Transport of Quinolone Antibacterial Drugs by Human P-Glycoprotein Expressed in a Kidney Epithelial Cell Line, LLC-PK₁

TATSUYA ITO, IKUKO YANO, KUMIKO TANAKA and KEN-ICHI INUI
Department of Pharmacy, Kyoto University Hospital, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606-01, Japan
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ABSTRACT
The purpose of this study was to characterize the transport mechanisms involved in the renal tubular secretion of quinolones. The contribution of P-glycoprotein to the transport of quinolones was elucidated using a kidney epithelial cell line, LLC-PK₁, and its transfectant derivative cell line, LLC-GA5-COL150, which expresses human P-glycoprotein on the apical membrane. The transcellular transport of levofloxacin, a quinolone antibacterial drug, from the basolateral to apical side was increased in LLC-GA5-COL150 compared with that in LLC-PK₁ monolayers. The apparent Michaelis constant and maximum velocity values for the saturable transcellular transport of levofloxacin from the basolateral to apical side in LLC-GA5-COL150 monolayers were 3.0 mM and 45 nmol/mg protein per 15 min, respectively. The increased basolateral-to-apical transport in LLC-GA5-COL150 monolayers was completely inhibited by cyclosporin A and quinidine to the level observed in LLC-PK₁ monolayers. In addition, 3 mM levofloxacin inhibited the basolateral-to-apical transport of daunorubicin in LLC-GA5-COL150 monolayers. The basolateral-to-apical transport of another quinolone antibacterial drug, DU-6859a, in LLC-GA5-COL150 monolayers greatly exceeded than that in LLC-PK₁ monolayers, and was inhibited by levofloxacin. These findings suggest that quinolone antibacterial drugs are transported by P-glycoprotein, and that P-glycoprotein may contribute at least in part to the renal tubular secretion of quinolones.

Quinolone antibacterial drugs are frequently used to treat various bacterial infections. Most quinolone antibacterial drugs are zwitterions at physiological pH, and are excreted into urine via renal tubular secretion. Probenecid has been shown to decrease the renal clearance of cinoxacin, norfloxacin and ciprofloxacin (Rodriguez et al., 1979; Shimada et al., 1983; Sörgel and Kinzig, 1993). Cimetidine has been shown to decrease the renal clearance of temafloxacin and enoxacin (Sörgel et al., 1992; Misiak et al., 1993). These findings suggested that quinolone antibacterial drugs undergo tubular secretion as either acids or bases (Sörgel and Kinzig, 1993). Levofloxacin, a zwitterion at physiological pH, also undergoes renal tubular secretion in man (Kamiya et al., 1992). We have shown that ofloxacin, a racemate of levofloxacin and its optical enantiomer, potently inhibits the H⁺-dependent tetraethylammonium uptake in renal brush-border membrane vesicles (Okano et al., 1990). We have also shown that levofloxacin potently inhibits the apical H⁺/organic cation antiporter expressed in the kidney epithelial cell line LLC-PK₁, and that the transcellular transport of levofloxacin would be mediated by systems that are distinct from those involved in tetraethylammonium transport (Ohtomo et al., 1996). The details of the transport mechanisms for quinolones have yet to be elucidated.

P-glycoprotein, which is a 170- to 180-kDa membrane glycoprotein, has been extensively investigated with regard to the multidrug resistance phenomenon in tumor cells (Gottesman and Pastan, 1988; Endicott and Ling, 1989). P-glycoprotein functions as an ATP-dependent drug-efflux pump, thereby actively excreting a variety of structurally unrelated anticancer drugs out of cells thus producing resistance. P-glycoprotein is also found in normal tissues such as on the brush-border membranes of proximal tubules of the kidney, the bile canicular membranes of hepatocytes, the apical membranes of mucosal cells in the intestine and the luminal membranes of endothelial cells in the blood-brain barrier sites (Cordon-cardo et al., 1989; Thiebaut et al., 1987). In our laboratory, the renal secretion of the commonly used drug digoxin by P-glycoprotein was elucidated using LLC-PK₁ cells transfected with human MDR1 cDNA, LLC-GA5-COL150 cells, which overexpress human P-glycoprotein on the apical membranes (Tanigawara et al., 1992). P-glycoprotein is localized on the brush-border membranes of the normal proximal tubular cells in the kidney, and has

