AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

PHARMACOLOGY

In Vitro Mechanistic Study of the Distribution of Lascufloxacin into Epithelial Lining Fluid

Kouhei Ohya,[a] Junichi Takano,[a] Shigeru Manita[a]

[a]Watarase Research Center, Kyorin Pharmaceutical Co. Ltd., Tochigi, Japan

ABSTRACT The present study aimed to clarify the mechanism underlying the high distribution of lascufloxacin in epithelial lining fluid (ELF). Involvement of transporters was examined by transcellular transport across Calu-3 and transporter-overexpressing cells; the binding of lascufloxacin to ELF components was examined by an organic solvent-water partitioning system that employed pulmonary surfactant and phospholipids. Transcellular transport across the transporter-overexpressing cells indicated lascufloxacin to be a substrate of both P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP); therefore, its transport across Calu-3 cells was inhibited by P-gp and BCRP inhibitors. However, permeability and efflux ratios of lascufloxacin were similar to those of the other quinolones with relatively low ELF distribution, indicating the existence of another mechanism for lascufloxacin distribution in ELF. Amongst pulmonary surfactants, which are a primary component of ELF, lascufloxacin preferentially bound to phosphatidylserine (PhS) from several phospholipids, and the binding was significantly greater than that for other quinolones. This binding was saturable with two apparent classes of binding sites and inhibited by some weakly basic drugs, indicating the presence of an ionic bond. In conclusion, the results of this study suggest that the binding of lascufloxacin to PhS in the pulmonary surfactant is the major mechanism of the high distribution of lascufloxacin in the ELF.

KEYWORDS lascufloxacin, pulmonary distribution, quinolones, transporters

The pulmonary distribution of antibacterial agents is considered to be an important factor in their ability to be effective agents in the treatment of respiratory tract infections. Several studies have reported antibiotic distribution of the lungs, specifically, the epithelial lining fluid (ELF) and alveolar macrophages (AMs), and have linked this distribution to pharmacological effects (1–3). The elucidation of the mechanism for the pulmonary distribution of antibacterial agents can be helpful in predicting the therapeutic effect of respiratory tract infections.

Lascufloxacin is a novel quinolone antibacterial agent that was recently developed by Kyorin Pharmaceutical Co., Ltd. (Tokyo, Japan); it exhibits a potent activity against various respiratory pathogens (4). In phase II and III studies that involved patients with respiratory tract infections, clinical and microbiological efficacy of >90% was achieved with the oral administration of 75 mg of lascufloxacin (once daily), which is about five times lower than the clinical doses of existing quinolones, such as levofloxacin (5). A clinical pharmacological study (phase I) revealed that ELF-to-free plasma area under the curve (AUC) ratio of lascufloxacin was remarkably higher than that of other quinolones. The ELF- and AM-to-free plasma AUC ratios for lascufloxacin were 61.7 and 163, respectively (6). The ELF-to-free plasma AUC ratios reported for existing quinolones, such as levofloxacin, garenoxacin, and grepafloxacin, were approximately 2.45 to 4.14, 5.38, and 24.1, and the AM-to-free plasma AUC ratios were 7.54 to 24.2, 55.6, and 352, respectively (7). Moreover, the lascufloxacin AUC in AMs were only 2.64 times higher

Citation Ohya K, Takano J, Manita S. 2019. *In vitro* mechanistic study of the distribution of lascufloxacin into epithelial lining fluid. Antimicrob Agents Chemother 63:e02208-18. https://doi.org/10.1128/AAC.02208-18.

Address correspondence to Kouhei Ohya, kouhei.ooya@mb.kyorin-pharm.co.jp.

Received 23 October 2018
Returned for modification 27 November 2018
Accepted 25 January 2019
Accepted manuscript posted online 4 February 2019
Published 27 March 2019

than that in ELF; in contrast, values for other quinolones were greater than that for lascufloxacin (levofloxacin, 1.82 to 9.07; garenoxacin, 10.3; grepafloxacin, 14.6). It has further been reported that drugs, such as levofloxacin, garenoxacin, and grepafloxacin, distributed in AMs essentially interfere with the accurate determination of their concentration in the ELF (7). The effect was observed to be prominent, when the concentration in AMs was much higher than that in the ELF. However, intracellular concentration of lascufloxacin in AMs was not high enough to affect its concentration in the ELF. These findings allude to the existence of specific mechanism(s) for the preferential distribution of lascufloxacin in the ELF.

Active transport mediated by transporters is generally known as one of the mechanisms for the pulmonary distribution of drugs. Some of efflux transporters, such as P-glycoprotein (P-gp), multidrug resistance-associated proteins (MRPs), and breast cancer resistance protein (BCRP), are highly expressed in the pulmonary epithelium (8). Regarding antimicrobial agents, active transport to the ELF compartment by such transporters has been suggested for several quinolone antibacterial agents (9–11).

The binding of drugs to ELF components has been considered to be another plausible mechanism. The pulmonary surfactant in ELF coats the interior surface of the airways under normal physiological conditions. Approximately 90% of the surfactant is composed of various phospholipids (12). Weakly basic drugs or quinolones, such as quinidine, propranolol, imipramine, and grepafloxacin, have been reported to distribute to specific tissues due to the binding to phosphatidylserine (PhS) (13–17).

The present study aimed to clarify the mechanisms that can account for the higher lascufloxacin concentration than that of other quinolone antibacterial agents in the ELF. Two possible mechanisms were examined. (i) The first was transporter-mediated secretion using the Calu-3 cell model that has been used as an effective *in vitro* lung epithelial cell model expressing tight-junction and drug efflux transporters, such as P-gp, BCRP, and MRPs, for predictive investigations (18–20). (ii) The second involved binding to the components of ELF using organic solvent-water partitioning system to examine the binding of lascufloxacin to surfactants or phospholipids (13).

(These data were presented in part at ASM Microbe, Boston, MA, June 16 to 20, 2016 [21].)

RESULTS

Transcellular transport of lascufloxacin across the monolayers expressing MDR1 and BCRP. The transcellular transport of lascufloxacin across the LLC-PK1 monolayers expressing MDR1 and BCRP was assessed. The permeability of lascufloxacin to the direction of basolateral (BL) to apical (AP) exceeded that to the opposite direction (AP to BL) in MDR1- and BCRP-expressing cells. Efflux ratios (ERs) of lascufloxacin were 1.4 and 4.4 in control cells and MDR1-expressing cells and 2.7 and 4.3 in control cells and BCRP-expressing cells, respectively. ERs in MDR1- and BCRP-expressing cells were higher than those in each control cells (Fig. 1). These results indicated that lascufloxacin was a substrate of MDR1 and BCRP.

