Transport Characteristics of Ebastine and Its Metabolites across Human Intestinal Epithelial Caco-2 Cell Monolayers

Yuichiro IMAMURA,a Kazuteru SHIMIZU,b Fumiyoshi YAMASHITA,a Kiyoshi YAMAOKA,b Yoshinobu TAKAKURA,a,b and Mitsuru HASHIDA*a

Departments of Drug Delivery Researcha and Biopharmaceutics and Drug Metabolism,b Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606–8501, Japan.

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The transport characteristics of a selective peripheral H1 receptor antagonist, ebastine, a substrate for cytochrome P450 3A4, and its three major metabolites, i.e., the hydroxyl metabolite of ebastine (M-OH), the pharmacologically active metabolite carbastine (Car), and the desbutyrophenone metabolite (des-BP), were studied in cultured human intestinal Caco-2 cells expressing a drug efflux pump, P-glycoprotein (P-gp), on the apical membrane. The polarized transport of [3H]cyclosporin A (CyA), mediated by P-gp in the basolateral to apical direction across the Caco-2 cell monolayers, was affected by the presence of ebastine in a concentration-dependent manner and significant inhibition was observed at high concentrations (>50 μM). M-OH (300 μM) also significantly inhibited whereas Car and des-BP did not. Although no marked polarized transport of [14C]ebastine in a secretory direction was observed in the Caco-2 systems, the flux in the basolateral to apical direction was slightly higher than that in the opposite direction at concentrations less than 30 μM. [14C]Ebastine (2 μM) uptake from the apical side was significantly increased in the presence of an excess of cold CyA, suggesting that the efflux process mediated by P-gp may be involved in the ebastine uptake by Caco-2 cells. Collectively, these results indicate that ebastine (and presumably M-OH) is transported via P-gp in Caco-2 cells, however, the affinity for P-gp is very low. It is unlikely that the secretory transport of ebastine mediated by P-gp will dramatically affect overall intestinal absorption in vivo because efficient passive diffusion of this drug should occur due to its high lipophilicity. However, it may be advantageous for its efficient first-pass metabolism.

Key words: P-glycoprotein; ebastine; Caco-2 cell; intestinal absorption; cyclosporin A; CYP3A4

The intestinal epithelial cells are the first site for the metabolism of orally administered drugs. It has been demonstrated that intestinal cytochrome P450s contribute significantly to the first-pass metabolism of a variety of drugs, although it is widely accepted that the liver plays the most important role.1) Cytochrome P450 3A4 (CYP3A4) appears to be the principal cytochrome P450 enzyme in the human intestine2)–4) with a very broad substrate specificity. The contribution of intestinal CYP3A4-mediated metabolism to poor oral drug bioavailability has been shown to be clinically important for some drugs.5) P-Glycoprotein (P-gp) is an active drug efflux pump that has been shown to transport a variety of structurally diverse compounds.6) It is expressed on the apical membrane of intestinal epithelial cells, where it can secrete drugs from the intracellular space back into the intestinal lumen.7) Recently, it has been shown that there is a strong overlap between substrates for gut metabolism by CYP3A4 and gut active efflux by P-gp, suggesting that these two processes may work together to affect significantly the intestinal absorption of a variety of drugs.8–9) Accordingly, an understanding of the likelihood of interaction of drugs of interest with both CYP3A4 and P-gp is essential for their rational.10–12)

The selective peripheral H1 receptor antagonist, ebastine, belongs to a new generation of antihistamines characterized by negligible anticholinergic and antiserotonergic properties.13,14) After oral administration of ebastine to animals and human subjects, it was well absorbed and underwent complete first-pass metabolism in the gastrointestinal tract to its pharmacologically active, acid metabolite, carbastine (Car), and other metabolites.15–17) Furthermore, a previous study has demonstrated a significant contribution by the small intestine to the metabolic conversion of ebastine to its active principle, Car, after oral administration in rats.18) Previous in vitro metabolism studies using human liver microsomes have also demonstrated that N-dealkylation to desbutyrophenone metabolite (des-BP) and oxidation to Car are predominantly mediated by CYP3A4, whereas hydroxylation to hydroxy metabolite of ebastine (M-OH) is mediated mainly by unidentified P450 isoform(s) other than CYP3A (Fig. 1).19) The purpose of the present study, therefore, was to clarify whether the P-gp efflux system in the intestinal epithelium is involved in the absorption of ebastine and its metabolites after oral administration. We investigated the transport characteristics of ebastine and its three major metabolites in vitro using a human intestinal epithelial Caco-2 cell system expressing P-gp on its apical surface.20)