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ABBREVIATIONS: HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; MRP, multidrug resistance-related protein.
wide substrate specificity. We hypothesized that P-glycoprotein may contribute to the renal tubular secretion of quinolone antibacterial drugs. We studied the transport of levofloxacin and another new quinolone, DU-6859a, using LLC-GA5-COL150 cells. Our results suggest that quinolone antibacterial drugs are transported by P-glycoprotein and that P-glycoprotein may contribute at least in part to the renal tubular secretion of quinolones.

Materials and Methods

Cell culture. LLC-GA5-COL150 cells established previously in our laboratory (Tanigawara et al., 1992; Ueda et al., 1992) and LLC-PK1 cells (ATCC, CRL-1392) as host cells were maintained by serial passage in plastic culture dishes. Complete medium consisted of Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum without antibiotics, and 150 ng/ml colchicine was added to the medium for LLC-GA5-COL150 cells. Monolayers were grown under an atmosphere of 5% CO2-95% air at 37°C, and were subcultured every 6 to 7 days using 0.02% EDTA and 0.05% trypsin (Saito et al., 1992). In general, the plastic dishes (100 mm) were inoculated with 1 x 10^5 cells in 10 ml of complete culture medium. For the transport studies, LLC-GA5-COL150 and LLC-PK1 cells were seeded on polycarbonate membrane filters (3-μm pores, 4.71 cm² growth area) inside Transwell cell culture chambers (Costar, Cambridge, MA) at a cell density of 5 x 10^3 and 3 x 10^5 cells/cm², respectively. Transwell chambers were placed in 35-mm wells of tissue culture plates with 2.6 ml of outside medium (basolateral side) and 1.5 ml of inside medium (apical side). Fresh medium was replaced every 2 days, and the cells were used on the 7th day after seeding. In our study, LLC-GA5-COL150 and LLC-PK1 cells were used between passages 7 and 16, and between passages 217 and 227, respectively.

Transport and cellular accumulation measurements. Transcellular transport and accumulation of [14C]levofloxacin, [3H]daunorubicin, and [14C]DU-6859a were measured using monolayer cultures grown in Transwell chambers (Saito et al., 1992). The composition of incubation medium was as follows: 145 mM NaCl, 3 mM KCl, 1 mM CaCl2, 1.5 mM MgCl2, 5 mM d-glucose, 5 mM HEPES (pH 7.4). The pH of the medium was adjusted with a solution of NaOH. Six hours before the transport experiments, the culture medium was replaced with fresh colchicine-free culture medium. After removal of the culture medium from both sides of the monolayers, the cell monolayers were preincubated with 2 ml of incubation medium in each side for 15 min at 37°C. Then, 2 ml of incubation medium containing the radioactive substrate were added to either the basolateral or apical side, 2 ml of nonradioactive incubation medium was added to the opposite side and the monolayers were incubated for specified periods at 37°C. d-[3H]Mannitol (5 μM, 37 kBq/ml), a compound that is not transported by the cells, was used to calculate the paracellular fluxes and the extracellular trapping of [14C]levofloxacin (5 μM, 5.4 kBq/ml) and [14C]DU-6859a (5 μM, 3.8 kBq/ml). [14C]Sucrose (21.2 μM, 3.6 kBq/ml) was used to calculate the paracellular fluxes and the extracellular trapping of [3H]daunorubicin (100 nM, 17.1 kBq/ml). For transport measurements, aliquots (50 μl) of the incubation medium on the other side were taken at specified times, and the radioactivity was counted.

