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Active intestinal secretion of new quinolone antimicrobials and the partial contribution of P-glycoprotein

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Abstract

Transport of quinolone antimicrobials and the contribution of the secretory transporter P-glycoprotein were studied in-vivo and in-vitro. In rat intestinal tissue (Ussing chambers method) and human Caco-2 cells (Transwell method), grepafloxacin showed secretory-directed transport. In both experimental systems, the secretory-directed transport was decreased by ciclosporin A, an inhibitor of P-glycoprotein, and probenecid, an inhibitor of anion transport systems. This suggested the contribution of P-glycoprotein and anion-sensitive transporter(s). The involvement of P-glycoprotein was investigated by using a P-glycoprotein over-expressing cell line, LLC-GA5-COL150, and P-glycoprotein-gene-deficient mice (mdr1a(−/−)/1b(−/−) mice). LLC-GA5-COL150 cells showed secretory-directed transport of grepafloxacin, while the parent cell line, LLC-PK1, did not. The secretory-directed transport of sparfloxacin and levofloxacin was also detected in LLC-GA5-COL150 cells. In the mdr1a(−/−)/1b(−/−) mice, the intestinal secretory clearance was smaller than that in wild-type mice after intravenous administration of grepafloxacin. Moreover, the absorption from an intestinal loop in mdr1a(−/−)/1b(−/−) mice was larger than that in wild-type mice. Accordingly, it appears that some quinolones are transported by secretory transporters, including P-glycoprotein. The involved transporters function in-vivo not only to transport grepafloxacin from blood to intestine but also to limit its intestinal absorption.

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Introduction

Quinolone antimicrobial drugs are widely used in the clinical setting, since they have considerable oral bioavailability with limited entry into the brain, resulting in minimal adverse effects in the central nervous system (Jaehde et al 1992; Murata et al 1999). Despite the moderate to excellent bioavailability of quinolones (e.g. 72% for grepafloxacin) in-vivo (Sörgel et al 1989), it has been reported that the quinolones, such as ciprofloxacin (Griffiths et al 1993; Dautrey et al 1999), norfloxacin (Griffiths et al 1994), pefloxacin and sparfloxacin (Cormet-Boyaka et al 1998), are transported preferentially in the secretory direction in Caco-2 cells. However, the intestinal secretory transport mechanisms of quinolones and the effect of such transport on in-vivo absorption have not yet been fully elucidated and absorptive mechanisms of quinolones in the intestine are still controversial.

So far, the secretory mechanisms of quinolones have been examined at the epithelial level. It has been reported that sparfloxacin secretion in Caco-2 cells was

inhibited by verapamil, suggesting a contribution of P-glycoprotein as a secretory transporter to this secretion in the intestine (Cavet et al 1997). Moreover, HSR-903, another quinolone, has been demonstrated to have limited entry into the brain due to the presence of P-glycoprotein at the blood–brain barrier (Murata et al 1999). Thus, quinolones are considered to be secreted by P-glycoprotein. We have recently demonstrated that brain distribution of several quinolones is restricted by multiple efflux transporters including P-glycoprotein in-vivo (Tamai et al 2000). As for the intestinal secretion of drug, Mayer et al (1996) demonstrated the role of P-glycoprotein in the pharmacokinetics of digoxin. They found that digoxin, a model substrate for P-glycoprotein, is secreted in the small intestine in considerable amounts in normal mice, and digoxin secretion was significantly decreased in mice with a defect of the mdr1a gene that encodes P-glycoprotein. We have also demonstrated that P-glycoprotein acts as an absorption barrier by transporting various drugs from intestinal cells into the lumen. Active secretion of several β-blockers from the small-intestinal epithelium in rats by P-glycoprotein functions as an absorption barrier, resulting in decreased blood concentrations of the drugs (Terao et al 1996), and absorptive transport of a serotonin antagonist, azasetron, is regulated by P-glycoprotein in-vitro (Tamai et al 1997). Thus, P-glycoprotein plays an important role in intestinal absorption and there is a possibility that absorption of quinolones in the intestine is also affected by P-glycoprotein. However, the contribution of P-glycoprotein to the intestinal secretion of quinolones has not yet directly been demonstrated in-vivo.

In this study, we first investigated absorptive and secretory mechanisms of quinolones, especially grepafloxacin, in rat intestinal tissue and Caco-2 cells in-vitro, focusing on the contribution of P-glycoprotein as a secretory transporter by using a P-glycoprotein over-expressing cell line, LLC-GA5-COL150. Then, mdr1a/1b gene-deficient mice were used to demonstrate the contribution of P-glycoprotein to the intestinal transport of quinolones in-vivo.

Materials and Methods

Chemicals

[^{14}C]Grepafloxacin, unlabelled grepafloxacin and [^{14}C]-levofloxacin were supplied by Otsuka Pharmaceutical Co., Ltd (Tokushima, Japan). [^{14}C]Sparfloxacin was purchased from Moravek Biochemicals (Brea, CA). All other reagents were commercial products of reagent grade, and were used without further purification.

Animals

Sprague-Dawley rats were purchased from Japan SLC (Hamamatsu, Japan). Male FVB/NJ and mdr1a(−/−)/1b(−/−) mice were purchased from Jackson Laboratories (Bar Harbor, ME) and Taconic Farms, Inc. (Germantown, NY), respectively. Animal studies were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals at the Takara-machi Campus of Kanazawa University.