Transcellular transport of quinolone antibiotics across Calu-3 cells. To explore the possibility of transporter-mediated lascufloxacin transport for lung distribution, transcellular transport was assessed using Calu-3 cells as a human lung epithelial cell model, whose AP and BL compartments represent ELF and blood, respectively. The amount of lascufloxacin transported over time increased linearly with time for up to 180 min. The permeability of lascufloxacin to the secretory direction (BL to AP) was superior to that of the absorptive direction (AP to BL), and the ER was 1.61 (Fig. 2 and Table 1). This vectorial transport disappeared in the presence of P-gp or BCRP inhibitors, such as 100 μM verapamil, 10 μM PSC-833, and 1 μM Ko143, and remained unaffected in the presence of MRP2 inhibitor, such as 100 μM probenecid (Table 1, experiments 1 and 2). These results suggested that an active transport of lascufloxacin through Calu-3 cell monolayer was mediated by P-gp and BCRP, but not by MRP2.

To evaluate the contribution of transporters to quinolone pulmonary distribution, the transcellular transport of lascufloxacin, levofloxacin, and garenoxacin was examined

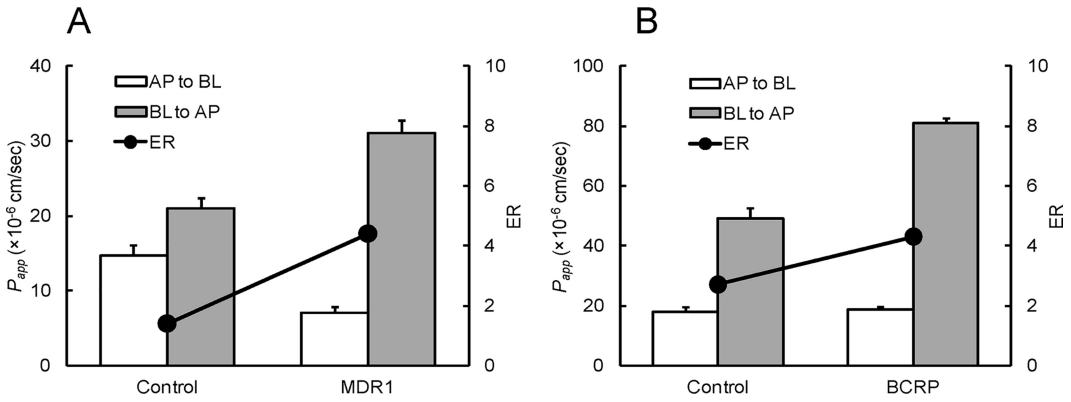

FIG 1 P_{app} and ER of lascufloxacin across MDR1 (A)- or BCRP (B)-overexpressing LLC-PK1 cells. The initial concentration of lascufloxacin in the donor was 3 μM. P_{app} data represent means ± the SD from three samples.

using Calu-3 cells. The P_{app} of the secretory direction (BL to AP) and the ER were observed with the increasing order of levofloxacin < garenoxacin ≤ lascufloxacin and levofloxacin < lascufloxacin < garenoxacin, respectively (Table 1, experiment 3). The P_{app} in the secretory direction (BL to AP) and the ER of lascufloxacin were not superior to those of the other quinolones.

Binding of quinolones to pulmonary surfactant and phospholipids. The binding study of quinolones to pulmonary surfactant was examined. The binding levels of levofloxacin, garenoxacin, and grepafloxacin to surfactant were 0.00181, 0.00476, and 0.00484 ml/mg surfactant, respectively. In contrast, the binding of lascufloxacin to surfactant was 0.0541 ml/mg surfactant, indicating that lascufloxacin had remarkably higher binding than the other quinolones (Fig. 3A).

To identify the pulmonary surfactant component bound to lascufloxacin, a binding study of lascufloxacin to various phospholipids was carried out. The binding of lascufloxacin to PhS was 15.2 ml/mg lipid, whereas those of the other phospholipids were 0.0825 to 0.733 ml/mg lipid (Fig. 3B). Furthermore, the binding of lascufloxacin to PhS was 10.6 ml/mg lipid, whereas binding of levofloxacin, garenoxacin, and grepafloxacin to PhS were 0.0925, 1.63, and 1.21 ml/mg lipid, respectively (Fig. 3C). Lascufloxacin preferentially bound to PhS rather than the other quinolones.

Characteristics for PhS binding of lascufloxacin and the other quinolones. Concentration dependency of lascufloxacin on PhS binding was examined. The direct plot of the bound lascufloxacin to PhS showed an apparent biphasic fitting curve with two

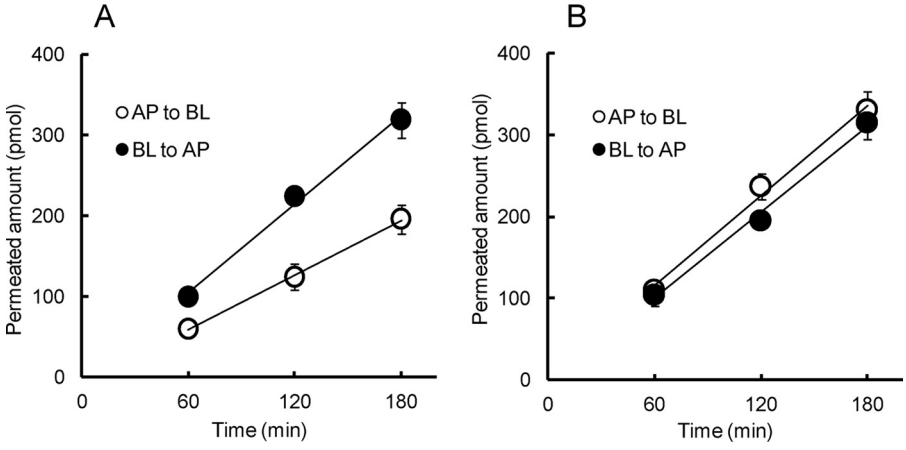

FIG 2 Amount of lascufloxacin that permeated across Calu-3 monolayers in the absence (A) or presence of 100 μM verapamil (B). The initial concentration of lascufloxacin in the donor side was 5 μM. Each plot represents the means ± the SD from four samples. The solid lines show regression lines for the data.

