Pharmacokinetic Analysis of Transcellular Transport of Levofloxacin across LLC-PK_1 and Caco-2 Cell Monolayers

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To characterize the membrane transport responsible for the renal excretion and intestinal absorption of levofloxacin, we performed pharmacokinetic analysis of transcellular transport across LLC-PK_1 and Caco-2 cell monolayers. Transcellular transport of levofloxacin in LLC-PK_1 cells was greater in the basolateral-to-apical direction than in the opposite direction. Pharmacokinetic analysis indicated that basolateral uptake was the direction-determining step for the transcellular transport of levofloxacin in LLC-PK_1 cells. The apical efflux clearance of levofloxacin in LLC-PK_1 cells was increased at the medium pH 6 as compared with at pH 8, suggesting that membrane transport characteristics of levofloxacin are apparently similar to those of a prototypical organic cation, tetraethylammonium. On the other hand, transcellular transport of levofloxacin in Caco-2 cells was only slightly greater in the basolateral-to-apical direction than in the opposite direction. The apical efflux clearance of levofloxacin in Caco-2 cells was greater than basolateral efflux clearance, and apical influx clearance was greater than any other membrane transport clearance. In addition, the apical uptake of levofloxacin as well as quinidine in Caco-2 cells was inhibited significantly by nicotine and imipramine. The findings indicated that some transporters are responsible not only for the efflux but also for the influx of levofloxacin at the apical membrane of Caco-2 cells.

Key words levofloxacin; quinidine; intestinal absorption; Caco-2 cell; renal secretion; LLC-PK_1, cell

The human intestinal epithelial Caco-2 cell line forms confluent monolayers of well-differentiated enterocyte-like cells with functional properties of transporting epithelia, and retains various influx and efflux transporters expressed in the intestine, such as H^+ -coupled oligopeptide transporter 1 (PEPT1), P-glycoprotein (P-gp), multi-drug resistance proteins (MRPs), and breast cancer resistance protein (BCRP). Caco-2 cells have been widely used as a model to investigate the intestinal absorption or secretion of various drugs and other xenobiotics. Caco-2 cell monolayers grown on plastic dishes are useful to mainly investigate the uptake of drugs at the apical membrane. On the other hand, Caco-2 cell monolayers grown on porous membrane filters allow us to investigate transcellular absorption and the secretion of drugs across intestinal epithelial cells. For example, transcellular transport of digoxin was investigated using Caco-2 cells grown on porous membrane filters, and the results indicated that P-gp is involved in the intestinal secretion of digoxin. Chiu et al. investigated the transcellular transport of another P-gp substrate, cyclosporin A, across Caco-2 cell monolayers to examine the jejunal permeability of the drug. Watanabe et al. investigated the effects of progestosterone and norethisterone on the apical-to-basolateral and basolateral-to-apical transport of cephalaxin, a typical PEPT1 substrate, using Caco-2 cell monolayers cultured on permeable membranes. Irie et al. compared the recognition characteristics of the basolateral peptide transporter for several nonpeptidic compounds, such as valacyclovir, with those of apical PEPT1 using Caco-2 cells grown on porous membrane filters.

We previously performed pharmacokinetic analysis of transcellular transport of quinidine across Caco-2 cell monolayers grown on porous membrane filters in order to investigate the mechanism responsible for the intestinal absorption of lipophilic organic cation. The transcellular transport of quinidine was greater in the apical-to-basolateral direction than in the opposite direction. The calculated efflux clearance of quinidine at the apical membrane was 6.1-fold greater than that at the basolateral membrane, suggesting that P-gp is at least partly responsible for the apical efflux of the drug. The influx clearance value at the apical membrane was much greater than that at the basolateral membrane. Therefore, we further investigated the uptake of quinidine at the apical membrane using Caco-2 cells grown on plastic dishes. The apical uptake of quinidine was markedly increased by alkalization of the apical medium, and was diminished by the decrease in temperature (4 °C) of the medium. In addition, the uptake of quinidine in Caco-2 cells grown on plastic dishes was significantly inhibited by the presence of levofloxacin and cationic drugs, such as imipramine. These findings indicated that influx at the apical membrane was the direction-determining step in the transcellular transport of quinidine across Caco-2 cell monolayers, and that some specific transport system was involved in the apical uptake of the drug.

