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Possible involvement of P-glycoprotein in renal excretion of pazufloxacin in rats

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Abstract

The present study aims to investigate whether pazufloxacin, a new quinolone antimicrobial agent, is a substrate for P-glycoprotein in vitro, and whether it is excreted from kidney by P-glycoprotein and/or multidrug resistance-associated protein (Mrp2) in vivo. The in vitro experiments showed that the intracellular accumulation of pazufloxacin in adriamycin-resistant human chronic myelogenous leukemia cells (K562/ADR) overexpressing P-glycoprotein was significantly lower than that in human chronic myelogenous leukemia cells (K562/S) not expressing P-glycoprotein. When rats received an intravenous injection of pazufloxacin in combination with or without cyclosporine, cyclosporine significantly delayed the disappearance of pazufloxacin from plasma and decreased the systemic clearance and volume of distribution at steady state of pazufloxacin to 50% and 70% of the corresponding control values, respectively. Renal handling experiments revealed that the renal clearance of pazufloxacin was 75% of that corresponding to the systemic clearance, suggesting that the main route of pazufloxacin elimination is the kidney. Cyclosporine significantly increased the steady-state concentration of pazufloxacin in plasma by decreasing the tubular secretion clearance and glomerular filtration rate. These results suggest the possibility that pazufloxacin is excreted into the urine via P-glycoprotein. No significant differences in the renal and tubular secretion clearances of pazufloxacin were observed between normal rats and Eisai hyperbilirubinemic rats (EHBR), which have a hereditary deficiency in Mrp2, indicating the lack of the involvement of Mrp2 in the renal excretion of pazufloxacin. Sparfloxacin, a P-glycoprotein substrate, also significantly decreased the renal and tubular secretion clearances of pazufloxacin, suggesting that pazufloxacin and sparfloxacin share the same transporters, including P-glycoprotein. The present study at least suggests that pazufloxacin is excreted into the urine via P-glycoprotein and some active drug transporters other than Mrp2.

Keywords: Quinolone antimicrobial agent; Pazufloxacin; P-glycoprotein; Multidrug resistance-associated protein 2 (Mrp2); Renal excretion; (Rat)

1. Introduction

It is well known that drug transporters play a critical role in drug absorption, distribution, metabolism and excretion. One of the transporters, P-glycoprotein, an ATP-dependent transporter, acts as an efflux pump for various drugs such as *Vinca* alkaloid and anthracycline anticancer drugs, calcium channel blockers and immunosuppressive agents (Tsuruo et al., 1981, 1982; Twentyman et al., 1987; Naito et al., 1992). This transporter is located not only in anticancer drug-resistant cells, but also in normal tissues, including the brush-border membrane of

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renal proximal tubule cells, the bile canalicular membrane of hepatocytes, intestinal epithelial cells and blood–brain barrier (Thiebaut et al., 1987; Schinkel et al., 1996, 1997). Another known transporter, like P-glycoprotein, multidrug resistance-associated protein 2 (Mrp2), is also presented in almost the same tissues as P-glycoprotein, and plays an important role in excretion of various organic anions by an ATP-dependent mechanism (Oude Elferink et al., 1995; Borst et al., 1999; König et al., 1999). Thus, both drug transporters appear to act as an efflux transporter protein and have a protective function for endogenous and exogenous substances. It is also suggested that P-glycoprotein substrate and Mrp2 substrate overlap (Hidemura et al., 2003).

Quinolone antimicrobial agents are known to have potent activities against Gram–negative bacteria, and they are currently widely used for the treatment of various bacterial infections. The newly developed quinolone antimicrobial agent pazufloxacin mesilate (pazufloxacin) [(−)-(*S*)-10-(1-aminocyclopropyl)-9-fluoro-3-methyl-7-oxo-2,3-dihydro-7*H*-pyrido[1,2,3-*de*][1,4]benzoxazine-6-carboxylic acid monomethanesulfonate] has been produced as a formulation for injection and is currently under clinical use in Japan, although almost all quinolone antimicrobial agents are usually used as oral formulations. It is suggested that pazufloxacin has broad-spectrum, potent antibacterial activity and lower toxicity than conventional quinolone antimicrobial agents (Rubinstein, 2001; Nomura et al., 2002). We previously reported that pazufloxacin was mainly excreted into the urine, its renal clearance correlated well with creatinine clearance in patients, and that it has no effect on the pharmacokinetics of theophylline in rats (Hasegawa et al., 1995; Yamaki et al., 1997).