TABLE 1 Inhibitory effect of various transporter inhibitors on P_{app} of lascufloxacin (experiments 1 and 2) and comparison of P_{app} and ER values between quinolones across Calu-3 cells (experiment 3)

Expt[a] and inhibitor (or antibiotic)	Inhibited transporter	Direction	P_{app} ($\times 10^{-6}$ cm/s)[b]	ER
Expt 1				
Control (1% DMSO)	None	AP to BL	11.5 ± 1.14	1.61
		BL to AP	18.5 ± 1.47	
100 μM verapamil	P-gp, MRP2	AP to BL	18.7 ± 1.55**	0.952
		BL to AP	17.8 ± 1.40	
10 μM PSC-833	P-gp, MRP1 (weak)	AP to BL	23.6 ± 1.99**	1.06
		BL to AP	24.9 ± 2.00*	
10 μM elacridar	P-gp, BCRP	AP to BL	25.5 ± 1.40**	0.718
		BL to AP	18.3 ± 4.13	
1 μM Ko143	BCRP	AP to BL	19.6 ± 0.591**	0.827
		BL to AP	16.2 ± 2.93	
Expt 2				
Control (1% DMSO)	None	AP to BL	11.7 ± 0.690	1.61
		BL to AP	18.8 ± 1.35	
100 μM verapamil	P-gp, MRP2	AP to BL	19.1 ± 2.88**	0.953
		BL to AP	18.2 ± 1.86	
100 μM probenecid	MRP2	AP to BL	9.46 ± 0.871	1.54
		BL to AP	14.6 ± 2.06*	
Expt 3				
Lascufloxacin		AP to BL	7.16 ± 0.479	2.40
		BL to AP	17.2 ± 1.89	
Levofloxacin		AP to BL	5.94 ± 0.233	1.38
		BL to AP	8.22 ± 0.479	
Garenoxacin		AP to BL	3.74 ± 0.203	4.30
		BL to AP	16.1 ± 1.49	

[a]Experiments 1 and 2 were performed on other dates.
[b]P_{app} data represent the means ± the SD of four samples. Dunnett's test was used to compare data against P_{app} of control data. *, $P < 0.05$; **, $P < 0.001$. For the three antibiotics in experiment 3, each P_{app} result represents the means ± the SD of four samples.

class binding sites in a saturable manner in the range of 3 to 300 μM lascufloxacin (Fig. 4A). The association constants K_1 and K_2 were 0.252 and 0.000141 μM^{-1}, and the number of binding sites (n_1 and n_2) were 83.6 and 4,832 nmol/mg lipid. The binding of lascufloxacin to PhS was inhibited in the presence of weakly basic drugs, such as propranolol, quinidine, and imipramine, in a concentration-dependent manner (Fig. 4B).

Effect of surfactant or PhS on the activities of antibacterial agents. The MICs of quinolones and daptomycin against *Staphylococcus aureus* ATCC 29213 in the presence of PhS or surfactant were examined. Daptomycin was known to interact *in vitro* with the pulmonary surfactant, resulting in inactivation of antibacterial activity (22). In the

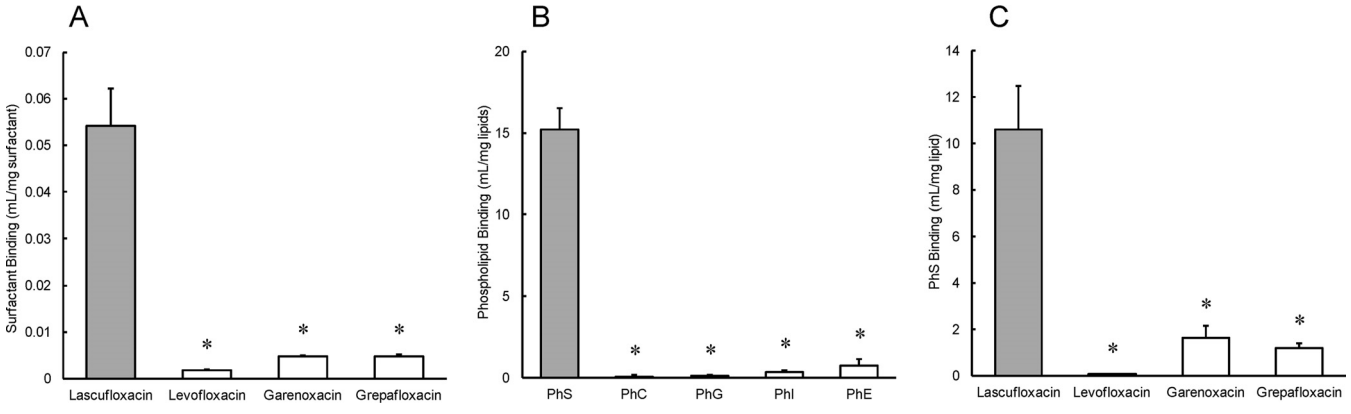

FIG 3 Binding of lascufloxacin and other quinolones to the surfactant and phospholipids. (A) Binding of quinolones to the surfactant. (B) Binding of lascufloxacin to various phospholipids. (C) Binding with PhS among quinolones. The binding data represent means ± the SD from three samples. Dunnett's test was used to compare data against surfactant or PhS binding of lascufloxacin. *, $P < 0.001$. Abbreviations: PhC, phosphatidylcholine; PhG, phosphatidylglycerol; PhI, phosphatidylinositol; PhE, phosphatidylethanolamine.