In the present study, we performed pharmacokinetic analysis of the transcellular transport of levofloxacin in Caco-2 cells grown on porous membrane filters in order to elucidate whether the specific transport system responsible for the apical uptake of cationic drugs is also involved in the intestinal absorption of levofloxacin. That is, when transcellular drug transport is examined under the condition where the unlabeled drug concentration in the monolayer is equilibrated with that of the incubation medium in the apical and basolateral chambers, the transport data for a small amount of a radio-labeled drug can be analyzed using a linear pharmacokinetic model. In addition, we compared the transport characteristics of levofloxacin in Caco-2 cells with those in the renal epithelial LLC-PK_1 cell line, which is known to express the transporters for organic cations.
MATERIALS AND METHODS

Materials Levofloxacin hydrochloride, imipramine hydrochloride, nicotine tartrate dihydrate, tetraethylammonium chloride, cimetidine, procainamide hydrochloride, and propranolol hydrochloride were obtained from Nacalai Tesque Inc. (Kyoto, Japan). Quinidine hydrochloride monohydrate and metoprolol tartrate were purchased from Sigma (St. Louis, MO, U.S.A.). Diphenhydramine hydrochloride and clonidine hydrochloride was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Bisoprolol hemifumarate was obtained from Tanabe Pharmaceutical Co. (Osaka, Japan). [14C]levofloxacin (2.43 MBq/mg) was obtained from Daiichi Pure Chemicals Co. (Ibaraki, Japan). [3H]Quinidine (740 GBq/mmol) and [3H]mannitol (740 GBq/mmol) were purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO, U.S.A.). All other chemicals were of the highest purity available.

Cell Culture and Preparation of Monolayers LLC-PK₁ cells at passage 197 were obtained from the American Type Culture Collection (Manassas, VA, U.S.A.), and Caco-2 cells at passage 43 were obtained from the Riken Biorepository (Tsukuba, Japan). All experiments were carried out with LLC-PK₁ cells between passages 204—208 and with Caco-2 cells between passages 54—62. The cells were maintained by serial passage in plastic dishes with Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Valley Biochemical Inc., Winchester, VA, U.S.A.) in an atmosphere of 5% CO₂-95% air at 37°C. The medium was changed every second or third day, and when the cells reached 80—90% confluence, they were sub-cultured using a 0.02% EDTA/0.05% trypsin solution. LLC-PK₁ cells were seeded at a density 5×10⁵ cells/cm² on a 1.12 cm² porous membrane (3 µm pore size) in a polyester membrane Transwell®-Clear insert (Coster, Cambridge, MA, U.S.A.) to evaluate transcellular transport of levofloxacin. The volume of the medium was 0.75 and 1.5 ml for inside (apical side) and outside (basolateral side) of the insert, respectively. The seeded cells were maintained for 6 d to prepare differentiated cell monolayers. The maturity of the monolayer was judged by transepithelial electrical resistance (TEER). TEER was measured using a Millicell-ERS resistance system (Millipore, Bedford, MA, U.S.A.). LLC-PK₁ cell monolayers were used for experiments of the transcellular transport of levofloxacin in LLC-PK₁ and Caco-2 cell monolayers prepared on a 0.4 m pore size) in a Falcon® multwell™ plate (BD Bioscience), and were maintained for 21 d to characterize the uptake of levofloxacin and quinidine at the apical membrane.