Transport experiments (Ussing-chamber method)

Rat intestinal tissue sheets were prepared as described previously (Tamai et al 1997). The tissue preparation, consisting of the mucosa and most of the muscularis mucosa, was made by removing the submucosa and tunica muscularis with fine forceps. The tissue sheets were mounted vertically in an Ussing-type chamber that provided an exposed area of 0.5 cm^2. The volume of bathing solution on each side was 5 mL, and the solution temperature was maintained at 37°C. Test solution (5 mL), with or without grepafloxacin (10 μM), was added to donor or acceptor compartment, respectively. The duration of the transport experiment was 130 min and samples (0.2 mL) were taken from the acceptor chamber at designated times and replaced with the same volume of drug-free test solution. The test solution was composed of (mM): 128 NaCl, 5.1 KCl, 1.4 $CaCl_2$, 1.3 $MgSO_4$, 21 $NaHCO_3$, 1.3 KH_2PO_4, 10 NaH_2PO_4 and 5 glucose at pH 7.4, and was gassed with 95% O_2/5% CO_2 before and during the transport experiment.

Cell culture

Caco-2 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% non-essential amino acids, 2 mM L-glutamine, 100 units mL^{-1} penicillin G and 100 μg mL^{-1} streptomycin, as described previously (Tamai et al 1997). For the transport experiments, Caco-2 cells were grown on dishes and Transwell microporous polycarbonate membranes (Costar, Bedford, MA). Caco-2 cells were cultured for 21–23 days before use for the transport experiments.

LLC-PK1 or LLC-GA5-COL150 cells (Tanigawara et al 1992, Ueda et al 1992) obtained from Riken Cell

Bank (Tsukuba Japan) were grown in M199 medium containing 10% fetal calf serum 14.3 mM $NaHCO_3$ and 3% L-glutamine, without or with 150 ng mL^{-1} colchicine, as described previously (Kusunoki et al 1998). For the transport experiments, LLC-PK1 and LLC-GA5-COL150 cells were grown on Transwell microporous polycarbonate membranes, and cultured for 3 or 4 days before use.

Transport experiments with cultured cells (Transwell method)

The transport study was performed with Caco-2 cells or LLC-PK1/LLC-GA5-COL150 cells grown on Transwells as described previously (Tsuji et al 1994). The confluent cells were washed with Hanks' balanced salt solution (HBSS) (composition (mM): 0.952 $CaCl_2$, 5.36 KCl, 0.441 KH_2PO_4, 0.812 $MgSO_4$, 136.7 NaCl, 0.385 Na_2HPO_4, 25 D-glucose and 10 HEPES, pH 7.4 unless otherwise noted; the osmolarity was 315 mOsm kg^{-1}), and 0.5 mL and 1.5 mL of HBSS were added on the apical and basolateral sides, respectively, of a cell insert. To measure apical-to-basolateral or basolateral-to-apical flux, a test compound was included in the apical or basolateral side, respectively. At the designated times, samples (0.5 mL of basolateral- or 0.2 mL of apical-side solution) were withdrawn from the acceptor compartment and replaced with an equal volume of HBSS. The radioactivity in each solution was measured.

Intestinal secretion study (loop method)

Mice were anaesthetized with pentobarbital, the intestine was exposed by midline abdominal incision, and the bile duct was ligated. A 10-cm closed loop of ileum was prepared by ligation at both ends after clearing the gut by passing warmed isotonic 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (composition (mM): 5 KCl, 100 NaCl, 10 MES, 85 mannitol, polyethylene glycol 0.01 %; pH 6.4; the osmolarity was 290 mOsm kg^{-1}) slowly until the effluent was clear, and expelling the remaining solution by means of air pumped using a syringe. Each loop was filled with 0.4 mL of isotonic MES buffer. After the preparation was completed, the mouse was kept on a warm plate at 37°C for a 10-min recovery period. [^{14}C]Grepafloxacin was given intravenously at a dose of 1 μCi per animal. Blood was collected from the intra-orbital venous plexus using heparinized capillary tubes at designated times and the plasma was prepared by centrifugation. One hour after administration, the solution in the loop was collected and the loop was rinsed with ice-cold isotonic MES buffer to make a total effluent volume of 5 mL.

Intestinal absorption study (loop method)

A 5-cm ileal loop was prepared using the same procedure as described above. Isotonic MES buffer (0.2 mL) containing unlabelled grepafloxacin (10 μM) was introduced into the loop. The mouse was kept on a warm plate at 37°C. After 15 min, the solution in the loop was collected and the loop was rinsed with isotonic MES buffer to give a total effluent volume of 5 mL. The concentration of grepafloxacin in the effluent was measured by HPLC to estimate the remaining amount of grepafloxacin.

Analytical methods

To assay radioactivity, all samples were transferred into counting vials, mixed with scintillation fluid (Cleasol I; Nacalai Tesque, Kyoto, Japan) and quantified in a liquid scintillation counter (Aloka, Tokyo, Japan).

Non-radioactive grepafloxacin was used in the Ussing-chamber method and the intestinal absorption study by the in-situ loop method, and the amount was measured by HPLC. The HPLC system consisted of a constant-flow pump (980-PU; Japan Spectroscopic Co., Tokyo, Japan), a fluorescence detector (RF-550; Shimadzu Co., Kyoto, Japan), integrator (Chromatopac CR3A; Shimadzu Co.) and an automatic sample injector (AS-950; Japan Spectroscopic Co.), and a TSK gel ODS-80Ts (150 × 4.6 mm i.d., Tosoh, Tokyo, Japan) column was used. The excitation and emission wavelengths were 282 and 448 nm, respectively, and the column was kept at 50°C. The mobile phase consisted of acetonitrile–water–phosphoric acid (25:75:0.1, v/v/v), and the flow rate was 0.8 mL min^{-1}. The assay was shown to be linear over the concentration range studied.