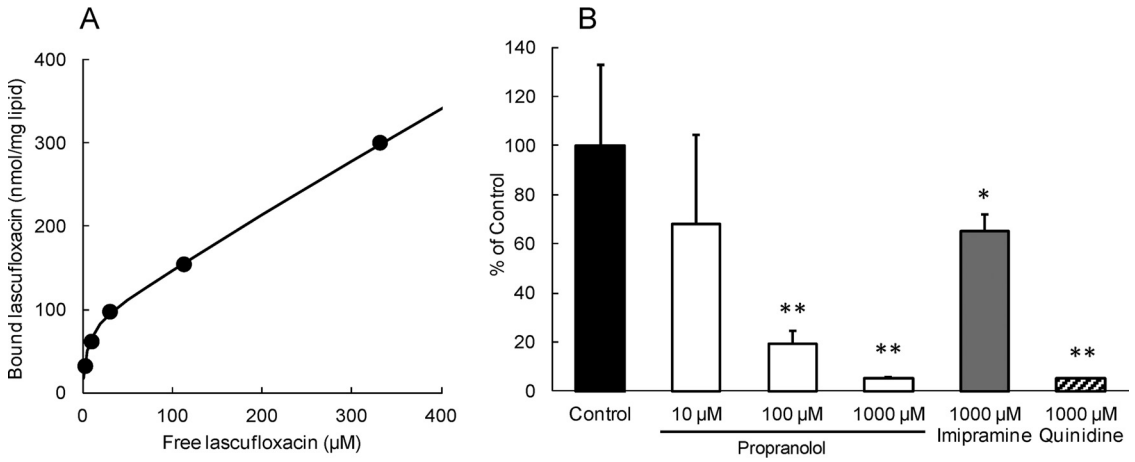

FIG 4 Characteristics for PhS binding of lascufloxacin. (A) Concentration-dependent binding of lascufloxacin to PhS. The solid line shows the fitting curve calculated using equation 2. (B) Inhibitory effect of weakly basic drugs on PhS binding of lascufloxacin. Binding data represent means ± the SD from five samples. Dunnett's test was used to compare data against the control. *, $P < 0.05$; **, $P < 0.001$.

presence of a surfactant, MIC of daptomycin demonstrated a 16-fold loss of potency, whereas the MIC of lascufloxacin remained unchanged in the presence of a surfactant and PhS (Table 2).

DISCUSSION

The results from the Calu-3 cell model suggested that the contribution of active transport toward the distribution of lascufloxacin in the ELF played a minor role, although lascufloxacin could be a substrate of MDR1 and BCRP (Fig. 1). In transcellular transport, lascufloxacin was actively transported in the secretory direction across Calu-3 cells (Fig. 2), and the active transport was inhibited by P-gp and BCRP inhibitors (Table 1, experiments 1 and 2). On the other hand, the ELF-to-free plasma AUC ratios reported for lascufloxacin (61.7) exceeded those for existing quinolones, such as levofloxacin, garenoxacin, and grepafloxacin (2.45 to 4.14, 5.38, and 24.1, respectively) (6, 7), and the P_{app} in the secretory direction (BL to AP) and the ER of lascufloxacin were comparable to those of the other quinolones (Table 1, experiment 3). Therefore, the active transport did not reflect a higher pulmonary distribution of lascufloxacin than that of other existing quinolones. Brillault et al. indicated that several quinolones can be actively transported by P-gp across Calu-3 cells. This active transport is relatively important for compounds with low lipophilicity but minor for those with high lipophilicity because of high passive permeability (10). Lascufloxacin has a higher lipophilicity (the log *D* was 0.81 at pH 7) than other quinolones (log *D* of levofloxacin, −1.35 at pH 7; log *D* of garenoxacin, −0.90 at pH 7; and log *P* of grepafloxacin, −0.23 at pH 6.4 and 0.03 at pH 7.4) (23, 24). Grepafloxacin, which has the second highest log *D*, is also a substrate of P-gp, but the ELF-to-plasma concentration ratio of grepafloxacin in the P-gp knockout mouse is almost identical to that in the wild-type mouse, indicating that the contribution of P-gp to the distribution of grepafloxacin in ELF may be minor (25, 26). Limited

TABLE 2 *In vitro* antibacterial activity of antibacterial agents in the presence or absence of surfactant or PhS against *S. aureus* ATCC 29213

Condition	MIC (μg/ml) against *S. aureus* ATCC 29213				
	Lascufloxacin	Levofloxacin	Garenoxacin	Grepafloxacin	Daptomycin
Control	0.015	0.25	0.03	0.03	1
+ 1% surfactant	0.015	ND[a]	ND	ND	16
+ 8 μg/ml PhS	0.015	0.25	0.03	0.03	ND
+ 16 μg/ml PhS	0.015	0.25	0.03	0.03	ND

[a]ND, not determined.

contribution of the transporters may be caused by the relatively higher lipophilicity of lascufloxacin than that of the other quinolones.

The binding of lascufloxacin to the bovine-derived pulmonary surfactant (Surfacten; Beractant), which has a similar composition and a function equivalent to that of natural mammalian pulmonary surfactant (27, 28), was significantly higher than that of the other quinolones (Fig. 3A). The binding of lascufloxacin to PhS was significantly higher than those to other phospholipids; this binding was superior to those of other quinolones (Fig. 3B and C). The corrected binding indices of lascufloxacin to each phospholipid in the physiological surfactant were determined to estimate the amount of lascufloxacin bound to each phospholipid in the surfactant. The corrected binding indices of lascufloxacin were calculated by multiplying the binding to each phospholipid and the composition of each phospholipid; the physiological phospholipid composition of human pulmonary surfactant was 67.5% PhC, 10.0% PhG, 5.3% PhE, 3.6% PhI, and 1.6% PhS (29). The corrected binding index of lascufloxacin to PhS (0.243 ml/mg lipid) was higher than those for PhC, PhE, PhG, and PhI (0.0557, 0.0388, 0.0153, and 0.0124 ml/mg lipid). Moreover, the corrected surfactant binding value was determined to verify whether the PhS contained in Surfacten contributed to the binding of lascufloxacin to the surfactant. The corrected surfactant binding value (8.37 ml/mg lipid) was calculated by dividing the binding to surfactant value by the PhS (18:0/18:1) concentration in Surfacten (6.46 μg lipid/mg surfactant [in-house data, Kyorin Pharmaceutical Co., Ltd.]) (30). The corrected binding value was close to the binding to PhS alone (15.2 or 10.6 ml/mg lipid, Fig. 3B and C). Despite the small amounts of PhS in the pulmonary surfactant, PhS is considered to be a predominant component for the binding of lascufloxacin to surfactant in the ELF. Therefore, it was suggested that the binding of lascufloxacin to PhS in pulmonary surfactant was involved in the higher distribution of lascufloxacin in the ELF than other quinolones.

