat intestinal loop (18) where at least two nonlinear events are suggested to be involved in the intestinal absorption of azasetron. If this is also the case for CAM transport, the increase in a → b permeability observed at lower concentration corresponds to the saturation of P-gp efflux. At a higher concentration, a transporter of CAM in the absorptive direction was saturated, decreasing a → b permeability. The saturated fraction detected in the cell-to-basal transport of CAM (Fig. 5), and the enhanced b → a permeability of CAM by verapamil (Fig. 2) might relate to transporters other than P-gp. However, we have at present no further evidence to support this hypothesis. Identification of the absorptive transporter of CAM in Caco-2 cells is now proceeding.

In conclusion, we have demonstrated both EM and CAM are substrates of P-gp, and that both are actively effluxed in cell-to-apical direction in Caco-2 monolayers. In the presence of P-gp, CAM exhibited high permeability in the absorptive direction due to the high partition to the cell membrane which saturated the active efflux system. In addition, multiple transporter mechanisms appeared to participate in the transmural transport of CAM.

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