Pharmacokinetic Analysis of Transcellular Transport of Levofloxacin The transcellular transport of levofloxacin in LLC-PK₁ and Caco-2 cell monolayers prepared on a porous membrane was examined as described previously. In brief, the monolayer was pre-incubated for 1 h at 37°C with culture medium (pH 8 or 6) containing unlabeled levofloxacin (100 µm) to equilibrate the drug concentration. After the 1-h equilibration period, [14C]levofloxacin (0.2—0.4 µCi/well) was applied to the apical chamber to examine the apical-to-basolateral transcellular transport of levofloxacin. A volume (50 µl) of medium in the basolateral chamber was then collected after 1, 2, and 3 h. Following the last collection, the cell monolayers on the porous membrane were immediately washed three times with ice-cold phosphate buffer, and the cells were collected. The amounts of [14C]levofloxacin in the medium and cells were determined using a liquid scintillation counter. The time course of the transport of levofloxacin in the opposite direction (basolateral-to-apical) was examined in the same manner. To estimate the paracellular transport and extracellular trapping of levofloxacin, we also examined the transport and extracellular trapping of [3H]mannitol. The transcellular transport of levofloxacin was analyzed in a model-dependent manner using NONMEM software running on a mainframe UNIX machine at the Kyoto University Data Processing Center, as described previously. The following mass balance equations were prepared for the pharmacokinetic analysis:

\[
\frac{dX_A}{dt} = -\frac{CL_{A\rightarrow C}}{V_A} \cdot X_A + \frac{CL_{C\rightarrow A}}{V_C} \cdot X_C - \frac{CL_{A\rightarrow B}}{V_A} \cdot X_A + \frac{CL_{A\rightarrow B}}{V_B} \cdot X_B
\]

(1)

\[
\frac{dX_B}{dt} = -\frac{CL_{B\rightarrow C}}{V_B} \cdot X_B + \frac{CL_{C\rightarrow B}}{V_C} \cdot X_C - \frac{CL_{A\rightarrow B}}{V_A} \cdot X_A - \frac{CL_{A\rightarrow B}}{V_B} \cdot X_B
\]

(2)

\[
\frac{dX_C}{dt} = \frac{CL_{A\rightarrow C}}{V_A} \cdot X_A + \frac{CL_{B\rightarrow C}}{V_B} \cdot X_B - \frac{(CL_{C\rightarrow A} + CL_{C\rightarrow A'})}{V_C} \cdot X_C
\]

(3)

where \(X_A, X_B,\) and \(X_C\) are the amount of levofloxacin in the apical chamber, the basolateral chamber, and the monolayer determined at time \(t\), respectively. \(V_A\) and \(V_B\) indicate the volume of the apical medium and the basolateral medium, respectively. \(V_C\) indicates the cell volume (1.12 µl/cm² for LLC-PK₁ cells and 2.50 µl/cm² for Caco-2 cells), measured with sulfuramidine as described previously. The influx and efflux clearance of levofloxacin at the apical membrane of the cells was designated as \(CL_{A\rightarrow C}\) and \(CL_{C\rightarrow A}\), respectively. The influx and efflux clearance of levofloxacin at the basolateral membrane of the cells was designated as \(CL_{B\rightarrow C}\) and \(CL_{C\rightarrow B}\), respectively. Paracellular transport clearance \((CL_{A\rightarrow B})\) was estimated by analyzing the transport profile of radio-labeled mannitol using the following mass balance equations:

\[
\frac{dX_A}{dt} = -\frac{CL_{A\rightarrow B}}{V_A} \cdot X_A + \frac{CL_{A\rightarrow B}}{V_B} \cdot X_B
\]

(4)

\[
\frac{dX_B}{dt} = \frac{CL_{A\rightarrow B}}{V_A} \cdot X_A - \frac{CL_{A\rightarrow B}}{V_B} \cdot X_B
\]

(5)

In addition, the results of preliminary experiments showing...
that the amount of \(^{14}\)C-mannitol transported in the apical-to-basolateral direction was not significantly different from that transported in the opposite direction, indicated that the paracellular clearance of mannitol in the apical-to-basolateral direction is equivalent to that in the opposite direction.