Data analysis

Permeation was estimated by the amount transported (pmol cm^{-2}). The permeation coefficient (cm s^{-1}) was obtained from the slope of the linear portion of the plots of permeation against time (s). All data are expressed as means ± s.e.m. and statistical analysis was performed by using the analysis of variance test, followed by the Bonferroni test when significance was observed. A dif-

Table 1 Regional differences of grepafloxacin transport in rat intestinal tissue evaluated by the Ussing-chamber method.

		Duodenum	Jejunum	Ileum
Permeability coefficient ($\times 10^{-5}$ cm s^{-1})	Mucosal-to-serosal	0.323 ± 0.100	0.662 ± 0.167*	1.27 ± 0.17*
	Serosal-to-mucosal	0.277 ± 0.057	1.52 ± 0.45*	5.41 ± 0.79*†
Net secretion ($\times 10^{-5}$ cm s^{-1})	(Serosal-to-mucosal)–(Mucosal-to-serosal)	-0.138 ± 0.100	0.862 ± 0.450*	4.15 ± 0.66*
Secretory ratio	(Serosal-to-mucosal)/(Mucosa-to-serosal)	0.723 ± 0.152	2.52 ± 0.88	4.32 ± 0.36*

The time course of the transport of grepafloxacin (10 μM) across the intestinal tissue was evaluated. The tissue was isolated from duodenum, jejunum or ileum. The experimental solution was adjusted to pH 7.4 and the temperature was maintained at 37°C. Each datum represents the mean ± s.e. of 4–6 experiments. *$P < 0.05$, vs corresponding duodenum datum. †$P < 0.05$, vs corresponding serosal-to-mucosal datum.

Table 2 Regional differences of grepafloxacin absorption from rat intestine by the loop method.

	Duodenum	Jejunum	Ileum
Disappearance (%)	77.0 ± 3.6	41.8 ± 4.0*	40.5 ± 4.3*

In the loop absorption method, 0.5 mL of isotonic MES buffer containing 10 μM grepafloxacin (pH 6.0) was introduced into a 10-cm loop of duodenum, jejunum or ileum. Luminal fluid in each loop was collected 15 min after administration. Each datum represents the mean ± s.e. of 4–6 experiments. *$P < 0.05$, vs corresponding duodenum datum.

ference between means was considered to be significant when the P-value was less than 0.05.

Results

Regional differences of grepafloxacin transport in rat intestinal tissue and absorption from rat intestine

Permeation of grepafloxacin in rat intestinal tissue was studied by using the Ussing-chamber method. Permeability coefficients of grepafloxacin in both absorptive and secretory directions showed a regional dependence with an increase in the order of duodenum < jejunum < ileum (Table 1). The order of secretory ratios calculated by dividing serosal-to-mucosal by mucosal-to-serosal permeability coefficients was duodenum < jejunum < ileum, indicating more pronounced secretion in the lower region (ileum). Accordingly, the secretory transport activity is high in the lower region of the small intestine.

The absorption was estimated in terms of the disappearance from the rat intestinal loop in 15 min. The major metabolite of grepafloxacin is its glucuronide conjugate. The sample was treated with glucuronidase and analysed by HPLC. There was essentially no difference in values with or without glucuronidase treatment, indicating that grepafloxacin in the intestinal loop was unlikely to be affected by metabolism in this experiment. The fraction lost from the loop in 15 min showed regional dependence with a decrease in the order of duodenum > jejunum ⩾ ileum (Table 2).

Transport of quinolones in Caco-2 cells

To determine whether transport of [^{14}C]grepafloxacin (1.0 μM) across Caco-2 cells was unidirectional, monolayers of Caco-2 cells grown on Transwells were used (Figure 1). The apical-to-basolateral permeability coefficient was $2.60 \pm 0.32 \times 10^{-5}$ cm s^{-1} and this value is 74 times higher than the value of mannitol transport (0.035×10^{-5} cm s^{-1}), which represents paracellular transport. Nonetheless, the basolateral-to-apical permeability coefficient of [^{14}C]grepafloxacin ($4.80 \pm 0.08 \times 10^{-5}$ cm s^{-1}) was significantly higher than the apical-to-basolateral value. These results suggest that [^{14}C]grepafloxacin transport in the secretory direction could also be ascribed to carrier-mediated transport.

The effect of increasing concentrations of unlabelled grepafloxacin and other compounds on the transport of [^{14}C]grepafloxacin (1.0 μM) in Caco-2 cell monolayers was studied. The results are summarized in Table 3. Unlabelled grepafloxacin, 0.1 mM, significantly increased the apical-to-basolateral permeability coefficient and did not affect the basolateral-to-apical permeability coefficient. A higher concentration of

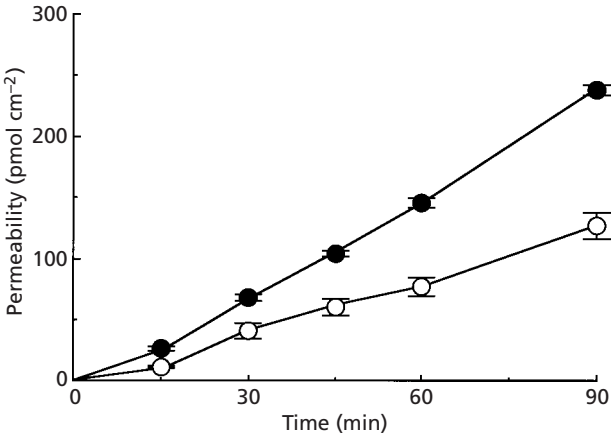

Figure 1 Transcellular transport of [^{14}C]grepafloxacin in Caco-2 cells evaluated by the Transwell method. Permeability of [^{14}C]grepafloxacin (1 μM) was measured at 37 °C for 90 min in HBSS buffer (pH 7.4). Open and closed circles represent apical-to-basolateral and basolateral-to-apical transport, respectively. Each point represents the mean ± s.e.m. of 3 experiments.