For accumulation studies, the medium was removed by aspiration at the end of the incubation period, and the monolayers were rapidly washed twice with 2 ml of ice-cold incubation medium on each side. The filters with monolayers were detached from chambers, the cells on the filters were solubilized with 0.5 ml of 1 N NaOH and the radioactivity in aliquots of 50 μl were counted. The radioactivity of the collected medium and the solubilized cell monolayers was determined in 3 ml of ACS II (Amersham International, Buckinghamshire, UK) by liquid scintillation counting.

Protein assay. The protein contents of the cell monolayers solubilized in 1 N NaOH were determined by the method of Bradford (1976) using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA) with bovine γ-globulin as a standard. The protein contents of LLC-GA5-COL150 and LLC-PK1 monolayers were 0.7 to 0.8, and 1.0 to 1.2 mg/filter, respectively.

Statistical analysis. Statistical analysis was performed by non-paired t test or Scheffe’s F test when multiple comparisons were needed. Differences were considered significant when P < .05.

Materials. d-[3H]Mannitol (828.8 GBq/mmol), [14C]sucrose (170.0 MBq/mmol), and [3H]daunorubicin (170.2 GBq/mmol) were purchased from Du Pont-New England Nuclear Research Products (Boston, MA). [14C]Levofloxacin (1.07 GBq/mmol), [14C]DU-6859a (769 MBq/mmol), unlabeled levofloxacin and DU-6859a were kindly supplied by Daiichi Seiyaku Co. (Tokyo, Japan) (fig. 1). Cyclosporin A was a gift from Sandoz Pharmaceutical Co. Ltd. (Tokyo, Japan). Cimetidine, colchicine, quinidine, tetracylammonium and p-aminobiphenyl were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). All other chemicals used were of the highest purity available.

Results

Transcellular transport and cellular accumulation of levofloxacin. We first measured the transcellular transport and cellular accumulation of levofloxacin (100 μM) by LLC-GA5-COL150 and LLC-PK1 monolayers. The basolateral-to-apical flux rate of levofloxacin by LLC-PK1 monolayers was larger than that in the opposite direction (fig. 2A). The basolateral-to-apical transport of levofloxacin was significantly increased (P < .01) in LLC-GA5-COL150 compared with that in LLC-PK1 monolayers. The cellular accumulation of levofloxacin from the basolateral side in LLC-GA5-COL150 monolayers was significantly increased (P < .05) at 15 and 30 min compared with that in LLC-PK1 monolayers. The cellular accumulation of levofloxacin from the apical side in LLC-GA5-COL150 monolayers was significantly decreased (P < .01) compared with that in LLC-PK1 monolayers (fig. 2B).

Concentration dependence of levofloxacin transcellular transport. We next examined the transcellular transport of varying concentrations of levofloxacin by LLC-GA5-
COL150 monolayers. Figure 3 shows the results of kinetic analysis of the transcellular transport of levofloxacin from the basolateral to apical side by LLC-GA5-COL150 monolayers as a function of substrate concentration ranging from 0.05 to 5 mM. The transport rates were estimated at 15 min because findings were linear on incubation up to 60 min (fig. 2A). The relationship between concentration and the basolateral-to-apical flux rate in LLC-GA5-COL150 monolayers approached saturation. We evaluated the apparent kinetic parameters by nonlinear least squares regression analysis according to the sum of the saturable transport indicated by Michaelis-Menten equation and the nonsaturable transport. The apparent Michaelis constant ($K_m$) and maximum velocity ($V_{max}$) values for the saturable transport of levofloxacin were 3.0 mM and 45 nmol/mg protein per 15 min, respectively.