MATERIALS AND METHODS

Chemicals [3H]Cyclosporin A (CyA) (specific activity = 8.00 Ci/mmol) was purchased from Amersham (Tokyo, Japan). [14C]Mannitol (specific activity = 51.5 mCi/mmol) was purchased from NEN™ Life Science Products, Inc. (Boston, MA, U.S.A.). Ebastine, its three major metabolites, i.e., M-OH, Car, des-BP, and [14C]ebastine (16.49 mCi/ mmol) were kindly supplied by Dainippon Pharmaceutical Co., Ltd.

Cell Culture Human colon adenocarcinoma Caco-2 cells (ATCC HTB37), obtained from the American Type Culture Collection (Rockville, MD, U.S.A.), were used between 38 to 48 passages. The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids

* To whom correspondence should be addressed. e-mail: hashidam@pharm.kyoto-u.ac.jp © 2001 Pharmaceutical Society of Japan
(NEAA), 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified 37 °C incubator with 5% CO₂. For subculture, cells were split using trypsin-EDTA every week at a split ratio of 1:3. The medium was changed every 2—3 d. For transport experiments, the cells were seeded at a density of 0.5 × 10⁵ cells/ml onto the membranes of culture inserts, Transwell (Corning Costar Cambridge, MA, U.S.A.) permeable polycarbonate inserts (0.4 μm pore size). Subsequent medium changes were at 2-d intervals and 14—21-d-old cell monolayers were used for transport studies.

Transport Experiments with [³H]CyA

Transport of CyA across Caco-2 cell monolayers was evaluated by applying [³H]CyA either to the apical (A) (luminal) or basolateral (B) (serosal) side of the cell monolayer grown to confluence on polycarbonate membrane inserts of Transwell. The volume of the apical cell layer (insert) was 0.5 ml, and that of the basolateral side (well) was 1.5 ml. Before experiments, the cells were washed twice with warm HBSS, pH 7.4. The effects of ebastine (1 to 300 μM), M-OH (300 μM), Car (300 μM), and des-BP (300 μM) on the transport of [³H]CyA (280 nM), a typical substrate for P-gp, 21,22 across the Caco-2 cell monolayers were studied in the presence of ebastine and its three metabolites in the same side as [³H]CyA. The final concentration of ethanol used to dissolve ebastine and its metabolites in buffer was less than 1%. The integrity of the tight junctions of Caco-2 cell monolayers was confirmed by measuring the transepithelial electrical resistance (TER, normally ≥300 Ω cm²) of cell monolayers during the experiment with a Millicell-ERS meter (Millipore Corp., Bedford, MA, U.S.A.). In each set of experiments, the transport of [¹⁴C]mannitol was monitored using Caco-2 cell monolayers cultured under the same conditions. The cell monolayers were considered tight when the mannitol transport was <0.5% of the dose/h. Samples of [³H]CyA were taken from the contralateral side at 30, 60, 90, 120, and 180 min. Overall transport rates were determined by measuring the total radioactivity present on the desired Transwell side by liquid scintillation counting after addition of Clear-sol I (Nacalai Tesque, Kyoto, Japan).

Transport and Uptake Experiments with [¹⁴C]Ebastine

The transport characteristics of [¹⁴C]ebastine were also examined in the same Caco-2 system. Before experiments, the cells were washed three times with warm HBSS, pH 7.4, and TER values were measured after the last wash. The buffer was then replaced with fresh HBSS buffer on one side of the cell layer and [¹⁴C]ebastine (10—100 μM) in HBSS buffer on the other side. Samples of [¹⁴C]ebastine were taken from the contralateral side periodically up to 6 h and their radioactivity measured by counting in a liquid scintillation counter after the addition of Clear-sol I. In a separate experiment, [¹⁴C]ebastine (2 μM) uptake by Caco-2 cells from the apical side was also assessed in the presence or absence of CyA (10 μM) on both the apical and basolateral sides. After 120 min incubation at 37 °C, medium was aspirated from both the apical and basolateral side and each membrane was washed eight times with cold HBSS at 4 °C. The radioactivity taken up by Caco-2 cells on the membrane was measured in a liquid scintillation counter.

