The Transport of Ciprofloxacin in Cultured Kidney Epithelial Cells LLC-PK₁

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In this study, the transport characteristics of ciprofloxacin (CPFX) were investigated in a LLC-PK₁, kidney epithelial cell line. CPFX uptake from the apical medium into LLC-PK₁ cells on plastic dishes was shown to be temperature-dependent. Guanidine and cimetidine inhibited the uptake of CPFX, whereas tetraethylammonium chloride (TEA) and N¹-methylnicotinamide (NMN) did not. CPFX transport across LLC-PK₁ cell monolayers cultured on permeable supports was about 1.8 times larger in the basolateral-to-apical direction than in the apical-to-basolateral direction. Both the basolateral-to-apical and apical-to-basolateral transport of CPFX were inhibited by guanidine, whereas CPFX transport in both directions was not inhibited by TEA, NMN, or cimetidine. The basolateral-to-apical transport of CPFX was sensitive to the pH of the apical medium, and increased in an acidic pH. Enoxacin (ENX) as well as guanidine, inhibited the basolateral-to-apical transport of CPFX, and the inhibitory effect of ENX was sensitive to the pH of the apical side of the monolayers.

Key words ciprofloxacin (CPFX); LLC-PK₁, cell; organic cation transport; guanidine; enoxacin (ENX)

Recently, many new fluorinated quinolone derivatives have been developed and introduced into clinical use for the treatment of various infectious diseases. Ciprofloxacin (CPFX), a new fluorinated quinolone derivative, is a zwitterionic compound with pKₐ values of 6.0 and 8.8. The pharmacokinetics of CPFX have been reviewed extensively.¹,² With regard to the handling of CPFX in the kidney, the renal clearance of CPFX has been demonstrated to be far in excess of creatinine clearance, suggesting that tubular secretion must be involved.³,⁴ However, little information has been available on the mechanism of the tubular secretion of CPFX.

Previously, we reported a specialized transport system for enoxacin (ENX), which is another new fluorinated quinolone derivative and structurally close to CPFX (Chart 1), in cultured kidney epithelial cells (LLC-PK₁).⁵ The uptake of ENX by LLC-PK₁ cells cultured on plastic dishes was temperature- and concentration-dependent. We also demonstrated that the basolateral-to-apical transport of ENX across LLC-PK₁ cell monolayers on permeable supports was about two times greater than the apical-to-basolateral transport, and the transport in both directions was inhibited by guanidine.⁵¹

In this study, we attempted to clarify the uptake of CPFX by LLC-PK₁ cells cultured on plastic dishes, as well as the cellular mechanism of the transcellular transport of CPFX by LLC-PK₁ cell monolayers grown on collagen-coated microporous membrane filters. We also examined the interaction between ENX and CPFX on the transcellular transport by LLC-PK₁ cell monolayers.

MATERIALS AND METHODS

Materials CPFX, ENX and N¹-methylnicotinamide (NMN) were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). Tetraethylammonium chloride (TEA), guanidine, and bovine serum albumin (BSA) were from Wako Pure Chemical Industries (Osaka, Japan). Cimetidine was from Tokyo Kasei Hanbai Company (Tokyo, Japan). Medium 199 and fetal calf serum (FCS) were from Gibco Laboratories (Grand Island, NY, U.S.A.). All other chemicals used were of the highest purity commercially available.

Cell Culture LLC-PK₁ cells at passage 191 were kindly provided by the JCRB cell bank (Tokyo, Japan). The cells at passage 199—219 were seeded on 60-mm plastic culture dishes coated with rat tail collagen type I (Becton Dickinson, Bedford, MA, U.S.A.) at a density of about 4 × 10⁵ cells in 5 ml culture medium. The culture medium used was Medium 199 supplemented with 5% FCS. The medium was replaced with a fresh one every 2—3 d after inoculation, and was developed to confluence on 6—7 d of culture in an atmosphere of 5% CO₂ in air at 37°C. Subcultures were carried out every 5—6 d using 0.02% EDTA and 0.25% trypsin.

Uptake of CPFX For the uptake of CPFX by LLC-PK₁ cells, 60-mm dishes were inoculated with 4 × 10⁵ cells of complete culture medium (Medium 199 supplemented with 5% FCS). The cells were given fresh medium every 2—3 d after inoculation, and were used between 6—7 d on confluence. The uptake of CPFX (0.1 mm) was measured at 37 or 4°C, as described by Takano et al.⁶ The uptake assays were performed in Dulbecco’s phosphate-buffered saline (PBS, pH 7.4) [137 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 1 mM CaCl₂, 0.5 mM MgCl₂]. After the cultured medium was removed, the cells were washed three times with 5 ml of PBS buffer and pre-

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incubated at 37 or 4°C for 10 min. Then, 2 ml of PBS buffer containing CFPX was added to each dish, and the cells were incubated for specified periods. After incubation, the medium was immediately aspirated and the cells were washed three times with 5 ml of ice-cold PBS buffer. The cells were scraped with a cell scraper into 2 ml of ice-cold PBS buffer and homogenized with polytron (Kinematica, Kriens-Luzern, Switzerland) at a setting of 7 for 1 min. CFPX was determined by HPLC.