**Inhibitory effects of P-glycoprotein modulators on levofloxacin transport.** The inhibitory effects of P-glycoprotein modulators on the transcellular transport of levofloxacin were examined. As shown in figure 4, the transcellular transport of levofloxacin in LLC-GA5-COL150 monolayers was significantly inhibited by 5 and 10 μM cyclosporin A ($P < .05$) and 50 μM quinidine ($P < .01$), and was decreased to the level observed in LLC-PK1 monolayers. The transcellular transport of levofloxacin by LLC-PK1 monolayers was not affected by these P-glycoprotein modulators. Cimetidine, tetraethylammonium and p-aminohippurate did not influence the transcellular transport of levofloxacin by LLC-GA5-COL150 or LLC-PK1 monolayers. Cyclosporin A and quinidine were used as 1% ethanol solutions, which did not affect the transcellular transport of levofloxacin (data not shown).

**Inhibitory effect of levofloxacin on daunorubicin transport.** We have shown that daunorubicin, an antineoplastic drug, is transported by P-glycoprotein in LLC-GA5-COL150 monolayers (Tanaka et al., 1996). To further characterize the interaction of levofloxacin with P-glycoprotein, the inhibitory effect of levofloxacin on the transport of daunorubicin was examined. As shown in figure 5A, the transcellular transport of daunorubicin from the basolateral to apical side was inhibited by 3 mM levofloxacin, while transport in the opposite direction was not affected. The cellular accumulation of daunorubicin from both sides was increased, because levofloxacin inhibited the expulsion of daunorubicin across the apical membranes via P-glycoprotein (fig. 5B).

**Transcellular transport and cellular accumulation of DU-6859a.** To elucidate the general contribution of P-glycoprotein for the transport of quinolones, we studied the transport of another quinolone antibacterial drug, DU-6859a (100 μM), which is also mainly excreted via the kidneys in man (Nakashima et al., 1995). The basolateral-to-apical flux rate of DU-6859a by LLC-PK1 monolayers was larger than the apical-to-basolateral flux rate (basolateral-to-apical, $2.7 \pm 0.5$; apical-to-basolateral, $1.3 \pm 0.5$, nmol/mg protein per 60 min, mean ± S.E. of three monolayers). The basolateral-to-apical transport in LLC-GA5-COL150 greatly exceeded that in LLC-PK1 monolayers (fig. 6A). The basolateral-to-apical transport of DU-6859a in LLC-GA5-COL150 monolayers was suppressed and the cellular accumulation was significantly increased ($P < .05$) by 3 mM levofloxacin (fig. 6B).

**Discussion**

To investigate the participation of P-glycoprotein in the renal tubular secretion of quinolone antibacterial drugs, we
examined the transport of levofloxacin and DU-6859a by LLC-GA5-COL150 monolayers that overexpress P-glycoprotein on the apical membranes. Our results clearly indicated that these quinolone antibacterial drugs are transported by P-glycoprotein, and that P-glycoprotein-mediated transport may contribute at least in part to the renal tubular secretion of quinolones.

The basolateral-to-apical transport of levofloxacin was significantly increased in LLC-GA5-COL150 relative to that in LLC-PK₁ monolayers, but the cellular accumulations from the basolateral side were almost same between these two cell lines, although significantly different (fig. 2). In general, most substrates for P-glycoprotein are extensively transported by LLC-GA5-COL150 compared with LLC-PK₁ monolayers, accompanied by a decrease in cellular accumulation (Saeki et al., 1993a; Tanaka et al., 1996). Our results may be due to the characteristics of levofloxacin with low cellular accumulation by LLC-PK₁ monolayers similarly to diltiazem.
(Saeki et al., 1993b). Therefore, we used the transcellular transport of levofloxacin as an index of P-glycoprotein-mediated transport.