Data Analysis

For each transport experiment, the apparent permeability coefficients (Papp) were estimated from the slope of the linear portion of the time course of drug transport across the Caco-2 cell monolayers according to the equation: Papp=\((dC/dt)/AC_0\), where A is the area of the membrane (cm²), C₀ is the initial concentration of the solute in the donor sample (μM), and dC/dt is the permeability rate (μM/s). In addition, ΔPapp was determined from the equation: ΔPapp=Papp (basolateral to apical flux)−Papp (apical to basolateral flux) to characterize secretory flux from the basolateral to apical side of the Caco-2 monolayers.

RESULTS

Effects of Ebastine and Its Metabolites on [³H]CyA Transport across Caco-2 Cell Monolayers

Figure 2 illustrates the effect of ebastine (1 to 300 μM) on the bidirectional transport of [³H]CyA across confluent Caco-2 cell monolayers. In the absence of ebastine, the [³H]CyA flux from the basolateral to apical side was significantly higher than that from the apical to basolateral side, indicating that functional expression of P-gp took place in the Caco-2 cell system.20 The
net secretory flux of [3H]CyA was affected by the presence of ebastine, a CYP3A4 substrate, in a concentration-dependent manner (Fig. 2). Although the increase in the [3H]CyA flux from the apical to basolateral side was not observed at higher ebastine concentration (100 and 300 μM), the reason for this observation is unknown. The effects of the three metabolites of ebastine, M-OH, Car and des-BP, on the polarized [3H]CyA transport across the Caco-2 monolayers are shown in Fig. 3. M-OH (a substrate for CYP3A4) significantly inhibited the secretory flux of [3H]CyA at a high concentration (300 μM). In contrast, the pharmacologically active metabolite, Car, and the N-dealkylation product by CYP3A4, des-BP, had no significant inhibitory effect at the same concentration. Figure 4 summarizes the effect of ebastine (A) and its metabolites (B) on the ΔPapp values for polarized [3H]CyA transport (apparent [3H]CyA efflux) in these inhibition experiments. The ΔPapp values decreased with an increase in ebastine concentration and the efflux was significantly inhibited at higher concentrations (>50 μM), suggesting that the affinity for P-gp as an inhibitor was low. Although the ΔPapp at 300 μM was negative value, only passive diffusion of [3H]CyA would occur at this concentration. Among the ebastine metabolites, M-OH acted as an inhibitor of secretory transport of [3H]CyA at a concentration of 300 μM.

Transport and Uptake of [14C]Ebastine in Caco-2 Cell Monolayers Figure 5 shows the flux of [14C]ebastine across Caco-2 monolayers after apical (A) and basolateral (B) application over the concentration range 10 to 100 μM. The Papp values for bidirectional transport of [14C]ebastine are shown in Fig. 6. Although similar Papp values were calculated at all the concentrations studied, the Papp values for the flux from the basolateral to apical side were slightly higher than those from the apical to basolateral side at concentrations of 10, 15 and 30 μM. In the uptake experiment, [14C]ebastine (2 μM) uptake by Caco-2 cells from the apical side in the presence of cold CyA was significantly (p<0.01) higher than that in the absence of the drug (Fig. 7). This re-
result suggests that the uptake of ebastine, which was pumped out of the Caco-2 cells by P-gp, was increased by inhibition of the efflux transporter by CyA.

**DISCUSSION**

The first step in the metabolism of ebastine in human liver microsomes involves its conversion to two major metabolites, des-BP and M-OH. Des-BP, the N-dealkylation product, is pharmacologically inactive and is excreted after undergoing further metabolism. M-OH undergoes further oxidation to Car, an active compound possessing potent H1 receptor antagonist activity. Efficient first-pass metabolism of ebastine appears likely because the peripheral plasma concentration of unchanged ebastine was almost below the de-

**Fig. 4. ΔPapp Values for 280 nm [3H]Cyclosporin A (CyA) Transport across Caco-2 Cell Monolayers**

A; ΔPapp value of CyA in the presence of ebastine. B; ΔPapp value of CyA in the presence of ebastine metabolites. ΔPapp = Papp (B to A) − Papp (A to B). Error bars represent S.D. (n = 3 or 4). * ΔPapp is significantly higher (p < 0.01) than that of control.