It is reported that some quinolone antimicrobial agents may be transported via drug transporters such as P-glycoprotein and/or Mrp2 (Oude Elferink et al., 1995; Cormet-Boyaka et al., 1998; Murata et al., 1999; De Lange et al., 2000; Tamai et al., 2000; Naruhashi et al., 2001; Yamaguchi et al., 2002; Zhao et al., 2002). Although there is a possibility that pazufloxacin is a substrate for P-glycoprotein and/or Mrp2, there is no detailed information whether it is excreted from kidney by P-glycoprotein and/or Mrp2. Also, little is known about the renal handling characteristics of pazufloxacin.

The present study aims to clarify the involvement of the drug transporters P-glycoprotein and/or Mrp2 in the renal excretion of pazufloxacin. First, we investigated the effect of cyclosporine, a P-glycoprotein inhibitor, on the renal excretion of pazufloxacin in Sprague–Dawley rats possessing Mrp2. Second, we studied the role of Mrp2 in the renal excretion of pazufloxacin using Eisai hyperbilirubinemic mutant rats (EHBR) lacking Mrp2. Third, we investigated the competitively inhibitory effect of sparfloxacin, a substrate for P-glycoprotein, on the renal excretion of pazufloxacin in Sprague–Dawley rats.

2. Materials and methods

2.1. Chemicals

Pazufloxacin mesilate (pazufloxacin) was kindly donated by Taisho Toyama Pharmaceutical (Tokyo, Japan). The structure of pazufloxacin mesilate is illustrated in Fig. 1. Sparfloxacin was donated from Dainippon Pharmaceutical (Tokyo, Japan). Grepafloxacin was kindly donated by Otsuka Pharmaceutical (Tokyo, Japan). Cyclosporine and inulin were purchased from Novartis Pharma (Tokyo, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. All other reagents are commercially available, of analytical grade, and used without further purification.

2.2. Cell culture and in vitro cellular accumulation of pazufloxacin in K562/S and K562/ADR cells

Human chronic myelogenous leukemia cell line (K562/S) and its adriamycin-resistant subline (K562/ADR) were kindly provided by Professor Ken-ichi Miyamoto (Kanazawa University School of Medicine). These cells were grown in RPMI medium supplemented with 1% penicillin/streptomycin, 1 mM L-glutamine and 10% fetal calf serum (Equitex-Bio, Kerrville, TX, USA) at 37 °C in 5% CO_2 humidified atmosphere. Adriamycin (400–500 nM) was added every 2 weeks to the culture medium of K562/ADR cells. The resistant K562/ADR cells were grown for 7 days in the presence of adriamycin prior to use in the experiments. Cell viability was assessed by trypan blue dye exclusion.

K562/S and K562/ADR cells (2×10^6 cells/ml) were suspended in phosphate buffered saline (PBS) solution (pH 7.2), and were incubated with pazufloxacin (50 μM) at 4 or 37 °C. After incubation, the cells were washed three times with cold PBS solution, and the obtained cell pellets were kept at −30 °C until analysis. The pellets were suspended in 50 μl of water containing grepafloxacin (0.6 μg/ml) as an internal standard, and then ultrasonicated with an ultrasonic disrupter (UD-2000, Tomy Seiko, Tokyo, Japan). The ultrasonicated solution was added to methanol and then shaken vigorously. Acetonitrile was added to the ultrasonicated solution and was shaken vigorously. After centrifugation by 3000×*g* at 4 °C for 10 min, the methanol layer was collected into glass tubes and was evaporated under a nitrogen gas stream at 40 °C. The residue was

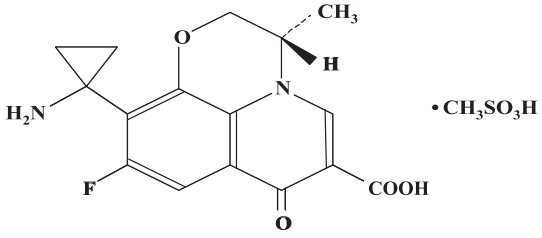

Fig. 1. Chemical structure of pazufloxacin mesilate.

reconstituted with the mobile phase to measure pazufloxacin by HPLC.

2.3. Animal experiments

Male Sprague–Dawley rats and Eisai hyperbilirubinemic mutant rats (EHBR), which have a hereditary deficiency in multidrug resistance associated protein 2 (Mrp2), were purchased from SLC Japan (Hamamatsu, Japan). The body weight of the rats was standardized (270–285 g). The animals were housed under controlled environmental conditions (temperature 23±1 °C and humidity 55±5%) with a commercial food diet and water freely available. All animal experiments were carried out according to the Guidelines of Nagoya University School of Medicine for the Care and Use of Laboratory Animals.