The apparent $K_m$ value for the P-glycoprotein-mediated transport of levofloxacin was 3.0 mM from the kinetic analysis to examine saturable transcellular transport (fig. 3). Therefore, the affinity of levofloxacin to P-glycoprotein was lower than that of cyclosporin A (Saeki et al., 1993a). The increased transcellular transport of levofloxacin in LLC-GA5-COL150 monolayers was almost completely inhibited by the P-glycoprotein modulators cyclosporin A and quinidine, and was reduced to the level observed in LLC-PK$_1$ monolayers. More than half of the increased transcellular transport of 100 $\mu$M levofloxacin in LLC-GA5-COL150 monolayers were inhibited by only 5 $\mu$M cyclosporin A. This result was consistent with the reported apparent $K_m$ value of cyclosporin A for P-glycoprotein, 8.4 $\mu$M (Saeki et al., 1993a). These findings clearly showed that the increased saturable transcellular transport of levofloxacin by LLC-GA5-COL150 monolayers is mediated by P-glycoprotein.

The occurrence of multidrug resistance is a major obstacle for the treatment of cancer, and identification of clinically usable agents that can effectively reverse multidrug resistance is a current issue. We examined the inhibitory effect of levofloxacin on the P-glycoprotein-mediated transport of daunorubicin. The basolateral-to-apical transport of daunorubicin was inhibited by 3 mM levofloxacin, accompanied by a 93% increase in cellular accumulation (fig. 5). The serum concentration after oral administration of 200 mg levofloxacin was reported to be 1.2 $\mu$g/ml (3.2 $\mu$M) at the peak time in man (Nakashima et al., 1992). Therefore, levofloxacin does not appear to be a clinically usable agent to reverse P-glycoprotein-associated multidrug resistance. Some multidrug-resistant cells overexpress MRP that shares minor sequence homology with P-glycoprotein and is also a member of the ATP-binding cassette transmembrane transporter protein superfamily (Krishnamachary and Center, 1993). Gollapudi et al. (1995) showed that difloxacin increases the sensitivity of HL-60/AR cells, which overexpress MRP mRNA compared with HL-60 cells, to daunorubicin at clinically achievable concentrations. The affinity of quinolones might be different between P-glycoprotein and MRP.

Griffiths et al. (1994) reported the active transepithelial secretion of quinolone derivatives, such as ciprofloxacin and norfloxacin, from the basolateral to apical side by human intestinal Caco-2 cells. Ciprofloxacin was also studied in the isolated perfused rat liver, and an active transport mechanism was shown to be involved in the biliary excretion of this drug (Abadia et al., 1995). In addition, the accumulation of quinolones into the brain is low, which may be caused either by the relative low influx permeability at the blood-brain barrier and blood-cerebrospinal fluid barrier and/or by active efflux at both barriers (Oue et al., 1996). Considering the distribution of P-glycoprotein in normal tissues, P-glycoprotein might be a common transport system for the elimination of quinolone antibacterial drugs.

We have shown that levofloxacin potently inhibits the apical H$^+$/organic cation antiporter expressed in LLC-PK$_1$ cells, but that the transport of levofloxacin would be mediated by systems that are distinct from those involved in tetraethylammonium transport (Ohtomo et al., 1996). In this study, levofloxacin and DU-6859a were unidirectionally transported by LLC-PK$_1$ monolayers, and the basolateral-to-apical transcellular transport of levofloxacin was not inhibited by P-glycoprotein modulators, cyclosporin, cimetidine, tetraethylammonium or p-aminohippurate. We considered that there are single or multiple pathways for renal tubular secretion of these quinolone antibacterial drugs in addition to that via P-glycoprotein. The mechanisms for renal elimination of quinolones need to be examined further.

In conclusion, our results suggest that quinolone antibacterial drugs are transported by P-glycoprotein and that P-glycoprotein may contribute at least in part to the renal tubular secretion as well as other elimination processes. The degree to which P-glycoprotein contributes to the in vivo pharmacokinetics of quinolones should be evaluated in future studies.

References


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Send reprint requests to: Dr. Ken-ichi Inui, Department of Pharmacy, Kyoto University Hospital, Sakyo-ku, Kyoto 606-01, Japan.