Lascufloxacin bound to PhS with two apparent classes of binding sites in a saturable manner (Fig. 4A). These observations were similar to those for weakly basic drugs, which have been reported to ionically bind to PhS (13, 14, 17). The binding of lascufloxacin to PhS was inhibited in the presence of several weakly basic drugs (Fig. 4B). These results suggested that lascufloxacin was bound to PhS mainly by the ionic bond, although the partial contribution of hydrophobic interaction to the binding of lascufloxacin to PhS cannot be neglected. Lascufloxacin is a zwitterion with carboxyl (pKa = 6.24) and secondary amine (pKa = 7.99) groups. Under physiological conditions, the positively charged amine of lascufloxacin interacted with the negatively charged carboxyl group of PhS (pKa = 4 $\sim$ 5.5) (14). Lascufloxacin has a higher log *D* under physiological conditions than the other quinolones.

Furthermore, it is possible that the binding of lascufloxacin to PhS will affect its antibacterial activity. Silverman et al. reported that the irreversible binding of daptomycin to the pulmonary surfactant led to the inactivation of its antibacterial activity (22, 31). On the contrary, the addition of surfactant and PhS had no effect on the antibacterial activity of lascufloxacin against *S. aureus* (Table 2). This result suggests that daptomycin and lascufloxacin have different modes of binding to surfactants. Further investigations, including MIC studies against various bacterial strains, are necessary.

Lascufloxacin and the other quinolones have high bioavailability and long plasma half-lives (bioavailability and half-life of lascufloxacin, 96% and 16.1 h; levofloxacin, 99% and 6.9 h; garenoxacin, 92% and 9.8 to 14.2 h; and grepafloxacin, 72% and 11.1 h, respectively) (4, 32, 33). Although the clinical dose and AUC in free plasma (*f*AUC) of lascufloxacin were lower than those of the existing quinolones (dosage and *f*AUC of lascufloxacin, 75 mg and 2.65 μg · h/ml; levofloxacin, 500 mg and 33.1 μg · h/ml; garenoxacin, 400 mg and 15.0 μg · h/ml; and grepafloxacin, 400 mg and 6.35 μg · h/ml) (lascufloxacin [in-house data of Kyorin Pharmaceutical Co., Ltd.]) (33), a >90% clinical efficacy rate was achieved in patients with respiratory tract infections (6). The target value of lascufloxacin against pathogens of respiratory tract infection, estimated as the *f*AUC/MIC ratio, was 4 h, which was remarkably lower than those of the other quinolones (*f*AUC/MIC of levofloxacin, 60.9 h; *f*AUC/MIC of garenoxacin, 28 h; *f*AUC/MIC of

grepafloxacin, 42.8 h) (6, 34–36). Therefore, the high pulmonary distribution of lascufloxacin could be responsible for its clinical antimicrobial efficacy despite its lower systemic exposure in comparison with those of the other quinolones.

In conclusion, mediated transport of lascufloxacin can function in a minor role, but the binding of lascufloxacin to PhS in the pulmonary surfactant would be a major mechanism for the high distribution of lascufloxacin in the ELF.

MATERIALS AND METHODS

Materials. Lascufloxacin and garenoxacin were synthesized by Kyorin Pharmaceutical Co., Ltd. (Tokyo, Japan). Levofloxacin was purchased from Sigma-Aldrich (St. Louis, MO). Grepafloxacin was purchased from Bosche Scientific (New Brunswick, NJ). The following standard phospholipids were purchased from Sigma-Aldrich and used immediately after dissolution without further purification: 1,2-diacyl-*sn*-glycero-3-phospho-L-serine (PhS; derived from bovine brain), 1,2-dihexadecanoyl-rac-glycero-3-phospho-rac-(1-glycerol) ammonium salt (PhG; synthesized), L-α-phosphatidylinositol sodium salt (PhI; derived from *Glycine max* [soybean]), 1,2-diacyl-*sn*-glycero-3-phosphocholine (PhC; derived from egg yolk), and L-α-phosphatidylethanolamine (PhE; derived from egg yolk). Surfacten (Beractant) was purchased from Mitsubishi Tanabe Pharma Corporation (Osaka, Japan). All other reagents were commercially available and were of reagent grade.

Calu-3 cell culture. Calu-3 cells were purchased from the American Type Culture Collection (ATCC; Rockville, MD). Eagle minimal essential medium supplemented with 10% fetal bovine serum (FBS), penicillin (200 IU/ml), and streptomycin (200 μg/ml) was used for Calu-3 cell culture. The cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C. The cells were subcultured in 150-cm^2 cell culture flasks to allow them to reach the required confluence by using a 0.25% trypsin and EDTA solution. The medium was changed every 2 to 3 days.

Transcellular transport across calu-3 cells. Prior to transport assay, Calu-3 cells passaged among 23 to 28 were seeded at a density of 5×10^5 cells/well on Transwell (24-well 0.4-μm-pore-size polyester; Corning). The medium on the AP side (0.25 ml) was removed after 24 h from seeding, and that in BL side (1 ml) was changed every 2 to 3 days and cultured 14 days from seeding. At this point, the MDR1 and BCRP mRNA expression in Calu-3 cells was confirmed in the present study (data not shown).

The transcellular transport was performed in Hanks balanced salt solution (HBSS). The formation of the functional tight junction was checked by measuring the transepithelial electrical resistance (TEER) by Millicell ERS-2 (Merck Millipore, Darmstadt, Germany). Monolayers with TEER values of $>300\ \Omega \cdot cm^2$ were used (37). Cells were preincubated for 15 min at 37°C HBSS in the absence or presence of inhibitors. The concentrations of inhibitors were as follows: verapamil, 100 μM; PSC-833, 10 μM; elacridar, 10 μM; and Ko143, 1 μM. To start the transport assay, HBSS containing each quinolone (5 μM) was added to the AP or BL compartments. An aliquot of sample (AP, 50 μl; BL, 200 μl) was taken from opposite side at 60, 120, and 180 min, and the same volume of fresh HBSS was added to compensate for the volume sampled.