grepafloxacin (5 mM) increased the apical-to-basolateral permeability coefficient and significantly decreased the basolateral-to-apical permeability coefficient. These results indicate that the secretory-directed transport of [^{14}C]grepafloxacin was less marked at higher concentrations of grepafloxacin, suggesting the participation of secretory transporter(s) in grepafloxacin transcellular transport. On the other hand, SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid) and DIDS (4,-4′-diisothiocyanatostilbene-2,2′-disulfonic acid), which are inhibitors of anion exchangers, decreased both the apical-to-basolateral and basolateral-to-apical permeability coefficients. Ciclosporin A, an inhibitor of P-glycoprotein, and genistein and probenecid, inhibitors of anion transport systems, increased the apical-to-basolateral permeability coefficient, but did not affect the basolateral-to-apical permeability coefficient. *p*-Aminohippuric acid, a representative substrate for anion transporter(s) in kidney, did not affect the apical-to-basolateral or basolateral-to-apical permeability coefficient. The increase of apical-to-basolateral permeability coefficient can be accounted for by inhibition of efflux transporters, such as P-glycoprotein and other anion-sensitive transporter(s). These results suggest that efflux transporters are involved in grepafloxacin transport across Caco-2 cells.

To examine whether the efflux transporters observed in this study act commonly on other quinolones, the transepithelial transport and effects of ciclosporin A and probenecid were studied with [^{14}C]sparfloxacin (3.3 μM) and [^{14}C]levofloxacin (0.57 μM). Both showed significantly higher basolateral-to-apical than apical-to-basolateral permeation (Figure 2). Moreover, ciclosporin A or probenecid, or the combination of the two, showed no significant difference between apical-to-basolateral and basolateral-to-apical permeability coefficients. It appears that the quinolones are transported in the secretory direction by common efflux transporters,

Table 3 Inhibitory effects of various compounds on [^{14}C] grepafloxacin transport in Caco-2 cells evaluated by the Transwell method.

Inhibitor	Concn	Relative permeability coefficient (% of control)	
		Apical-to-basolateral	Basolateral-to-apical
Control		100.00	100.00
Grepafloxacin	0.1 mM	134.92 ± 0.59*	103.30 ± 9.85
Grepafloxacin	5 mM	190.62 ± 6.54*	78.22 ± 2.92*
SITS	1 mM	49.18 ± 2.61*	67.38 ± 2.11*
DIDS	1 mM	83.10 ± 3.23*	88.84 ± 4.93*
Ciclosporin A	10 μM	152.48 ± 2.54*	104.23 ± 0.84
Probenecid	5 mM	136.58 ± 3.77*	106.90 ± 3.16
Indometacin	0.1 mM	155.10 ± 8.11*	98.51 ± 4.90
Genistein	200 μM	115.81 ± 3.34*	109.89 ± 3.08
p-Aminohippuric acid	5 mM	92.81 ± 2.40	108.85 ± 7.02

Cells were pre-incubated at 37°C for 20 min in HBSS in the presence or absence of inhibitor, and permeation of [^{14}C]grepafloxacin (1 μM) was measured for 90 min at 37°C in HBSS (pH 7.4) in the presence or absence of various compounds. Each value represents the mean ± s.e.m. of 3 experiments. *$P < 0.05$, vs control.