Transcellular Transport and Cellular Accumulation of CFPX The transcellular transport and cellular accumulation of CFPX was measured in the LLC-PK₁ cell monolayers cultured in Transwell chambers (Becton Dickinson, Bedford, MA, U.S.A.). LLC-PK₁ cell monolayers were seeded at a density of 4 × 10⁵ cells/cm² on a polycarbonate membrane filter (0.45 μm pore) in Transwell chambers (4.2 cm² area), and the chamber was placed in a six-well cluster. The volume of the medium inside and outside the monolayers was 2.0 and 2.5 ml, respectively. Fresh medium was replaced every 2–3 d, the cell monolayers were used between 7–8 d, and the transepithelial resistance ranged over 180 Ω cm². The cell monolayers were washed three times with 2 ml PBS buffer and preincubated at 37 or 4°C for 10 min. The transcellular transport measurement of CFPX was initiated by adding PBS buffer containing CFPX (25 μM) either to the apical or basolateral side of the monolayers (the volume of the PBS buffer inside and outside monolayers was 1.5 and 2.2 ml). After incubation for specified periods, the medium in the opposite side was taken as the sample solution and the CFPX concentration was measured by HPLC. For the cellular accumulation measurement, the medium was immediately aspirated and the cell monolayers were washed three times with 2 ml of ice-cold PBS buffer in both sides. The filters with cell monolayers were detached from the chambers, and the cells on these filters were immersed in 0.5 ml of the extraction solution [0.03 M phosphate buffer (pH 7.0): MeOH (1:1)] for 1 h at room temperature. CFPX in the extracts was measured by HPLC as follows.

Analytical Methods CFPX was measured by a high performance liquid chromatograph LC-6A (Shimadzu, Kyoto, Japan) equipped with a UV detector SPD-6A (Shimadzu). The conditions used for HPLC of CFPX were as follows: column, Inertsil C8 (GL Sciences Inc., 5 μm, 5 mm i.d. × 250 mm); mobile phase, acetonitrile:0.03 M citric acid containing 0.2% triethylamine (1:6); flow rate, 1.0 ml/min; wavelength, 286 nm; temperature, 50°C. In the measurement of the uptake sample, 1 ml of MeOH and 40 μl of 100 μM pipemidic acid as internal standard were added to 1 ml of homogenate. After shaking for 10 min, the sample was centrifuged at 3000 rpm for 10 min, and the supernatant was applied to HPLC. In the measurement of the transcellular transport sample, 20 μl of pipemidic acid (100 μM) as an internal standard was added to 0.1 ml of sample solution and applied to HPLC. In the measurement of the cellular accumulation sample, 20 μl of pipemidic acid (100 μM) as an internal standard was added to 0.5 ml of extracts, the sample was centrifuged at 7000 rpm for 25 min, and the supernatant was applied to HPLC. Protein was determined by the method of Bradford, using a Bio-Rad Protein Assay Kit, with BSA as a standard.

Statistical Analysis Statistical differences were investigated using one-way ANOVA followed by the Tukey test for multiple comparisons. Differences between group means were judged significant at p < 0.05.

RESULTS

Uptake of CFPX by LLC-PK₁ Cells The time course of CFPX uptake from the apical medium by LLC-PK₁ cells cultured on plastic dishes was compared at 37 and 4°C to observe the general transport properties. As shown in Fig. 1, the uptake of CFPX from the apical medium reached a nearly steady-state up to 45 min. The uptake of CFPX at 4°C was remarkably reduced. Thus, the uptake of CFPX by LLC-PK₁ cells was temperature-dependent.

The time course of CFPX efflux from LLC-PK₁ cells preincubated with CFPX for 60 min into the apical medium is shown in Fig. 2. At 37°C, the efflux of CFPX was rapid, and intracellular CFPX was almost undetectable.

**Fig. 1.** Time Course of CFPX Uptake from the Apical Medium by LLC-PK₁ Cells

**Fig. 2.** Efflux of CFPX from LLC-PK₁ Cells into the Apical Medium
able at 60 min. At 4°C, on the other hand, about 90% of CPFX still remained in the cells, even after 60 min.

We examined the effect of several organic cations on the uptake of CPFX by LLC-PK₁ cells from the apical medium (Fig. 3). CPFX uptake was inhibited by cimetidine and guanidine, but not by TEA and NMN.