For investigating the effect of cyclosporine on the pharmacokinetics of pazufloxacin, rats were anesthetized with sodium pentobarbital (25 mg/kg), and the right jugular vein was cannulated with polyethylene tubes for drug administration and blood sampling. Pazufloxacin was injected intravenously at a dose of 10 mg/kg at 5 min after intravenous injection of cyclosporine (30 mg/kg) or saline (Control rats). Blood samples were collected at designated intervals (3, 5, 10, 20, 30, 45, 60, 90 and 120 min after the injection of pazufloxacin).

For elucidating the renal handling of pazufloxacin and the effects of cyclosporine and sparfloxacin on the renal handling of pazufloxacin, Sprague–Dawley rats or EHBR under light anesthesia with sodium pentobarbital were cannulated with polyethylene tubes into the right jugular vein, left carotid artery and the urinary bladder for drug administration, blood collection and urine collection, respectively. All the experiments were done under pentobarbital anesthesia, and the body temperature was maintained at 37 °C with a heat lamp. The rats received a bolus injection of pazufloxacin and inulin in a loading dose of 0.37 mg/kg and 25 mg/kg followed by a constant-rate infusion, using Harvard infusion pump (PHD 2000, South Natick, MA, USA), of a 4% mannitol solution delivering doses of 0.42 mg/h/kg of pazufloxacin and 20 mg/h of inulin at a rate of 4 ml/h until the end of the study. Steady-state plasma concentration of pazufloxacin was attained 60 min after starting the infusion. Mannitol was used to obtain sufficient and constant urine flow rate. After 60-min infusion, urine was collected in preweighed tubes at 20-min intervals for 60 min throughout the experiment. Blood samples were taken at the midpoints of the urine collection periods (70, 90 and 110 min after starting of infusion). After 120-min infusion, the rat received a single intravenous injection of cyclosporine (30 mg/kg) or sparfloxacin (20 mg/kg), and urine and blood samples were collected for 60 min using the same methods as described above. Plasma samples were obtained by centrifugation of the blood samples at 1200×g for 10 min. The volume of urine samples was measured gravimetrically with the specific gravity assumed to be 1.0. Plasma and urine samples were stored at −40 °C until analysis.

2.4. Protein binding experiments

To estimate the differences in protein binding of pazufloxacin in Sprague–Dawley rats and EHBR, the protein binding experiment was done by ultrafiltration with an Ultrafree-MC ultrafiltration device (Amicon, Bedford, MA). Under light anesthesia with ethyl ether, blood samples were obtained from the abdominal aorta of the rats, and plasma samples were immediately obtained by centrifugation. Four hundred microliters of plasma sample containing 0.2 μg/ml of pazufloxacin was poured into the device and centrifuged at 2000×g for 30 min at 20 °C. The ultrafiltrate was then assayed for measurement of the unbound fraction of pazufloxacin. Adsorption of pazufloxacin to the device was negligible. The total and unbound (ultrafiltrate) concentrations of pazufloxacin were measured by HPLC.

2.5. Drug analysis

Concentrations of pazufloxacin in plasma, urine and cells were determined by HPLC. Urine samples were appropriately diluted in distilled water, and brains were homogenized in a threefold volume of PBS solution. Plasma and diluted urine sample (50 μl) and 300 μl of acetonitrile were mixed and centrifuged at 12,000×g for 5 min. After centrifugation, the supernatant (50 μl) was injected directly into the HPLC apparatus.

The apparatus used for HPLC was a Shimadzu LC-10A system (Kyoto, Japan) equipped with a fluorescence detector (RF-10AXL, Shimadzu) (excitation, 330 nm; emission, 505 nm) consisting of an LC-10A liquid pump and an SIL-10A autoinjector. The conditions were as follows: column, a Cosmocil $5C_{18}$ column (4.6 by 150 mm, Nacalai Tesque, Kyoto, Japan); mobile phase, 20 mM Na_2SO_4/acetonitrile=80:20 [vol/vol] solution containing 0.1% H_3PO_4 (pH 4.0); column temperature (OTC-6A, Shimadzu), 40 °C; flow rate, 1.0 ml/min. These assays were shown to be linear for the concentrations studied with a correlation coefficient of 0.999. No interference with the peak of pazufloxacin was observed in any samples. The within- and between-day coefficients of variation for this assay were less than 8%. Concentration of inulin in plasma and urine was measured by the colorimetric method (Kato et al., 2002).