**Inhibition of Cellular Uptake of Levofloxacin and Quinidine by Organic Cations and so Forth** The cellular uptake of \(^{14}\)C-levofloxacin was examined using Caco-2 cell monolayers grown on plastic dishes of a twofold plate, as described previously.\(^{11}\) The composition of the incubation medium was as follows: 125 mM NaCl, 4.8 mM KCl, 5.6 mM d-glucose, 1.2 mM CaCl\(_2\), 1.2 mM KH\(_2\)PO\(_4\), 1.2 mM MgSO\(_4\), 7H\(_2\)O, 25 mM HEPES, and 100 \(\mu\)M levofloxacin (pH 7.4). The monolayers were first pre-incubated for 60 min at 37 °C with 2 mL of incubation medium. In order to evaluate the effect of organic cations on the cellular uptake of \(^{14}\)C-levofloxacin, the incubation medium was replaced with fresh incubation medium supplemented with 5 mM organic cations at 5 min before the addition of \(^{14}\)C-levofoxacin (0.4 \(\mu\)Ci/well). After the cells were incubated with \(^{14}\)C-levofoxacin for another 15 min at 37 °C, they were immediately washed three times with ice-cold phosphate buffer, and collected. The amounts of \(^{14}\)C-levofoxacin in the cells were determined using a liquid scintillation counter.

The effect of organic cations on the apical uptake of \(^{3}\)H-quinidine in Caco-2 cells was also evaluated at 37 °C in the presence of 100 \(\mu\)M quinidine. The incubation medium was replaced with fresh incubation medium supplemented with 5 mM organic cations at 5 min before the addition of \(^{3}\)H-quinidine (0.5 \(\mu\)Ci/well). The cells were incubated with \(^{3}\)H-quinidine for 15 min at 37 °C, and the amounts of \(^{3}\)H-quinidine in the cells were determined as described above.

**Statistical Analysis** Values are expressed as the mean±S.E. In all figures, when error bars are not shown, they are smaller than the symbol. The statistical significance of differences between mean values was tested using Student’s \(t\)-test provided that the variances of the groups were similar. If this was not the case, the Mann–Whitney \(U\)-test was applied. Multiple comparisons were performed using Scheffe’s test following a one-way ANOVA. \(p<0.05\) was considered to be statistically significant.

**RESULTS AND DISCUSSION**

**Transcellular Transport and Membrane Transport Characteristics of Levofloxacin in LLC-PK\(_1\) Cell Monolayers**

We first evaluated the transcellular transport and membrane transport clearance of levofloxacin in LLC-PK\(_1\) cell monolayers grown on porous membrane filters. Figure 1 shows the profiles of transcellular transport and cellular accumulation of levofloxacin in LLC-PK\(_1\) cells. The amount of levofloxacin transported in the basolateral-to-apical direction was greater than that in the opposite direction at medium pH 8 and 6 (Fig. 1). The amount of levofloxacin accumulated in cells from the basolateral medium within 3 h was greater than that from the apical medium at pH 8, and was decreased by 32% at pH 6 as compared with that at pH 8 (Fig. 1). We performed a pharmacokinetic analysis of the data on the transcellular transport and cellular accumulation of levofloxacin to characterize the membrane transport of the drug. Table 1 shows membrane transport clearance of levofloxacin in LLC-PK\(_1\) cells. Influx clearance at the basolateral membrane \((CL_{B\rightarrow A})\) was much greater than any other clearance values at medium pH both 8 and 6 (Table 1). In particular, the value of \(CL_{B\rightarrow A}\) at the medium pH 8 was 2.8-fold greater than influx clearance at the apical membrane \((CL_{A\rightarrow B})\) (0.766 vs. 0.276 \(\mu\)mol/min/cm\(^2\)). These findings indicated that the influx of levofloxacin at the basolateral membrane \((CL_{B\rightarrow A})\) was the primary factor responsible for the greater basolateral-to-apical transport across LLC-PK\(_1\) cell monolayers. In addition, the efflux clearance of the apical membrane \((CL_{C\rightarrow A})\) was

![Fig. 1. Transcellular Transport and Accumulation of Levofloxacin in LLC-PK\(_1\) Cell Monolayers Grown on Porous Filters](image)