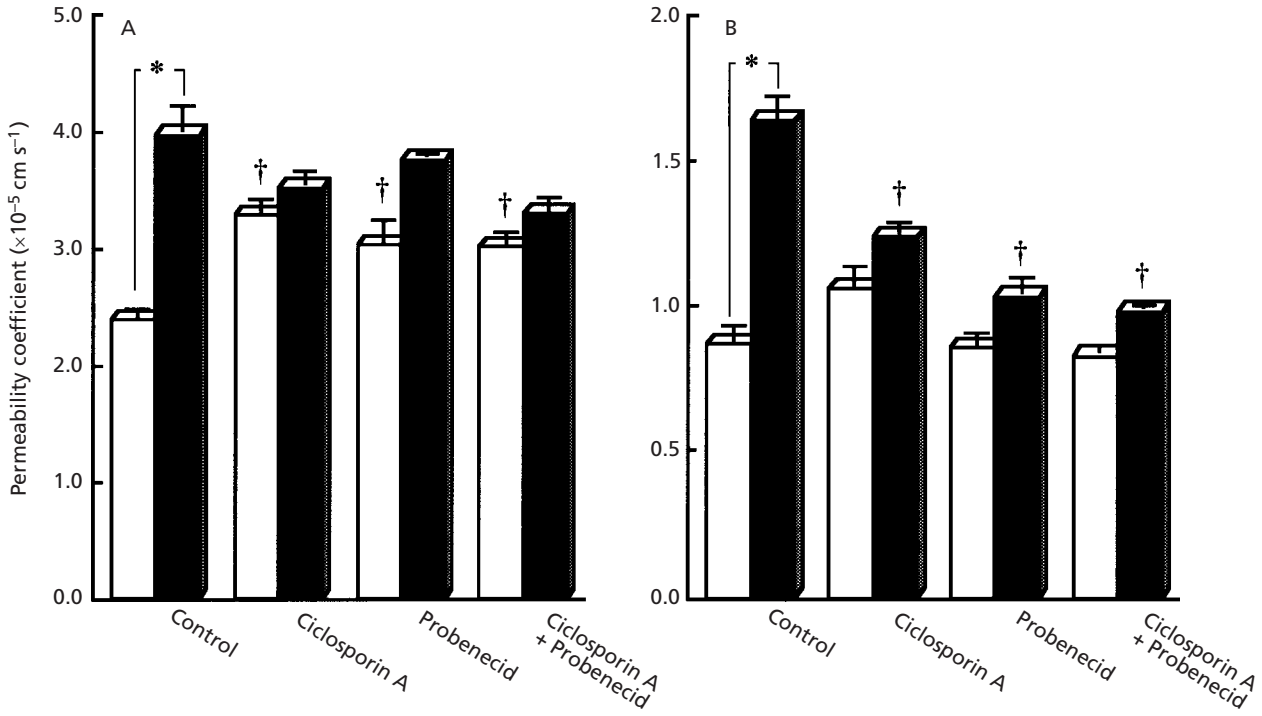

Figure 2 Inhibitory effects of ciclosporin A and probenecid on permeability of [^{14}C]sparfloxacin (A) and [^{14}C]levofloxacin (B) in Caco-2 cells evaluated by the Transwell method. Cells were pre-incubated at 37°C for 20 min in HBSS in the presence or absence of inhibitors (ciclosporin A (5 μM), probenecid (5 mM) or ciclosporin A (5 μM) + probenecid (5 mM)), and the permeability coefficient of [^{14}C]sparfloxacin (3.3 μM) or [^{14}C]levofloxacin (0.57 μM) in Caco-2 cells was measured at 37°C for 90 min in HBSS (pH 7.4) in the presence or absence of various compounds. White and black columns represent the apical-to-basolateral and basolateral-to-apical permeability, respectively, of [^{14}C] quinolones. Each column represents the mean ± s.e.m. of 3 experiments. *$P < 0.05$, apical-to-basolateral vs basolateral-to-apical permeability coefficients. †$P < 0.05$, vs corresponding control.

presumably at least, P-glycoprotein and anion-sensitive transporter(s).

Transport of quinolones in LLC-GA5-COL150 cells

LLC-GA5-COL150 cells were used to examine directly whether the quinolones studied here are substrates for P-glycoprotein, since LLC-GA5-COL-150 cells over-express P-glycoprotein on the apical membrane (Ueda et al 1992). The transepithelial transports of [^{14}C]grepafloxacin, [^{14}C]sparfloxacin and [^{14}C]levofloxacin were measured in LLC-GA5-COL150 cells and the parental LLC-PK1 cells, whose P-glycoprotein expression is reported to be negligible (Tanigawara et al 1992; Ueda et al 1992). The three quinolones showed only small differences between apical-to-basolateral and basolateral-to-apical permeations in LLC-PK1 cells (Figure 3). The basolateral-to-apical permeation in LLC-GA5-COL150 cells was greater than that in LLC-PK1 and the apical-to-basolateral permeation in LLC-GA5-COL150 cells was smaller than that in LLC-PK1 cells for each quinolone. These results indicate that the three quinolones are substrates for P-glycoprotein.

Grepafloxacin secretion into intestinal lumen of mouse small intestinal loop

It is important to demonstrate that the above findings reflect the in-vivo situation. We used P-glycoprotein-deficient mice to examine whether the intestinal secretion of [^{14}C]grepafloxacin occurs via P-glycoprotein. Figure 4 shows the time course of plasma concentration of [^{14}C]grepafloxacin after intravenous administration at a dose of 34 nmol per mouse. Apparently, there was no significant difference in pharmacokinetic parameters between wild-type FVB mice and mdr1a(−/−)/1b(−/−) mice within the experimental