2.6. Pharmacokinetic analysis

Plasma concentration–time data for pazufloxacin after a single administration were analyzed using a noncompartmental model. The area under the curve (AUC) and the area under the first-moment curve (AUMC) were calculated by the trapezoidal rule with extrapolation to infinity. The system clearance (CL_{SYS}) was determined as dose/AUC.

The mean residence time (MRT) was calculated as MRT=AUMC/AUC. The volume of distribution at steady state (V_{SS}) was calculated as $V_{SS}=CL_{SYS}\times MRT$.

For the renal handling experiments, the renal clearance (CL_R) of drugs (pazufloxacin and inulin) during each urine collection period was calculated by dividing the urinary excretion rate by the steady-state plasma concentration (C_{SS}) determined for that collection period. The renal clearance of drug unbound to plasma protein (CL_{RU}) was calculated by dividing CL_R by the unbound fraction of the drug (f_U). The glomerular filtration rate (GFR) was taken to represent the inulin clearance. The clearance ratio of pazufloxacin was calculated as CL_R/GFR. Assuming that the renal tubular reabsorption of pazufloxacin is negligible, the tubular secretion clearance of unbound drug, which represents the net tubular secretion, was calculated as CL_R/f_U minus GFR. Each parameter was calculated using the mean value of three points during 60 min.

2.7. Statistical analysis

Results are expressed as means±standard errors. Statistical differences between means were assessed by Student's *t*-test or one-way analysis of variance (ANOVA). When *F* ratios were significant ($P<0.05$), Scheffe's post hoc tests between the groups were done, and *P* values of <0.05 were considered statistically significant differences.

3. Results

3.1. In vitro accumulation of pazufloxacin in K562/S and K562/ADR cells

Western blot analysis revealed that P-glycoprotein was overexpressed in K562/ADR, but not in the K562/S cells as reported previously (Asakura et al., 2004). The time-courses of the intracellular accumulation of pazufloxacin into K562/S and K562/ADR cells are shown in Fig. 2. The concentration of pazufloxacin accumulated in both cells was significantly higher at all time points at 37 °C than 4 °C, and the amount of pazufloxacin accumulated in K562/S cells for 60 min was significantly higher than that in K562/ADR cells (11.7 and 7.5 μmol/2×10^6 cells, respectively).

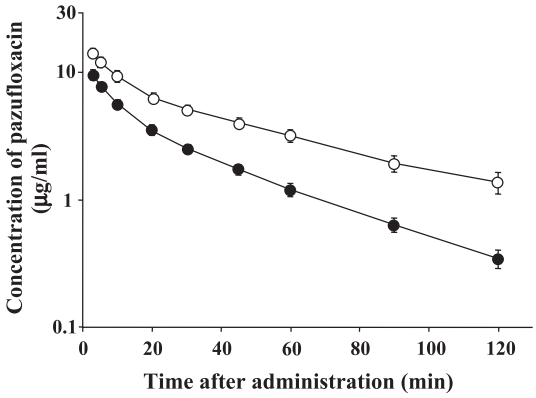

Fig. 3. Effect of cyclosporine on plasma concentrations of pazufloxacin after a single intravenous injection in Sprague–Dawley rats. Cyclosporine (30 mg/kg) was administered intravenously 5 min before injection of pazufloxacin (10 mg/kg). Each symbol represents the mean±S.E.M. (n=4). When the S.E.M. is small, it is included in the symbol. Symbols: ●, control; ○, cyclosporine. Plasma concentration data of pazufloxacin in all sampling points were significantly higher in cyclosporine-treated rats than untreated rats ($P<0.05$).

3.2. Effect of cyclosporine on pharmacokinetics and renal handling of pazufloxacin in Sprague–Dawley rats

First, we examined the effect of cyclosporine on the pharmacokinetics of pazufloxacin in Sprague–Dawley rats. Semilogarithmic plots of plasma concentration–time data for pazufloxacin in the control and cyclosporine-treated rats following a single intravenous injection of pazufloxacin (10 mg/kg) are illustrated in Fig. 3. Pretreatment with cyclo-