**Table 1. Transport Clearance (\(\mu\)l/min/cm\(^2\)) of Levofloxacin in LLC-PK\(_1\) and Caco-2 Cell Monolayers**

<table>
<thead>
<tr>
<th>Cell</th>
<th>(pH)</th>
<th>(CL_{A\rightarrow B})</th>
<th>(CL_{C\rightarrow A})</th>
<th>(CL_{C\rightarrow B})</th>
<th>(CL_{B\rightarrow C})</th>
<th>(CL_{A\rightarrow B})</th>
</tr>
</thead>
<tbody>
<tr>
<td>LLC-PK(_1)</td>
<td>8</td>
<td>0.276±0.008</td>
<td>0.251±0.020</td>
<td>0.435±0.043</td>
<td>0.766±0.053</td>
<td>0.048±0.007</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.410±0.015*</td>
<td>0.414±0.019*</td>
<td>0.341±0.022</td>
<td>0.532±0.018*</td>
<td>0.043±0.008</td>
</tr>
<tr>
<td>Caco-2</td>
<td>8</td>
<td>0.392±0.026</td>
<td>0.347±0.024</td>
<td>0.151±0.009</td>
<td>0.249±0.008</td>
<td>0.025±0.001</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.547±0.044*</td>
<td>0.289±0.016</td>
<td>0.121±0.009*</td>
<td>0.301±0.012*</td>
<td>0.032±0.001*</td>
</tr>
</tbody>
</table>

\(p<0.05\): significantly different from pH 8 in each cell monolayers.
significantly increased at the medium pH 6 as compared with that at pH 8, whereas the value of CL_{B→C} was significantly decreased at pH 6 (Table 1).

The transport characteristics of levofloxacin in LLC-PK_{1} cells were apparently similar to those of a prototypical organic cation, tetraethylammonium (TEA). However, the transporters responsible for the transcellular transport of levofloxacin in LLC-PK_{1} cells may not be identical to those for TEA. Ohtomo et al. and Matsuo et al. investigated the transcellular transport of TEA and levofloxacin in LLC-PK_{1} cells. TEA and another prototypical organic cation, cimetidine, did not alter the transcellular transport and cellular accumulation of levofloxacin in LLC-PK_{1} cells. TEA is transported by organic cation transporter 2 (OCT2) and chloride/bicarbonate antiporter 1 (MATE1) on the brush-border membrane. Okuda et al. demonstrated that the transport activity of TEA in MATE1-expressing Xenopus laevis oocytes was markedly elevated compared to the water-injection control, but the uptake of levofloxacin was not stimulated by OCT2. On the other hand, Tanihara et al. demonstrated that the transport activity of TEA in MATE1-expressing cells was stimulated in the presence of oppositely directed H^{+}-gradient, but that levofloxacin was only slightly transported by MATE1.

Transcellular Transport and Membrane Transport Characteristics of Levofloxacin in Caco-2 Cell Monolayers

We next evaluated the transcellular transport and membrane transport clearance of levofloxacin in Caco-2 cell monolayers grown on porous membrane filters. Figure 2 shows the profiles of transcellular transport and cellular accumulation of levofloxacin in Caco-2 cell monolayers. The amount of levofloxacin transported in the basolateral-to-apical direction was only slightly greater than that in the opposite direction with the medium pH 8 and 6. In contrast to the drug accumulation in LLC-PK_{1} cells, the cellular accumulation of levofloxacin from the apical medium in Caco-2 cells was greater than that from the basolateral medium (Fig. 2). Table 1 also shows membrane transport clearance of levofloxacin in Caco-2 cells. The efflux clearance of levofloxacin at the apical membrane (CL_{C→A}) in Caco-2 cells was greater than that at the basolateral membrane (CL_{C→B}). In addition, influx clearance at the apical membrane (CL_{A→C}) was greater than any other membrane transport clearance with the medium pH 8 and 6, and increased at pH 6 as compared with that at pH 8 (0.547 vs. 0.392 μl/min/cm², Table 1). The findings suggested that the some transport system is involved not only in the efflux and but also in the influx of levofloxacin at the apical membrane of Caco-2 cells.