Transcellular Transport of CPFX The apical-to-basolateral and basolateral-to-apical transport of CPFX across LLC-PK₁ cell monolayers was measured by adding CPFX into either the apical or basolateral medium and then determining the amount of CPFX which appeared in the opposite medium. Fig. 4 shows the time course of CPFX transport in the both directions. The basolateral-to-apical transport of CPFX was about 1.8 times greater than the apical-to-basolateral transport, and this basolateral-to-apical/apical-to-basolateral transport ratio was nearly constant for 60 min.

Table 1 shows the effect of temperature on CPFX transport across the LLC-PK₁ cell monolayers in the both directions. The basolateral-to-apical transport of CPFX at 4°C was remarkably reduced as compared with the transport at 37°C, and almost reached the same level of the apical-to-basolateral transport of CPFX at 4°C. Therefore, the basolateral-to-apical transport of CPFX across LLC-PK₁ cell monolayers, which predominated over the apical-to-basolateral transport, was greatly temperature-dependent, suggesting an involvement of a specialized transport system in the basolateral-to-apical transport of CPFX.

Effect of Various Organic Cations on Transcellular Transport of CPFX To examine the substrate specificity of both the basolateral-to-apical and apical-to-basolateral transport of CPFX in the LLC-PK₁ cell monolayers, the cis-inhibitory effects of several organic cations were evaluated by adding them to either the basolateral or apical side of the LLC-PK₁ cell monolayers. As shown in Fig. 5, only guanidine significantly inhibited both the basolateral-to-apical and apical-to-basolateral transport of CPFX. Inhibition by guanidine was more remarkable in the basolateral-to-apical transport.

Effect of ENX and Guanidine on Transcellular Transport

Table 1. Effect of Temperature on CPFX Transport across LLC-PK₁ Cell Monolayers

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Flux (pmol/cm²)</th>
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<tbody>
<tr>
<td></td>
<td>Basolateral-to-apical</td>
</tr>
<tr>
<td>37°C</td>
<td>309.8 ± 5.974</td>
</tr>
<tr>
<td>4°C</td>
<td>69.2 ± 4.303</td>
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LLC-PK₁ cell monolayers were incubated at 37 or 4°C for 60 min with 25 μM CPFX added either to the basolateral or the apical side of the monolayers. The appearance of CPFX in opposite side was determined. Values are the mean ± S.E. of four to five determinations.

![Fig. 3. Effect of Various Organic Cations on CPFX Uptake from the Apical Medium by LLC-PK₁ Cells](image)

![Fig. 4. Time Course of Transport of CPFX across LLC-PK₁ Cell Monolayers](image)

![Fig. 5. Effect of Various Organic Cations on CPFX Transport across LLC-PK₁ Cell Monolayers](image)
and the Accumulation of CPFX at Various pH Values of Apical Medium. We studied the effect of apical medium pH on the basolateral-to-apical transport of CPFX. As shown in Fig. 6A, when the apical medium pH was varied from 5.5 to 8.4 (the pH of the basolateral medium was fixed at 7.4), the basolateral-to-apical transport of CPFX was remarkably varied, being the greatest at pH 5.5 and the smallest at pH 8.4. In contrast, the accumulation of CPFX was the lowest at pH 5.5 and the greatest at pH 8.4 (Fig. 6B). These observations show that the transport across the apical membranes can be stimulated by acidifying the medium on the apical side, i.e., the inward directed H⁺ gradient acts as a driving force for extruding CPFX out of cells into the apical medium.

We compared the effect of ENX and guanidine on the basolateral-to-apical transport and the intracellular accumulation of CPFX at various apical medium pH values ranging from 5.5 to 8.4. As shown in Fig. 6A, the enhanced appearance of CPFX into the acidic apical medium was strongly inhibited in the presence of ENX and guanidine. Intracellular accumulation of CPFX in the acidic pH range was increased in the presence of guanidine. However, there was no significant change of CPFX accumulation in the presence of ENX over the pH range tested.

**DISCUSSION**

It has been reported that the important routes of CPFX elimination include glomerular filtration and renal tubular secretion, and renal clearance mechanisms account for approximately two-thirds of the total serum clearance of CPFX. However, renal handling of CPFX and its interaction with the transport system present in the proximal tubules are still obscure. As CPFX is ionized as a cation or zwitterion at physiological pH, a cationic form of CPFX may play a significant role in interacting with a renal transport system because urinary pH is usually weakly acidic. LLC-PK₁ cells, originally derived from a pig kidney, have many characteristics of proximal renal tubules. It was demonstrated that LLC-PK₁ cell monolayers grown on porous membrane filters possess an organic cation transport system on both the basolateral and apical membranes and that the cell monolayers are useful for an evaluation of the interactions of various drugs with the organic cation transport system.