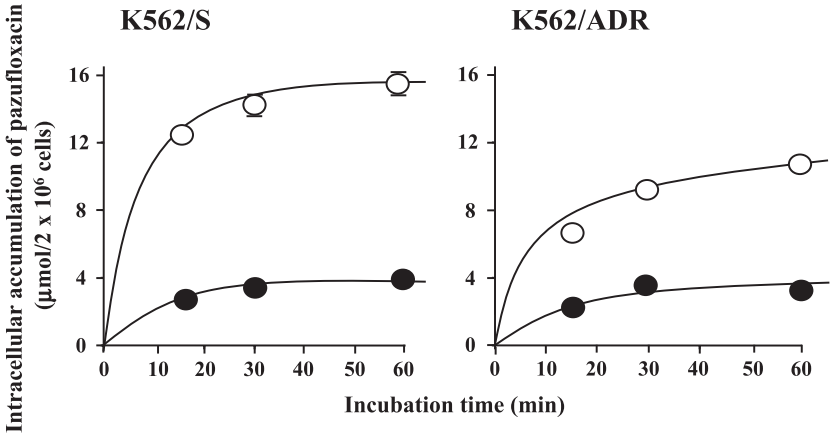

Fig. 2. Intracellular accumulation of pazufloxacin in K562/S and K562/ADR cells. The mean uptake of pazufloxacin in K562/S and K562/ADR cells for 60 min was 11.7 and 7.5 nmol/2×10^6 cells, respectively. Data are the mean±S.E.M. (n=3–5). When the S.E.M. is small, it is included in the symbol. Symbols: ●, 4 °C; ○, 37 °C. Significant differences were observed at all time points between 4 and 37 °C.

Table 1
Effect of cyclosporine on pharmacokinetic parameters of pazufloxacin in Sprague–Dawley rats

Parameter	Control	Cyclosporine
CL_{SYS} (l/h/kg)	2.21±0.16	1.03±0.11
V_{SS} (l/kg)	1.35±0.13	0.99±0.01
MRT (h)	0.61±0.05	0.99±0.10

Each values represents the mean±S.E.M. (n=4).
Cyclosporine (30 mg/kg) was administered intravenously 5 min before a single intravenous injection of pazufloxacin (10 mg/kg). CL_{SYS}, systemic clearance; V_{SS}, volume of distribution at steady state; MRT, mean residence time. Significant differences in all parameters were observed between control and cyclosporine (P<0.05).

sporine (30 mg/kg, 5 min earlier) significantly delayed the disappearance of pazufloxacin from plasma and increased its concentrations in plasma. The corresponding pharmacokinetic parameters of pazufloxacin are summarized in Table 1. Pretreatment with cyclosporine significantly decreased the systemic clearance and steady state volume of distribution of pazufloxacin to 50% and 70% of the respective control values.

Second, we examined the renal handling of pazufloxacin and the effect of cyclosporine on the renal excretion in Sprague–Dawley rats. The renal handling parameters of pazufloxacin in the control and cyclosporine-treated rats are summarized in Table 2. The steady state plasma concentrations of pazufloxacin after a bolus injection of cyclosporine (30 mg/kg) were significantly higher than that in the control rats (0.35 and 0.21 μg/ml, respectively). Significant decreases in the glomerular filtration rate and renal clearance of pazufloxacin were observed in cyclosporine-treated rats (1.5–1.1 and 8.0–3.6 ml/min, respectively). When the protein binding potency of pazufloxacin was measured using an ultrafiltration method, the plasma-unbound fraction was 0.58. Cyclosporine, which has a high protein binding potency, had no effect on the unbound fraction of pazufloxacin. To exclude the effect of the plasma protein binding on the renal excretion of pazufloxacin, the renal clearance for unbound drug to plasma protein was calculated. Cyclosporine significantly decreased the renal clearance for unbound pazufloxacin by 55%. Assuming that

Table 2
Effect of cyclosporine on renal handling of pazufloxacin in Sprague–Dawley rats

Parameter	Control	Cyclosporine
C_{SS} (μg/ml)	0.21±0.01	0.35±0.03
CL_R (ml/min)	8.01±0.51	3.59±0.21
CL_{RU} (ml/min)	13.7±0.88	6.17±0.36
GFR (ml/min)	1.48±0.09	1.06±0.06
CL_S (ml/min)	12.6±0.92	5.07±0.33

Each value represents the mean±S.E.M. (n=6–7).
C_{SS}, steady state plasma concentration; CL_R, renal clearance; CL_{RU}, renal clearance of unbound drug; GFR, glomerular filtration rate; CL_S, tubular secretion clearance. Each of these parameters represents the mean value of three points during 60 min. Significant differences in all parameters were observed between control and cyclosporine (P<0.05).

Table 3
Effect of cyclosporine on renal handling of pazufloxacin in EHBR

Parameter	Control	Cyclosporine
C_{SS} (μg/ml)	0.23±0.01	0.35±0.00
CL_R (ml/min)	8.24±0.29	4.47±0.10
CL_{RU} (ml/min)	14.1±0.49	7.66±0.18
GFR (ml/min)	1.38±0.13	1.07±0.05
CL_S (ml/min)	12.8±0.92	6.59±0.22

Each value represents the mean±S.E.M. (n=3).
C_{SS}, steady state plasma concentration; CL_R, renal clearance; CL_{RU}, renal clearance of unbound drug; GFR, glomerular filtration rate; CL_S, tubular secretion clearance. Each of these parameters represents the mean value of three points during 60 min. Significant differences in all parameters were observed between control and cyclosporine (P<0.05).

the renal tubular reabsorption of pazufloxacin is negligible, the net tubular secretion clearance of pazufloxacin was significantly decreased by injection of cyclosporine.