Transporter-overexpressing cells culture. MDR1-expressing cells (porcine kidney epithelial LLC-PK1 cells transfected with a vector containing human MDR1 cDNA) and control cells (LLC-PK1 cells transfected with the only vector) were used under a sublicense from BD Biosciences. BCRP-expressing cells (LLC-PK1 cells transfected with a vector containing human BCRP cDNA) and control cells (LLC-PK1 cells transformed with the only vector) were developed at the ADME and Tox. Research Institute, Sekisui Medical Co., Ltd. (Ibaraki, Japan). Medium199 supplemented with 10% FBS, 2.2 g/liter sodium hydrogen carbonate, and 50 mg/liter gentamicin was used for culture. To prepare a medium put into flasks for MDR1-expressing cells and control cells, 20 mg/ml hygromycin B (2.5 ml) was added, and then the solution was sterilized by filtration, followed by the addition of FBS (50 ml). FBS used for BCRP-expressing cells and control cells was inactivated by heating at 56°C for 30 min. For MDR1-expressing cells and control cells, inactivated FBS was used. The cells were cultured in a NAPCO automatic carbon dioxide cell culture system. The cells were subcultured in cell culture flasks every 4 to 5 days. The medium was changed every 2 to 3 days.

Transcellular transport across transporter-overexpressing cells. Prior to the experiments, the cells (passage number 24 to 36) were seeded at the density of 4×10^4 cells/well for MDR1-expressing cells and control cells and at 0.3×10^5 cells/well for BCRP-expressing cells and control cells in cell culture insert (MDR1, pore size, 3 μm; BCRP, pore size, 0.4 μm; area, 0.3 cm^2; PET porous filter [Corning]) and incubated in a NAPCO automatic carbon dioxide cell culture system to prepare the cell monolayers. The medium in both AP (MDR1, 100 μl; BCRP, 250 μl) and BL (MDR1, 600 μl; BCRP, 900 μl) compartments was changed every 2 to 3 days and cultured 14 days from seeding.

The transcellular transport was performed in HBSS–10 mM HEPES containing 1% bovine serum albumin (BSA-HBSS). Monolayers with TEER values of 100 to 800 $\Omega \cdot cm^2$ were used. The cells were preincubated for 1 h in 37°C BSA-HBSS containing 0.2% dimethyl sulfoxide (DMSO). To start the transport assay, the solution in the AP or the BL compartment was replaced with lascufloxacin solution (3 μM). After incubation for 30, 60, or 120 min, the solution (MDR1, 70 μl; BCRP, 100 μl) was collected from the opposite compartment of that spiked with lascufloxacin. To compensate for the volume sampled after incubation for 30 and 60 min, the same volume of BSA-HBSS was added. The collected samples were diluted with BSA-HBSS as necessary, and the samples were mixed with an equal volume of 0.1% formic acid in 50% acetonitrile.

Phospholipid-binding study. The binding of each quinolone to surfactant or phospholipids *in vitro* was performed by an organic solvent-water partitioning system (13). We selected an *n*-hexane (pH 7.4) buffer system in which all quinolones did not partition into the organic phase in the absence

of surfactant or phospholipids. Bovine-derived surfactant (Surfacten; Beractant) was dissolved by 4 ml of saline. To determine the binding to surfactant, 2 ml of *n*-hexane and 2 ml of the buffer solution (0.25 M sucrose, 0.1 M Tris-HCl buffer [pH 7.4]) supplemented with each quinolone and 1% surfactant (0.3 mg surfactant/ml) were mixed in a disposable glass tube at room temperature for 2 h. To determine binding to phospholipids, 2 ml of an *n*-hexane solution containing 8 μg/ml of phospholipid and 2 ml of buffer solution supplemented with each quinolone were mixed using the same method described above. To perform the inhibitory experiment, buffer solution supplemented with each quinolone in the absence or presence of weakly basic drugs as an inhibitor, such as propranolol, imipramine, and quinidine, were used. The concentrations of imipramine and quinidine were set at 1,000 μM, and those of propranolol were set at 10, 100, and 1,000 μM. For the concentration-dependent experiment, the lascufloxacin concentration was set from 3 to 300 μM. The mixture of *n*-hexane and buffer solution was centrifuged at 1,800 $\times$ *g* for 10 min, and separated organic and aqueous phases were collected.

Determination of quinolones in samples. Determination of lascufloxacin and the concentration of other quinolones in samples was performed by a validated high-performance liquid chromatography with a fluorescence detector (HPLC-FL) or by the liquid chromatography-tandem mass spectrometry (LC-MS/MS) method. *In vitro* samples were diluted appropriately using a mixture of 0.01 M phosphoric acid and acetonitrile (70:30 [vol/vol]). The mobile phase for HPLC-FL method consisted of a mixture of 0.01 M phosphoric acid and acetonitrile (70:30 [vol/vol]) containing 5 mM 1-octanesulfonic acid sodium salt. A part of the sample prepared as described above was pumped through a Kinetex XB-C_{18} column (2.6-μm particle size, 3.0 $\times$ 100 mm; Phenomenex, Inc., Torrance, CA) at a flow rate of 0.8 ml/min. In order to prevent interference by weakly basic drugs that function as inhibitors contained in samples, chromatography was carried out using a Poroshell 120 Phenyl-Hexyl column (2.7-μm particle size, 2.1 $\times$ 100 mm; Agilent, Santa Clara, CA). The excitation and emission wavelengths were set to 289 and 505 nm for lascufloxacin, 292 and 496 nm for levofloxacin, 290 and 418 nm for garenoxacin, and 285 and 448 nm for grepafloxacin. For the LC-MS/MS method, the mobile phase was a mixture of 0.1% formic acid and acetonitrile containing 0.1% formic acid. A part of the sample prepared as described above was pumped through an Inertsil ODS3 column (5-μm particle size, 2.1 $\times$ 150 mm; GL Science, Tokyo, Japan) at a flow rate of 0.3 ml/min. Quinolones were ionized prior to detection in multiple reaction monitoring modes, while monitoring the following transitions: *m/z* 440 to 422 for lascufloxacin, *m/z* 362 to 318 for levofloxacin, and *m/z* 427 to 366 for garenoxacin.

Measurement of the MIC. The antimicrobial activity of quinolones against *S. aureus* ATCC 29213 was determined by the broth microdilution method described by the Clinical and Laboratory Standards Institute (CLSI) (38). The MICs of each quinolone were measured in CAMHB containing 1% surfactant and 8 or 16 μg/ml PhS.