**Fig. 5. Transepithelial Flux of [14C]Ebastine across Caco-2 Cell Monolayers**

A, apical to basolateral flux; B, basolateral to apical flux. The ebastine concentrations were 10 μM ( ), 15 μM ( ), 30 μM ( ), 50 μM ( ), and 100 μM ( ). Error bars represent S.D. (n = 3 or 4).

**Fig. 6. Effect of the Ebastine Concentration on Caco-2 Cell Flux Rates**

[Diagram showing flux rates with different concentrations of ebastine and indicating statistical significance with symbols: * and **]. Error bars represent S.D. (n = 3 or 4).

**Fig. 7. Effect of CyA on the Apical Uptake of [14C]Ebastine by Caco-2 Cell Monolayers**

Uptake of [14C]ebastine (2 μM) was measured in the absence (closed bar) and presence (open bar) of 10 μM CyA at 30 and 120 min. *p < 0.05; **p < 0.01. Error bars represent S.D. (n = 4).
Ebastine is a substrate for P-gp and could be an inhibitor at the secretory level. Inhibit the secretory [3H]CyA transport mediated by P-gp at Caco-2 cell monolayers. Ebastine and M-OH were shown to be inhibitors, in the basolateral to apical direction across the membrane of Caco-2 cells was confirmed by polarized flux and its metabolites using the Caco-2 cell culture systems developed as a model for studying human intestinal drug absorption. The function of P-gp expressed on the apical membrane of Caco-2 cells was confirmed by polarized flux of [3H]CyA, a model drug for P-gp substrates as well as an inhibitor, in the basolateral to apical direction across the Caco-2 cell monolayers. Ebastine and M-OH were shown to inhibit the secretory [3H]CyA transport mediated by P-gp at very high concentrations while des-BP and Car did not (Figs. 2—4). These results indicate that ebastine or M-OH, but not the other two metabolites, are recognized by P-gp and act as inhibitors of transport mediated by this efflux pump. The affinity of ebastine or M-OH to P-gp might be low based on the observation that high concentration was required to inhibit the transport of CyA. It is interesting that Car, which is very similar in structure to ebastine and M-OH, has no significant inhibitory effect on [3H]CyA transport. Although knowledge of the structure–activity relationships of P-gp substrates and inhibitors is limited, the distinct physicochemical properties of these compounds, such as their hydrophobicity and negative charge derived from the carboxyl group, might be important. Further studies are required to confirm this.

On the other hand, no clear secretory transport of ebastine was found in the transport experiments using [14C]ebastine (Figs. 5 and 6). Only a slightly polarized flux from the basolateral to apical side was observed at low concentrations (10—30 μM), suggesting that the contribution of P-gp-mediated efflux to the bidirectional transport of ebastine itself is small under these conditions. Although data for concentration less than 10 μM would be informative for further characterization, i.e., estimation of Km value and contribution of passive diffusion, we were unable to estimate Papp values accurately due to the low specific activity of [14C]ebastine. Therefore, we carried out [14C]ebastine uptake experiments at a lower concentration (2 μM). The presence of cold CyA significantly increased [14C]ebastine uptake by Caco-2 cells after apical application of the drug (Fig. 7), implying that the efflux process mediated by P-gp may be involved in the uptake of ebastine by Caco-2 cells. These results indicate that ebastine is a substrate for P-gp. However, the affinity of ebastine for P-gp is expected to be low since an inhibitory effect on [3H]CyA transport was observed only at high concentrations and no marked secretory transport occurred across Caco-2 cell monolayers.

Taken together, the present study has demonstrated that ebastine is a substrate for P-gp and could be an inhibitor at very high concentrations. Among the metabolites of ebastine studied, only M-OH was shown to interact with P-gp. It is unlikely that the efflux mediated by P-gp dramatically affects the overall intestinal absorption of ebastine in vivo because efficient passive diffusion of this drug should occur due to its high lipophilicity (log Poctanol/water = 4.75). However, the secretory transport of ebastine, mediated by P-gp may be advantageous for its efficient in vivo first-pass metabolism to Car and other metabolites during absorption process, which might avoid absorption of unchanged drug with possible adverse cardiovascular effects. The present study also suggests possible drug–drug interactions between ebastine or M-OH and P-gp substrates during absorption. However, this possibility can be neglected in clinical situations because P-gp inhibition only takes place at very high ebastine concentrations (above 50 μM) in the luminal space of the intestine, which are not achieved in humans after oral administration of clinical doses.

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