3.3. Renal handling of pazufloxacin in EHBR

To clarify the involvement of Mrp2 in the elimination of pazufloxacin, we examined the renal handling of pazufloxacin and the effect of cyclosporine on the renal excretion in EHBR lacking Mrp2. As shown in Table 3, the control values of the renal clearance and steady state plasma concentration of pazufloxacin in EHBR were 8.2 ml/min and 0.23 μg/ml, respectively, which were not significantly different from those in Sprague–Dawley rats possessing Mrp2 (8.0 ml/min and 0.21 μg/ml, respectively). No significant difference in the unbound fraction to plasma protein was observed between Sprague–Dawley rats and EHBR. The observed glomerular filtration rate and tubular secretion clearance in EHBR (1.4 and 12.8 ml/min, respectively) were also insignificantly different from those in Sprague–Dawley rats (1.5 and 12.6 ml/min, respectively). Cyclosporine significantly decreased the renal clearance,

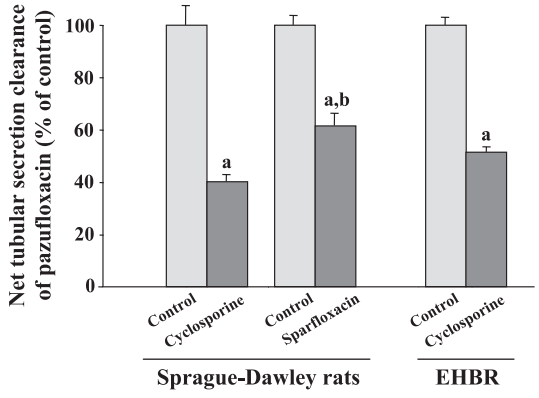

Fig. 4. Effects of cyclosporine and sparfloxacin on net tubular secretion of pazufloxacin. Each column represents the mean±S.E.M. (n=3–7). No significant differences in the control values of the tubular secretion of pazufloxacin were observed among cyclosporine- and sparfloxacin-treated Sprague–Dawley rats and cyclosporine-treated EHBR (12.6, 12.8 and 13.0 ml/min, respectively). [a,b]Significantly different from untreated and cyclosporine-treated Sprague–Dawley rats, respectively (P<0.05).

Table 4
Effect of sparfloxacin on renal handling of pazufloxacin in Sprague–Dawley rats

Parameter	Control	Sparfloxacin
C_{SS} (μg/ml)	0.25±0.01	0.33±0.01[a]
CL_R (ml/min)	8.48±0.24	5.53±0.34[a]
CL_{RU} (ml/min)	14.5±0.40	9.48±0.58[a]
GFR (ml/min)	1.52±0.08	1.46±0.04
CL_S (ml/min)	13.0±0.45	8.02±0.61[a]

Values are presented as the mean±the standard error (n=4).
C_{SS}, steady state plasma concentration; CL_R, renal clearance; CL_{RU}, renal clearance of unbound drug; GFR, glomerular filtration rate; CL_S, tubular secretion clearance. Each of these parameters represents the mean value of three points during 60 min.
[a] Significantly different from control (P<0.05).

renal clearance for unbound drug to plasma protein and tubular secretion clearance of pazufloxacin, and the glomerular filtration rate in EHBR, but significantly increased the steady state plasma concentration. As illustrated in Fig. 4, no significant differences in the degree of cyclosporine-induced decrease in the net tubular secretion clearance were observed between Sprague–Dawley rats and EHBR.

3.4. Effect of sparfloxacin on renal excretion of pazufloxacin

We examined the effect of sparfloxacin on the renal excretion of pazufloxacin in Sprague–Dawley rats. As shown in Table 4, sparfloxacin (20 mg/kg) significantly increased the steady state plasma concentration of pazufloxacin by about 1.3-fold and decreased the renal clearance by 35% compared with the control (0.25 μg/ml and 8.5 ml/min, respectively). Sparfloxacin significantly decreased the tubular secretion clearance of pazufloxacin with no change in the glomerular filtration, and the decreased percentage of the net tubular secretion of pazufloxacin induced by sparfloxacin (40%) was significantly smaller than that induced by cyclosporine (60%).