Data analysis. P_{app} values were obtained according to equation 1, as follows:

$$P_{app}(cm/s) = [dQ/dt]/A/C_0 \quad (1)$$

where [*dQ/dt*] is the transport rate (pmol/s) of the test agent (*Q*) appearing in the acceptor compartment as a function of time, obtained from the slope of the linear portion of the amount transported-versus-time plot; *A* is membrane surface area (cm^2); and C_0 is the initial donor concentration of each test agent (pmol/ml). The ER was calculated by the quotient of the P_{app} in the secretory (BL-to-AP) direction over the P_{app} in the absorptive (AP-to-BL) direction (9).

The binding value of quinolone to phospholipids or surfactant represents a ratio of bound to unbound quinolone concentration divided by the tested phospholipid (8 μg/ml) or surfactant (0.3 mg/ml) concentration. Bound and unbound quinolone concentrations are the concentrations of quinolone in the organic phase and in the aqueous phase, respectively. Binding parameters of lascufloxacin to PhS were calculated from data of concentration-dependent study by least-squares regression analysis according to equation 2 as given below:

$$\text{Bound lascufloxacin (nmol/mg lipid)} = (n_1 \times K_1 \times C_f)/(1 + K_1 \times C_f) + (n_2 \times K_2 \times C_f)/(1 + K_2 \times C_f) \quad (2)$$

where *n* and *K* are the number of binding sites and the association constant, respectively; C_f is the concentration of unbound lascufloxacin; and subscripts 1 and 2 indicate apparent high-affinity and apparent low-affinity binding sites, respectively (14). The fitting curve was calculated using WinNonlin ver. 7.0 (Certara G.K., Tokyo, Japan).

Statistical analysis. All values are indicated as means $\pm$ the standard deviations (SD). The statistical verification of the data were performed with Dunnett's test in the software EXSUS v8.0.0 (CAC Croit Corporation, Tokyo, Japan). A *P* value of <0.05 was considered statistically significant.

ACKNOWLEDGMENTS

We thank Mika Yamazaki and Ryuta Kishii for providing MIC data from experiments conducted in the presence of PhS and surfactant.

All authors are employees of Kyorin Pharmaceutical Co., Ltd.

REFERENCES

1. Baldwin DR, Wise R, Andrews JM, Gill M, Honeybourne D. 1993. Comparative bronchoalveolar concentrations of ciprofloxacin and lomefloxacin following oral administration. Respir Med 87:595–601. https://doi.org/10.1016/S0954-6111(05)80262-8.