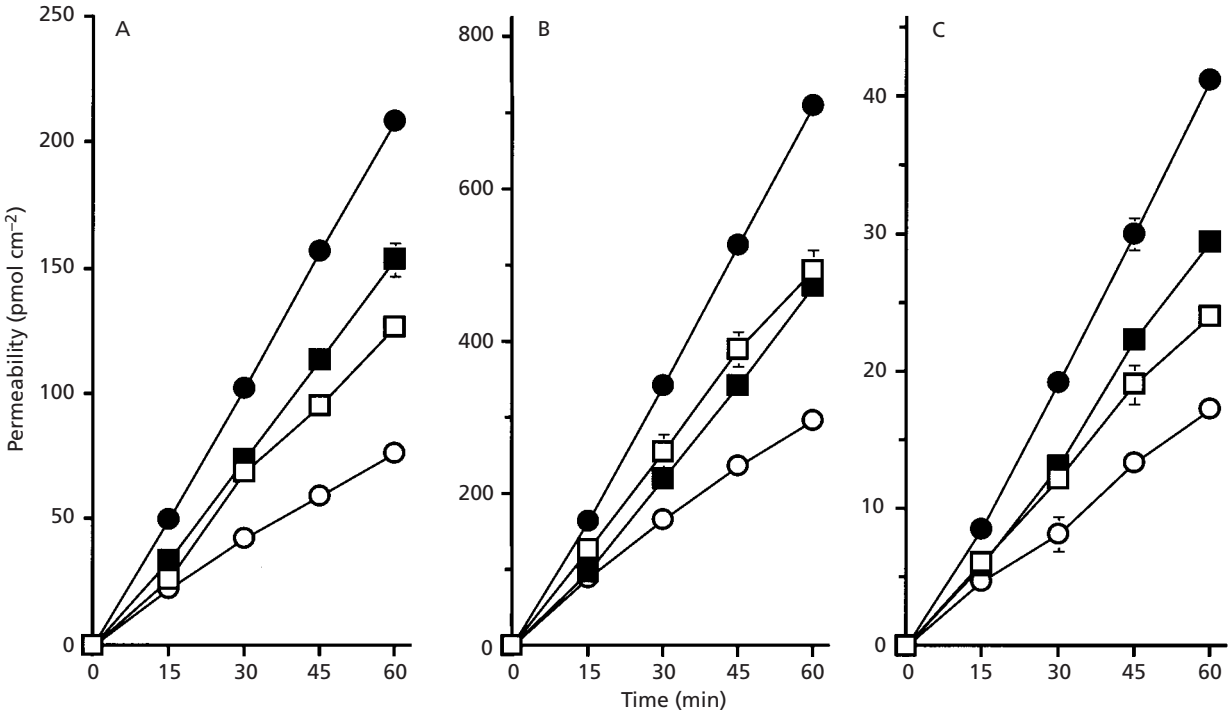

Figure 3 Transcellular transport of [^{14}C]quinolones in LLC-PK1 and LLC-GA5-COL150 cells evaluated by the Transwell method. Permeabilities of [^{14}C]grepafloxacin (1 μM; A), [^{14}C]sparfloxacin (3.3 μM; B) and [^{14}C]levofloxacin (0.57 μM; C) were measured at 37°C for 60 min in HBSS (pH 7.4) using LLC-PK1 (squares) and LLC-GA5-COL150 (circles) cells. Open and closed symbols represent, respectively, apical-to-basolateral and basolateral-to-apical transport. Each point represents the mean ± s.e.m. of 3 experiments.

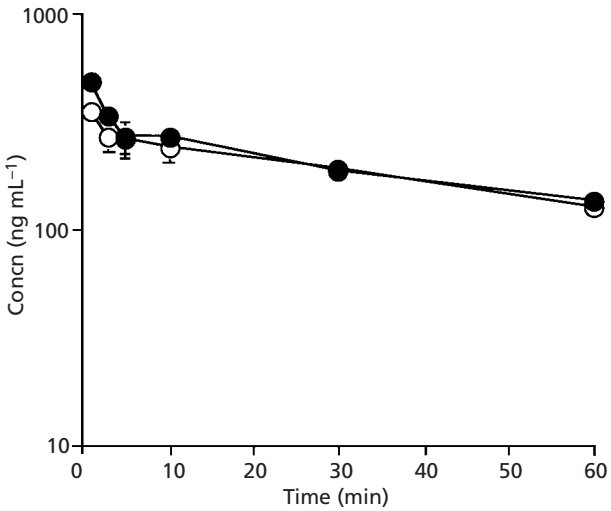

Figure 4 Time courses of plasma concentration of [^{14}C]grepafloxacin after intravenous administration to wild-type FVB mice and mdr1a(−/−)/1b(−/−) mice. [^{14}C]grepafloxacin (34 nmol) dissolved in saline solution was administered intravenously to wild-type FVB mice (open circles) or mdr1a(−/−)/1b(−/−) mice (closed circles). Serial blood samples were collected from the intra-orbital venous plexus using heparinized capillary tubes at designated time intervals in individual mice over the experimental period. Each point represents the mean ± s.e.m. of 4–6 animals.

time period (Table 4). The amount secreted into the 10-cm intestinal loop in wild-type FVB mice was 1.36 ± 0.14% of the dose in 60 min, while the corresponding value was 0.68 ± 0.08% in mdr1a(−/−)/1b(−/−) mice. In wild-type FVB mice and mdr1a(−/−)/1b(−/−) mice, the calculated value of intestinal secretory clearance was 12.56 ± 2.68 μL min^{-1} and 8.07 ± 1.57 μL min^{-1}, respectively (Table 4). This result demonstrates that P-glycoprotein also functions in the mouse for intestinal secretion of grepafloxacin.

Grepafloxacin absorption from mouse small intestinal loop

We examined the absorption of grepafloxacin from the small intestine in wild-type FVB mice and mdr1a(−/−)/1b(−/−) mice by the closed-loop method to determine whether the absorption of grepafloxacin from the intestine is affected by P-glycoprotein. The absorption was estimated in terms of the disappearance from the intestine at the end of the experimental period (15 min). The values of fractional ab-

Table 4 Intestinal absorption and secretion evaluated by the loop method in wild-type FVB mice and mdr1a(−/−)/1b(−/−) mice.

	Wild-type FVB mice	mdr1a(−/−)/ 1b(−/−) mice
Absorption and secretion		
Disappearance (%)	44.5 ± 4.0	66.4 ± 6.0*
Intestinal clearance (μL min^{-1})	12.56 ± 2.68	8.07 ± 1.57*
Pharmacokinetic parameters in the secretory loop experiment after intravenous administration of grepafloxacin		
ke (min^{-1})	0.0157 ± 0.0011	0.0142 ± 0.0005
AUC (μg mL^{-1} min)	12.8 ± 0.8	12.0 ± 1.4
CL_{tot}(mL min^{-1})	0.593 ± 0.054	0.625 ± 0.076
MRT (min)	23.8 ± 0.6	24.8 ± 0.2
Vd_{ss}(mL)	37.2 ± 2.3	44.1 ± 5.4

In the loop absorption method, 0.2 mL of isotonic MES buffer containing 1 μM grepafloxacin (pH 6.4) was administered into a 5-cm loop of ileum. Luminal fluid in the loop was collected 15 min after administration. In the loop secretion method, 0.4 mL of isotonic MES buffer was administered into a 10-cm loop of ileum, and then [^{14}C]grepafloxacin was administered intravenously at a dose of 34 nmol per mouse. Pharmacokinetic parameters were estimated according to model-independent moment analysis by WinNonlin. ke, linear terminal elimination rate; AUC, area under plasma concentration–time curve from 0 to 60 min; CL_{tot}, plasma total clearance; MRT, mean residence time from 0 to infinity; Vd_{ss}, distribution volume at steady state. Each value represents the mean ± s.e.m. from 4–6 mice. *$P < 0.05$, wild-type FVB mice vs mdr1a(−/−)/1b(−/−) mice.

sorption from the intestinal loop were 44.5 ± 4.0 % and 66.4 ± 6.0 % in wild-type FVB mice and mdr1a(−/−)/1b(−/−) mice, respectively, and the difference was statistically significant (Table 4). This result suggests that P-glycoprotein restricts the uptake of grepafloxacin from the small intestine.