4. Discussion

To date, it remains unclear whether pazufloxacin is excreted into urine by a P-glycoprotein- or other transporter-mediated transport mechanisms, although most quinolone antimicrobial agents are known to be excreted mainly into the urine by active transport systems. Therefore, in the present study the role of P-glycoprotein and/or Mrp2 in the renal excretion of pazufloxacin was investigated.

We previously confirmed that P-glycoprotein, but not Mrp2, is overexpressed in K562/ADR cells by Western blot analysis (Asakura et al., 2004). First, we examined the intracellular accumulation of pazufloxacin in K562/S cells not expressing P-glycoprotein and K562/ADR cells overexpressing P-glycoprotein in order to investigate whether pazufloxacin is a substrate for P-glycoprotein. The in vitro experiments revealed that the amount of pazufloxacin remaining in K562/S cells was higher than that in K562/ADR cells, suggesting that pazufloxacin is a substrate for P-glycoprotein and is pumped out of K562/ADR cells via P-glycoprotein. It is likely that the affinity of pazufloxacin for P-glycoprotein is lower than that of cyclosporine (Wang et al., 2000).

Second, we examined the effect of cyclosporine on the pharmacokinetics of pazufloxacin after a single intravenous injection in normal rats (Sprague–Dawley rats). The present study showed that cyclosporine significantly delayed the disappearance of pazufloxacin from plasma and decreased the systemic clearance and steady state volume of distribution. These results suggest that P-glycoprotein contributes to pazufloxacin clearance from plasma through P-glycoprotein-expressing tissues. Considering that pazufloxacin is predominantly excreted into the urine (Hayakawa et al., 2002), the cyclosporine-induced decrease in the systemic clearance of pazufloxacin can be attributed to the decrease in the function of P-glycoprotein and/or other transporters in the kidney.

Third, we examined the renal handling of pazufloxacin and the effect of cyclosporine on the renal excretion in Sprague–Dawley rats using renal clearance experiments. The contribution of the renal clearance to the systemic clearance, which was calculated by dividing the infusion rate by steady state plasma concentration, was relatively high (70%). These results agreed well with a previous report that pazufloxacin is mainly excreted into the urine (Hayakawa et al., 2002). The renal clearance of pazufloxacin (8.0 ml/min) was approximately fivefold higher than the measured glomerular filtration rate (1.48 ml/min). Considering that the unbound fraction of pazufloxacin to plasma protein was 0.58, the calculated clearance ratio, which represents the ratio of the renal clearance for unbound pazufloxacin (13.7 ml/min) to the glomerular filtration rate, was approximately 8, and the tubular secretion clearance was calculated to be 12.6 ml/min, suggesting that pazufloxacin is actively secreted into the urine by some active drug transporters. Since the kidney is the main route of pazufloxacin elimination, and it is thought that P-glycoprotein functions effectively in this tissue, we further demonstrated whether P-glycoprotein is involved in the renal secretion of pazufloxacin by using the well-known P-glycoprotein inhibitor cyclosporine. Cyclosporine significantly, but not completely, decreased the renal and tubular secretion clearances of pazufloxacin by 50% and 55%, respectively, and the glomerular filtration rate by 30%. These results suggest that the decreased renal clearance of pazufloxacin by cyclosporine would be partly involved in the decreased glomerular filtration rate by cyclosporine, which latter can be explained by reports that cyclosporine decreases glomerular filtration by decreasing the renal blood flow rate (Bloom et al., 1995; Verbeke et al., 1995). It is reported that cyclosporine at the same dose used in this study dramatically decreased P-glycoprotein-mediated renal tubular secretion of rhodamine 123, a typical P-glycoprotein

substrate (Huang et al., 2000). This would suggest that cyclosporine could inhibit the function of P-glycoprotein in the kidney, although the amount of unchanged cyclosporine excreted into the urine is reported to be small (Wagner et al., 1987). We may conclude that the decreased tubular secretion clearance of pazufloxacin by injection of cyclosporine is due mainly to the inhibition of pazufloxacin-sensitive transporters including P-glycoprotein, but not attributable to the decreased glomerular filtration rate because tubular secretion clearance is independent on glomerular filtration rate.