2. Furuie H, Saisho Y, Yoshikawa T, Shimada J. 2010. Intrapulmonary pharmacokinetics of S-013420, a novel bicyclolide antibacterial, in healthy Japanese subjects. Antimicrob Agents Chemother 54:866–870. https://doi.org/10.1128/AAC.00567-09.
3. Ishizuka H, Toyama K, Yoshiba S, Okabe H, Furuie H. 2012. Intrapulmonary distribution and pharmacokinetics of laninamivir, a neuraminidase inhibitor, after a single inhaled administration of its prodrug, laninamivir octanoate, in healthy volunteers. Antimicrob Agents Chemother 56:3873–3878. https://doi.org/10.1128/AAC.06456-11.
4. Kishii R, Yamaguchi Y, Takei M. 2017. *In vitro* activities and spectrum of the novel fluoroquinolone, lascufloxacin (KRP-AM1977). Antimicrob Agents Chemother 61:e00120-17.
5. Takashi K, Masaaki O, Shigeru K. 2017. Phase II clinical study of oral lascufloxacin in community-acquired bacterial pneumonia, abstr P1362. Abstr 27th ECCMID, Vienna, Austria.
6. Furuie H, Tanioka S, Shimizu K, Manita S, Nishimura M, Yoshida H. 2018. Intrapulmonary pharmacokinetics of lascufloxacin in healthy adult volunteers. Antimicrob Agents Chemother 62:e02169-17.
7. Kiem S, Schentag JJ. 2008. Interpretation of antibiotic concentration ratios measured in epithelial lining fluid. Antimicrob Agents Chemother 52:24–36. https://doi.org/10.1128/AAC.00133-06.
8. van der Deen M, de Vries EGE, Timens W, Scheper RJ, Timmer-Bosscha H, Postma DS. 2005. ATP-binding cassette (ABC) transporters in normal and pathological lung. Respir Res 6:59. https://doi.org/10.1186/1465-9921-6-59.
9. Brillault J, De Castro WV, Harnois T, Kitzis A, Olivier JC, Couet W. 2009. P-glycoprotein-mediated transport of moxifloxacin in a Calu-3 lung epithelial cell model. Antimicrob Agents Chemother 53:1457–1462. https://doi.org/10.1128/AAC.01253-08.
10. Brillault J, De Castro WV, Couet W. 2010. Relative contributions of active mediated transport and passive diffusion of fluoroquinolones with various lipophilicities in a Calu-3 lung epithelial cell model. Antimicrob Agents Chemother 54:543–545. https://doi.org/10.1128/AAC.00733-09.
11. Ong HX, Traini D, Bebawy M, Young PM. 2013. Ciprofloxacin is actively transported across bronchial lung epithelial cells using a Calu-3 air interface cell model. Antimicrob Agents Chemother 57:2535–2540. https://doi.org/10.1128/AAC.00306-13.
12. Goerke J. 1998. Pulmonary surfactant: functions and molecular composition. Biochim Biophys Acta 1408:79–89. https://doi.org/10.1016/S0925-4439(98)00060-X.
13. Suzuki T, Kato Y, Sasabe H, Itose M, Miyamoto G, Sugiyama Y. 2002. Mechanism for the tissue distribution of grepafloxacin, a fluoroquinolone antibiotic, in rats. Drug Metab Dispos 30:1393–1399. https://doi.org/10.1124/dmd.30.12.1393.
14. Yata N, Toyoda T, Murakami T, Nishiura A, Higashi Y. 1990. Phosphatidylserine as a determinant for the tissue distribution of weakly basic drugs in rats. Pharm Res 7:1019–1025. https://doi.org/10.1023/A:1015935031933.
15. Nishiura A, Higashi J, Murakami T, Higashi Y, Yata N. 1986. A possible contribution of phospholipids in tissue distribution of quinidine in rats. J Pharmacobiodyn 9:819–828. https://doi.org/10.1248/bpb1978.9.819.
16. Nishiura A, Murakami T, Higashi Y, Yata N. 1987. Role of acidic phospholipids in tissue distribution of quinidine in rats. J Pharmacobiodyn 10:134–141.
17. Murakami T, Yumoto R. 2011. Role of phosphatidylserine binding in tissue distribution of amine-containing basic compounds. Expert Opin Drug Metab Toxicol 7:353–364. https://doi.org/10.1517/17425255.2011.548805.
18. Hamilton KO, Backstrom G, Yazdanian MA, Audus KL. 2001. P-glycoprotein efflux pump expression and activity in Calu-3 cells. J Pharm Sci 90:647–658. https://doi.org/10.1002/1520-6017(200105)90:5<647::AID-JPS1021>3.0.CO;2-G.
19. Hamilton KO, Topp E, Makagiansar I, Siahaan T, Yazdanian M, Audus KL. 2001. Multidrug resistance-associated protein-1 functional activity in Calu-3 cells. J Pharmacol Exp Ther 298:1199–1205.
20. Paturi DK, Kwatra D, Ananthula HK, Pal D, Mitra AK. 2010. Identification and functional characterization of breast cancer resistance protein in human bronchial epithelial cells (Calu-3). Int J Pharm 384:32–38. https://doi.org/10.1016/j.ijpharm.2009.09.037.
21. Ohya K, Takano J, Manita S. 2016. Mechanism for high pulmonary distribution of lascufloxacin (AM-1977), a new fluoroquinolone antibiotic, abstr SUNDAY-459. ASM Microbe, Boston, MA.
22. Silverman JA, Mortin LI, Vanpraagh ADG, Li T, Alder J. 2005. Inhibition of daptomycin by pulmonary surfactant: *in vitro* modeling and clinical impact. J Infect Dis 191:2149–2152. https://doi.org/10.1086/430352.
23. Michot J-M, Seral C, Van Bambeke F, Mingeot-Leclercq M-P, Tulkens PM. 2005. Influence of efflux transporters on the accumulation and efflux of four quinolones (ciprofloxacin, levofloxacin, garenoxacin, and moxifloxacin) in J774 macrophages. Antimicrob Agents Chemother 49:2429–2437. https://doi.org/10.1128/AAC.49.6.2429-2437.2005.
24. Yamaguchi H, Yano I, Saito H, Inui K. 2001. Transport characteristics of grepafloxacin and levofloxacin in the human intestinal cell line Caco-2. Eur J Pharmacol 431:297–303. https://doi.org/10.1016/S0014-2999(01)01448-0.
25. Sasabe H, Kato Y, Suzuki T, Itose M, Miyamoto G, Sugiyama Y. 2004. Differential involvement of multidrug resistance-associated protein 1 and P-glycoprotein in tissue distribution and excretion of grepafloxacin in mice. J Pharmacol Exp Ther 310:648–655. https://doi.org/10.1124/jpet.104.065201.
26. Yamaguchi H, Yano I, Hashimoto Y, Inui KI. 2000. Secretory mechanisms of grepafloxacin and levofloxacin in the human intestinal cell line Caco-2. J Pharmacol Exp Ther 295:360–366.
27. Tanaka Y, Takei T. 1983. Lung surfactants. I. Comparison of surfactants prepared from lungs of calf, ox, dog and rabbit. Chem Pharm Bull (Tokyo) 31:4091–4099. https://doi.org/10.1248/cpb.31.4091.
28. Tanaka Y, Takei T, Aiba T, Masuda K, Kiuchi A, Fujiwara T. 1986. Development of synthetic lung surfactants. J Lipid Res 27:475–485.
29. Schmitz G, Müller G. 1991. Structure and function of lamellar bodies, lipid-protein complexes involved in storage and secretion of cellular lipids. J Lipid Res 32:1539–1570.
30. Kang YP, Lee WJ, Hong JY, Lee SB, Park JH, Kim D, Park S, Park C-S, Park S-W, Kwon SW. 2014. Novel approach for analysis of bronchoalveolar lavage fluid (BALF) using HPLC-QTOF-MS-based lipidomics: lipid levels in asthmatics and corticosteroid-treated asthmatic patients. J Proteome Res 13:3919–3929. https://doi.org/10.1021/pr5002059.
31. Silverman JA, Perlmutter NG, Shapiro HM. 2003. Correlation of daptomycin bactericidal activity and membrane depolarization in *Staphylococcus aureus*. Antimicrob Agents Chemother 47:2538–2544. https://doi.org/10.1128/AAC.47.8.2538-2544.2003.
32. Totsuka K, Odajima M, Nakauchi M, Sesoko S, Nakashima M. 2016. Phase I study to determine the safety and pharmacokinetics (PK) of single and multiple oral doses of lascufloxacin (AM-1977) in healthy subjects, abstr SUNDAY-467. ASM Microbe, Boston, MA.
33. Noreddin AM, Haynes VL, Zhanel GG. 2005. Pharmacokinetics and pharmacodynamics of the new quinolones. J Pharm Pract 18:432–443. https://doi.org/10.1177/0897190005282397.
34. Drusano GL, Preston SL, Fowler C, Corrado M, Weisinger B, Kahn J. 2004. Relationship between fluoroquinolone area under the curve: minimum inhibitory concentration ratio and the probability of eradication of the infecting pathogen, in patients with nosocomial pneumonia. J Infect Dis 189:1590–1597. https://doi.org/10.1086/383320.
35. Tanigawara Y, Nozawa K, Tsuda H. 2012. Optimal dose finding of garenoxacin based on population pharmacokinetics/pharmacodynamics and Monte Carlo simulation. Eur J Clin Pharmacol 68:39–53. https://doi.org/10.1007/s00228-011-1095-3.
36. Firsov AA, Lubenko IY, Vostrov SN, Portnoy YA, Zinner SH. 2005. Antistaphylococcal effect related to the area under the curve/MIC ratio in an *in vitro* dynamic model: predicted breakpoints versus clinically achievable values for seven fluoroquinolones. Antimicrob Agents Chemother 49:2642–2647. https://doi.org/10.1128/AAC.49.7.2642-2647.2005.
37. Foster KA, Avery ML, Yazdanian M, Audus KL. 2000. Characterization of the Calu-3 cell line as a tool to screen pulmonary drug delivery. Int J Pharm 208:1–11. https://doi.org/10.1016/S0378-5173(00)00452-X.
38. CLSI. 2015. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, 10th ed. CLSI document M07-A10. Clinical and Laboratory Standards Institute, Wayne, PA.