Discussion

Many quinolones are reported to have moderate to excellent bioavailability after oral administration (Turnidge 1999) even though the quinolones exist as ionic forms (e.g. zwitterionic form in the case of grepafloxacin) at the pH in the small intestine. In this study, we examined the mechanisms of transport of quinolones, especially grepafloxacin, in the small intestine and the contribution of secretory transporters, closely focusing on P-glycoprotein, by utilizing in-vitro and in-vivo experimental techniques.

First, we examined the transport and absorption of grepafloxacin by the Ussing-chamber method and the intestinal-loop method, respectively, in various rat intestinal regions (Table 1 and 2). The net secretory transport was estimated from the (serosal-to-mucosal)/(mucosal-to-serosal) ratio in the rat tissue. Secretory transport showed regional dependence with an increase in the order of duodenum < jejunum < ileum, while absorption showed a decrease in the order of duodenum > jejunum ⩾ ileum. That is, the secretory activity in the rat intestinal tissue correlated inversely with the absorption from rat intestinal loops in terms of regions. These results suggest that grepafloxacin transport activity in the secretory direction is high in the lower region of the small intestine and this secretory transport activity restricts, in part, the absorption rate of grepafloxacin from the intestine.

Next, we used Caco-2 cells grown on Transwells to focus closely on the transport mechanisms in the intestinal epithelial cell. The apical-to-basolateral permeability coefficient of [^{14}C]grepafloxacin was very high compared with that of mannitol (Naruhashi et al 2001), which represents the paracellular permeability. The basolateral-to-apical permeability coefficient was twice that of the apical-to-basolateral permeability coefficient (Figure 1), suggesting a contribution of secretory transporter(s) to the basolateral-to-apical transepithelial transport of [^{14}C]grepafloxacin. Secretory transport of grepafloxacin was observed not only in rat intestinal tissue but also in Caco-2 cells. Interestingly, DIDS and SITS, both of which are inhibitors of anion-exchange, decreased the apical-to-basolateral and basolateral-to-apical transport of [^{14}C]grepafloxacin. This result may indicate that there are DIDS- and SITS-sensitive transporters responsible for absorptive and secretory transport. The decrease in apical-to-basolateral [^{14}C]grepafloxacin transport by DIDS and SITS strongly supports the existence of an absorptive transporter for grepafloxacin, although this transporter could not be characterized in this study. As for an efflux transporter, Cavet et al (1997) have suggested the efflux of ciprofloxacin in Caco-2 cells involves a transporter sensitive to DIDS. In addition, it has been demonstrated by a brain perfusion method that DIDS inhibited efflux transport at the blood–brain barrier and increased the grepafloxacin distribution in rat brain (Tamai et al 2000). Therefore, there is a possibility that the intestine and brain share an anion-sensitive transporter responsible for grepafloxacin transport.

Increasing concentrations of unlabelled grepafloxacin, ciclosporin A, genistein and probenecid increased the apical-to-basolateral permeation of grepafloxacin, resulting in diminished net secretion. This finding supports the existence of efflux transporters

responsible for secretion of grepafloxacin. Ciclosporin A is a good substrate for P-glycoprotein, and genistein and probenecid inhibit anion transporters, thus, the secretory transport of grepafloxacin is presumably mediated by both P-glycoprotein and anion transporter(s) in addition to the DIDS/SITS-sensitive transporter. To determine whether these secretory transporters are common to other quinolones, we measured the transepithelial transport of [^{14}C]sparfloxacin and [^{14}C]levofloxacin, and carried out an inhibition study in Caco-2 cells. These two quinolones also showed a secretory-directed transport which was diminished by ciclosporin A, probenecid and the combination of the two compounds (Figure 2). Overall, the secretory-directed transport of the three quinolones was diminished, although the mode of inhibition by these compounds was apparently different for each quinolone. The apical-to-basolateral transport of [^{14}C]sparfloxacin was increased by these modulators, as was that of [^{14}C]grepafloxacin, while the basolateral-to-apical transport was decreased in the case of [^{14}C]levofloxacin. This difference is likely to be ascribed to the difference in the absolute permeability coefficient values. When the membrane permeation rate is high enough, other factors, such as unstirred layer, could affect the transport rate of drugs. We are now investigating the effects ascribed to the unstirred layer. The basolateral-to-apical permeation of [^{14}C]grepafloxacin and [^{14}C]sparfloxacin was rather high and may be unstirred-layer limited.