Several research groups have investigated the transporters responsible for the efflux of levofloxacin at the apical membrane of Caco-2 cells. Plausible candidates for efflux transporters of levofloxacin were P-gp, MRP, and BCRP. Naruhashi et al. reported that the secretory transport of levofloxacin in Caco-2 cells was diminished by a P-gp inhibitor cyclosporin A, a MRP modulator probenecid, and the combination of the two compounds. Yamaguchi et al., however, reported that cyclosporin A and probenecid did not affect the basolateral-to-apical transport of levofloxacin in Caco-2 cells. On the other hand, Xia et al. reported that BCRP was expressed at the apical membrane in Caco-2 cells, and transported its substrate, such as estrone-3-sulfate and methotrexate. Merino et al. demonstrated that BCRP weakly transported ofloxacin, a racemate of levofloxacin and its optical enantiomer, using BCRP-transduced cells. These findings suggested that BCRP is partly involved in the efflux of levofloxacin at the apical membrane in Caco-2 cells.

There are few reports concerning the transporter responsible for the influx of levofloxacin at the apical membrane of Caco-2 cells. Recently, however, Maeda et al. evaluated the levofloxacin uptake activity of Caco-2 subclones, and selected candidate solute carrier (SLC) transporter genes, for which the expression level is apparently correlated with levofloxacin uptake activity, based on the criterion of 2-fold or higher expression level in high-activity subclone versus low-activity subclone. They reported that the organic anion transporting polypeptide 1A2 (OATP1A2) may be at least partly involved in the apical uptake of levofloxacin in Caco-2 cells. Therefore, in the present study, we evaluated the effect of OATP inhibitors on the apical uptake of levofloxacin in Caco-2 cells grown on plastic dishes; however, the OATP inhibitors, rifamycin SV and erythromycin, had no significant effect on the apical uptake of levofloxacin (Fig. 3). We further evaluated the effect of organic cations on the apical uptake of levofloxacin and also quinidine in order to elucidate the characteristics of levofloxacin uptake at the apical membrane of Caco-2 cells. That is, TEA and cimetidine are the prototypical cations transported actively in renal epithelial cells, whereas the other drugs are hydrophobic/lipophilic organic cations (Fig. 4). The apical uptake of
quinidine was diminished by the decrease in temperature (4 °C) of the medium and presence of levofloxacin. TEA and cimetidine did not decrease the apical uptake of quinidine in Caco-2 cells (Fig. 4). In contrast, the apical uptake of quinidine was decreased to less than 65% of the control by the treatment with hydrophobic organic cations (clonidine, diphenhydramine, bisoprolol, metoprolol, propranolol, and quinidine) (Fig. 4). In particular, nicotine and imipramine diminished the uptake of the drug to 3.9 and 4.8% of the control, respectively (Fig. 4). Figure 5 shows the effect of organic cations on the apparent apical uptake (influx minus efflux) of levofloxacin in Caco-2 cell monolayers grown on plastic dishes. The apparent apical uptake of radio-labeled levofloxacin was not affected by 5 mM levofloxacin, suggesting that unlabeled levofloxacin inhibited not only influx but also efflux of the radio-labeled drug at the apical membrane of Caco-2 cells. On the other hand, the apparent apical uptake of levofloxacin was affected by several tested organic cations (Fig. 5). The apparent apical uptake of levofloxacin was increased to 175 and 184% of the control by quinidine and propranolol, respectively. The finding suggested that the efflux of levofloxacin at the apical membrane of Caco-2 cells was inhibited by quinidine and propranolol. In contrast, the apparent apical uptake of levofloxacin in Caco-2 cells was decreased to 30 and 36% of the control by nicotine and imipramine, respectively (Fig. 5). The apical uptake of levofloxacin in Caco-2 cells may be at least partly mediated by the specific transport system responsible for the apical influx and efflux of levofloxacin at the apical membrane of intestinal epithelial Caco-2 cells.

In conclusion, levofloxacin was transported directionally from basolateral side to apical side in LLC-PK1 cells, and basolateral uptake was the direction-determining step for the transcellular transport of the drug. On the other hand, the apical efflux clearance of levofloxacin in Caco-2 cells was greater than basolateral efflux clearance, and apical influx clearance was greater than any other membrane transport clearance. In addition, the apical uptake of levofloxacin as well as quinidine in Caco-2 cells was inhibited significantly by nicotine and imipramine. The findings indicated that some transporters are responsible not only for the efflux but also for the influx of levofloxacin at the apical membrane of Caco-2 cells.

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