Previously, we reported the transport of ENX in a LLC-PK₁ kidney epithelial cell line. ENX has two pKₐ values (pKₐ₁ = 6.2, pKₐ₂ = 8.8), being ionized as a cation or zwitterion at physiological pH and structurally close to CPFX. We reported that ENX interacts with an organic cation transport system on the basolateral membrane of LLC-PK₁ cells that guanidine shows a potent inhibitory effect on the transcellular transport of ENX.

The present results demonstrate that CPFX uptake from the apical medium by LLC-PK₁ cells cultured on plastic dishes was temperature-dependent. When the medium temperature was reduced to 4°C, CPFX uptake decreased drastically, suggesting the possibility that the uptake from the apical medium into the cell is a carrier-mediated process. We tried to examine the concentration dependence of CPFX uptake from the apical medium by LLC-PK₁ cells. However, the uptake was linear up to the concentration of 0.4 mM and it was impossible to determine Kₘ and V_max over the 0.4 mM due to the very low water solubility of CPFX. Therefore, kinetic parameters are unknown. As shown in Fig. 3, CPFX uptake from the apical medium was inhibited by the organic cations such as guanidine and cimetidine. However, TEA and NMM did not alter CPFX uptake. This result suggests that a specialized cation transport system(10, 11) is involved in CPFX transport across the apical membrane of LLC-PK₁ cells and that CPFX shares a common transport system with guanidine and cimetidine in LLC-PK₁ cells. This finding is consistent with our previous results on ENX.

The results from uptake experiments by LLC-PK₁ cells seemed compatible with those from the apical-to-basolateral transport experiment. Apical-to-basolateral CPFX transport across LLC-PK₁ cell monolayers was temperature-dependent (Table 1) and inhibited by guanidine (Fig. 5B), demonstrating again that guanidine and CPFX are commonly transported in the apical-to-basolateral direction by an organic cation transport system. In our previous study, cimetidine significantly inhibited apical-to-basolateral ENX transport. The reason why cimetidine failed to exhibit an inhibitory effect on the apical-to-basolateral transport of CPFX in this study is currently unclear.

As shown in Table 1, CPFX transport in both directions was almost identical at 4°C. Therefore, the significantly greater basolateral-to-apical CPFX transport than apical-to-basolateral transport at 37°C implied that the basolateral-to-apical transport of CPFX is much more mediated by a specialized process. The apical-to-basolateral CPFX transport exhibited several interesting features: 1) only guanidine exhibited an inhibitory effect on the basolateral-to-apical CPFX transport across LLC-PK₁ cell monolayers, 2) when the pH of the apical medium was varied from 5.5 to 8.4 (pH of the basolateral medium was...
fixed at 7.4), the basolateral-to-apical transport of CPFX was significantly varied, transport being the greatest at pH 5.5 and the lowest at pH 8.4, and 3) in the presence of ENX and guanidine, CPFX failed to exhibit the stimulated basolateral-to-apical transport in the acidic pH range. These results strongly suggest that a H⁺-organic cation antiport system on the apical membrane,\textsuperscript{12,13} which is amplified by the magnitude of an inward directed H⁺ gradient and common to guanidine, is involved in the basolaleral-to-apical CPFX transport. It is reasonable to consider that the increased intracellular accumulation of CPFX (Fig. 6B) is the result of the almost complete inhibition of the transport system by guanidine. Miyamoto \textit{et al.}\textsuperscript{14} previously reported that renal brush-border membranes possess multiple carrier systems for organic cations; one prefers TEA to guanidine as its substrate, and the other prefers guanidine to TEA. There was no interaction between TEA and CPFX in this study. Judging from our previous report and the present results, therefore, it may be concluded that new fluorinated quinolone derivatives such as ENX and CPFX are commonly transported by an organic cation transport system responsible for guanidine on the apical membrane.

Interestingly, the intracellular accumulation of CPFX was not increased when its appearance into the apical medium was inhibited by ENX. Although the reason is currently unclear, we assume that there is a possible interaction between ENX and CPFX in the transport across the basolateral membranes. Griffiths \textit{et al.}\textsuperscript{15} compared the transcellular transport of several fluorinated quinolone derivatives, including CPFX, using Caco-2 cell monolayers. They demonstrated the active transcellular secretion of CPFX, which was inhibited by ENX. At the same time, they implied the presence of a specialized transport system for new fluorinated quinolone derivatives on the basolateral membrane. Many new fluorinated quinolone derivatives are used widely as antibacterial drugs and many of them are excreted mainly by the kidney. In order to elucidate the correlation between chemical structures and renal transport mechanisms of the drugs, more intensive research is needed.

REFERENCES