P-glycoprotein seemed a good candidate for the ciclosporin-A-sensitive secretory transporter of quinolones. In the same experimental method (Caco-2 cells/Transwell), transport of daunorubicin, which is a representative substrate for P-glycoprotein, was inhibited by 10 μM of ciclosporin A. The concentration we used (5 or 10 μM ciclosporin A) is reported to inhibit P-glycoprotein relatively specifically (Höllo et al 1996). Therefore, we focused on the contribution of P-glycoprotein to the secretory transport of quinolones. To confirm that the quinolones studied are substrates for P-glycoprotein, we used the LLC-GA5-COL150 cell line and its parental LLC-PK1. LLC-GA5-COL150 cells over-express human P-glycoprotein as a result of transfection with human MDR1 cDNA. It has been clearly demonstrated that this transcellular transport model is suitable to examine whether or not a compound is a substrate for P-glycoprotein, by comparison of the results with those from LLC-PK1 (Tanigawara et al 1992; Ueda et al 1992). All three quinolones examined in this study showed greater secretory-directed transport in LLC-GA5-COL150 than in LLC-PK1 cells, with little or no difference among them (Figure 3), demonstrating clearly that all three quinolones are substrates for P-glycoprotein. It has been reported that levofloxacin is a substrate for P-glycoprotein by the same experimental method (Ito et al 1997; Matsuo et al 1998). Thus, it is clearly demonstrated that P-glycoprotein functions as a common secretory transporter for some quinolones in Caco-2 cells, although others, such as ciprofloxacin, may not be P-glycoprotein substrates (Cavet et al 1997).

It is important to investigate the significance of secretory transporter(s), especially P-glycoprotein, in the intestine in-vivo. P-glycoprotein-mediated digoxin secretion in the intestine after intravenous administration has been demonstrated using mdr1a-gene-defective mice (Mayer et al 1996). We used mice with a defect in both mdr1a and mdr1b genes. The intestinal secretion was evaluated by the use of [^{14}C]grepafloxacin, and the amount secreted into the 10-cm intestinal loop 60 min after intravenous administration was 1.36 ± 0.14% of the dose in wild-type FVB mice. In mdr1a(−/−)/1b(−/−) mice, the secreted amount was significantly decreased to about half of that in wild-type FVB mice. There were no significant differences in plasma blood levels during the 60-min experimental period between wild-type FVB mice and mdr1a(−/−)/1b(−/−) mice (Figure 4, Table 4). The major route of total clearance of grepafloxacin is biliary excretion of grepafloxacin and its conjugates via multidrug resistance-associated protein (MRP) 2 in rats (Sasabe et al 1998). FVB and mdr1a(−/−)/1b(−/−) mice retain MRP2, thus, no significant differences in apparent pharmacokinetic parameters were observed. Nevertheless, the values of intestinal clearance in wild-type FVB and mdr1a(−/−)/1b(−/−) mice were significantly different (Table 4). This observation strongly supports the involvement of P-glycoprotein in the efflux of grepafloxacin from blood into the intestinal lumen, and presumably other quinolones, in the intestine in-vivo. However, here we measured total radioactivity recovered in the intestinal loop, which may include both the parent grepafloxacin and glucuronidated metabolites. So we cannot tell exactly the relative contributions of the respective compounds to the total intestinal secretion. The values of secreted amounts seem very low, but this might be due to the reabsorption of the secreted grepafloxacin as Dautrey et al (1999) pointed out in the case of the intestinal elimination of ciprofloxacin, the oral bioavailability of which is smaller than that of grepafloxacin. P-glycoprotein functions as a barrier in the brain (i.e. P-glycoprotein limits the entry of substrates by pumping them out). It is reported that P-glycoprotein limits the bioavailability of certain drugs

after oral administration and co-administration of a P-glycoprotein inhibitor increases their bioavailability in-vivo (Terao et al 1996). Moreover, Transwell methods showed an increase of the apical-to-basolateral permeability of [^{14}C]grepafloxacin in Caco-2 cells by ciclosporin A, and in LLC-GA5-COL150 cells compared with LLC-PK1 cells. So, we further examined the effect of P-glycoprotein on the intestinal absorption of quinolones from the lumen into the body. The absorption from the intestinal loop in 15 min was high in wild-type FVB mice, while that in mdr1a(−/−)/1b(−/−) mice was significantly lower (Table 4). This result suggests that P-glycoprotein inhibits entry of grepafloxacin, and probably also some other quinolones, in the small intestine, which is reminiscent of P-glycoprotein's role as a component of the blood–brain barrier. Grepafloxacin inhibited secretory-directed transport of rhodamine123, which is a good substrate for P-glycoprotein, in Caco-2 cells (unpublished observation). Therefore, if quinolone is administered orally concomitant with drugs which are the substrates for P-glycoprotein, drug–drug interaction may occur at the absorption level and alter the oral bioavailability of either the quinolone or the concomitantly administered drug, or both.

As for the possibility that grepafloxacin is recognized as an anionic compound and transported via anionic transport mechanisms, identification of the anion transporter(s) that is (are) involved was difficult, because probenecid and genistein inhibit a variety of anion transporters. Recently it has been documented that MRP2 functions as an efflux transporter in the intestine and preferentially transports glutathione conjugates (Gotoh et al 2000). It has also been demonstrated that grepafloxacin, HSR-903 and their glucuronide conjugates are transported effectively via MRP2 across the canalicular membrane in rat liver (Sasabe et al 1998). The contribution of MRP2 to grepafloxacin transport in the small intestine is under investigation.

In conclusion, grepafloxacin is transported in the intestine by secretory transporters. P-glycoprotein plays a common role in the secretory transport of quinolones in the intestine and functions in-vivo not only to transport grepafloxacin from blood to intestine, but also to limit the absorption of grepafloxacin. Anion-sensitive transporter(s) is also suggested to contribute to the secretory transport of quinolones in the intestine. Furthermore, absorptive transporter(s) may also be involved in grepafloxacin transport in the intestine. The transporter/transport system is presumably anion sensitive, although the details remain to be clarified. These multiple transporters, both absorptive and secretory, may be one of the factors defining the bioavailability of quinolones, and quinolones could alter the oral bioavailability of other drugs, which share the transporters of quinolone, when administered simultaneously.

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