Mrp2 is known to exist in the kidney as well as in the liver, which functions as a potent efflux pump like P-glycoprotein. It is reported that hepatobiliary secretion of quinolone antimicrobial agents might be mediated via Mrp2 (Sasabe et al., 1999) and that cyclosporine inhibits not only P-glycoprotein but also Mrp2 (Chen et al., 1999). Therefore, it is possible that cyclosporine inhibited the Mrp2-mediated renal secretion of pazufloxacin to some extent. Fourth, to demonstrate the involvement of Mrp2 in the renal excretion of pazufloxacin, the renal excretion of pazufloxacin in Sprague-Dawley rats possessing Mrp2 was compared with that in EHBR lacking Mrp2. In this experiment, no significant difference in the tubular secretion clearance of pazufloxacin was observed between Sprague–Dawley rats and EHBR. These results indicate that the tubular secretion of pazufloxacin is mediated via some active drug transporters other than Mrp2, and that EHBR maintains other pazufloxacin-sensitive transporters than Mrp2 in the kidney because the contribution of Mrp2 to the renal excretion of organic anions is reported to be minor (Takenaka et al., 1995a,b; Morikawa et al., 2000; Terlouw et al., 2001). On the other hand, the cyclosporine-induced decrease in the tubular secretion clearance of pazufloxacin in EHBR (40%) was significantly smaller than that in Sprague–Dawley rats (60%). On the basis of these observations, we assume that pazufloxacin is partly transported by other active transporters in compensation for lack of Mrp2 in EHBR. Contrary to our results, Fukuda et al. (1995) have reported that probenecid, a known inhibitor of Mrp2, decreased the elimination rate constant and total body clearance of pazufloxacin in rabbit, and the renal clearance was approximately fourfold higher than the glomerular filtration rate, suggesting pazufloxacin was excreted into the urine by both glomerular filtration and renal tubular secretion. This finding can be explained by evidence that probenecid inhibits various types of organic anion transporting systems including Mrp2 (Terashita et al., 1995; Masereeuw et al., 1996; Ullrich and Rumrich, 1997). Summarizing the above data, pazufloxacin might be, at least in part, secreted into the urine by P-glycoprotein and other organic anion transporters rather than Mrp2.

It is well known that plasma protein binding is a limiting factor in drug disposition, since only the unbound drug portion is capable of being diffused across various biological membranes to be distributed in the body and is subject to metabolism and renal excretion. Bilirubin is known to bind strongly to albumin in plasma. Our previous studies have reported that the unbound fraction of enprofylline, a xanthine derivative highly bound to plasma proteins, is increased in EHBR when compared with that in Sprague–Dawley rats (Nadai et al., 1994). The protein binding experiments in this study showed no significant changes in the unbound fraction to plasma protein for pazufloxacin between Sprague–Dawley rats and EHBR, and cyclosporine had no effect on the protein binding of pazufloxacin. Therefore, the alteration of the renal excretion of pazufloxacin by injection of cyclosporine in Sprague–Dawley rats and EHBR may not be due to a change in the unbound fraction to plasma protein.

It has been reported that only sparfloxacin is transported by P-glycoprotein among various quinolone antimicrobial agents (norfloxacin, ciprofloxacin, pefloxacin, fleroxacin and sparfloxacin) (De Lange et al., 2000). We also reported that sparfloxacin has a much stronger inhibitory effect on P-glycoprotein-dependent anticancer drug resistance than other quinolone antimicrobial agents (norfloxacin, enoxacin, ofloxacin, lomefloxacin and grepafloxacin) (Zhao et al., 2002). Finally, we examined the effect of sparfloxacin, an established P-glycoprotein substrate, on the renal excretion of pazufloxacin. Sparfloxacin significantly decreased the tubular secretion clearance of pazufloxacin by 40%, and the decrease was significantly lower than that induced by cyclosporine (60%). In addition, the magnitude of cyclosporine-induced decrease in the tubular secretion clearance of pazufloxacin in Sprague–Dawley rats was not significantly different from that in EHBR. These results suggest that sparfloxacin competitively inhibits the tubular secretion of pazufloxacin by P-glycoprotein and/or sparfloxacin-sensitive transporters. Considering that cyclosporine is known to inhibit the function of both P-glycoprotein and Mrp2 (Chen et al., 1999; Hidemura et al., 2003) and that sparfloxacin is a substrate for P-glycoprotein, but not Mrp2 (De Lange et al., 2000), we presume that cyclosporine may inhibit other pazufloxacin-sensitive transporters besides P-glycoprotein. Further studies are needed to clarify other pazufloxacin-sensitive transporters, including organic anion or cation transport mechanisms.

In conclusion, the present study is, to our knowledge, the first demonstration that pazufloxacin is a substrate for P-glycoprotein and is, at least in part, excreted into the urine by P-glycoprotein and some other active transporters rather than Mrp2. Further studies, however, are needed to investigate which transporters are involved in the renal excretion of pazufloxacin.

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