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Accumulation of a Newly Developed Fluoroquinolone, OPC-17116, by Human Polymorphonuclear Leukocytes

KAZUSHIGE TAIRA, HIRONOBU KOGA,* AND SHIGERU KOHNO

Second Department of Internal Medicine, Nagasaki University School of Medicine, 1-7-1, Sakamoto, Nagasaki, 852, Japan

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The accumulation of OPC-17116 by human polymorphonuclear leukocytes was studied by comparison with three other new quinolones, ofloxacin, levofloxacin, and DR-3354. The intracellular/extracellular concentration ratio was the highest for OPC-17116, being 66.2, followed by 9.8 for levofloxacin, 7.6 for ofloxacin, and 5.8 for DR-3354. Furthermore, it was suggested that the accumulation of these new quinolones was partially related to their active transport system. The elution of OPC-17116 and ofloxacin from cells was rapid, but the elution of OPC-17116 decreased at a high extracellular pH and increased at a low extracellular pH. The accumulation of OPC-17116 as well as that of the other new quinolones was satisfactorily high, thus indicating that they are expected to be useful for the treatment of various kinds of infections, particularly infections caused by cytozoic bacteria.

New quinolones, which have been extensively developed in recent years, have potent, broad-spectrum antibacterial activity and are considered to be excellent antimicrobial agents with high efficacy by oral administration. Since the new quinolone derivatives show a high tissue and intracellular distribution, they are known to be useful for the treatment of infections caused by so-called cytozoic bacteria, such as *Legionella* spp., which grow in phagocytic cells (10, 14, 15).

The penetration of various antimicrobial agents into human polymorphonuclear leukocytes (PMNs) has been studied by a microbiological method (17), high-performance liquid chromatography (HPLC) (8), and radiolabeled antimicrobial agents (5–7, 12). However, since the fluorometric assay is simple and sufficiently sensitive to determine the intracellular accumulation of new quinolones (11), in this study the accumulation of a newly developed fluoroquinolone, OPC-17116, and three other new quinolones by human PMNs was investigated by the fluorometric assay.

MATERIALS AND METHODS

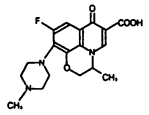
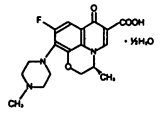
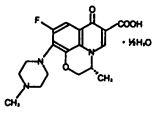
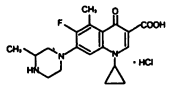
Isolation of PMNs. PMNs were isolated by the methods of Johnson et al. and other groups (5–8, 11, 12). Approximately 60 ml of venous blood was collected from healthy adult subjects with a heparinized syringe (final heparin concentration, 10 to 20 U/ml). PMNs were isolated from whole blood by using a Mono-Poly Resolving Medium (M-PRM, Flow Laboratories, Inc., McLean, Va.), as follows. Four milliliters of the M-PRM was placed in a sterile plastic tube (Becton Dickinson Labware). Five milliliters of fresh, heparinized blood was layered on the top of the M-PRM, and the tubes were then centrifuged at 300 × *g* for 30 min at room temperature. The PMN fraction was collected and washed with Hanks' balanced salt solution (HBSS). Any contaminating erythrocytes were lysed by the addition of 0.2% physiological saline for about 20 s, and then an equal volume of 1.6% physiological saline was added to make the medium isotonic. The PMNs were washed with HBSS twice and resuspended in the HBSS to make a 2×10^6 cells per ml suspension. The PMN fraction contained 95% or more PMNs, and their viability, determined by the trypan-blue exclusion method, was at least 99%.

Accumulation of antimicrobial agents by PMNs. OPC-17116 (Otsuka Pharmaceutical Co., Ltd., Osaka, Japan) and other new quinolones, ofloxacin (OFLX), levofloxacin (LVFX, DR-3355), and DR-3354 (Daiichi Seiyaku Co., Ltd., Tokyo, Japan), whose physicochemical characteristics are shown in Table 1, were studied. Although all four agents were dissolved in the HBSS at the six different concentrations of 2, 5, 10, 50, 100, and 200 μg/ml in the initial experiments, 10 μg/ml was regarded as the optimal concentration and was used in all further experiments. Five milliliters of the cell suspension containing 10^7 PMNs was mixed with 5 ml of the drug solution to make a final concentration of 5 μg/ml. The mixtures were incubated for a uniform time under various conditions and then centrifuged at 250 × *g* for 10 min at 4°C. All but 0.5 ml of the supernatant was discarded, and the PMNs were resuspended. To a microcentrifuge tube, 0.5 ml of silicone oil was added, and the PMN suspension was layered on top and then centrifuged at 12,000 × *g* for 3 min to separate completely the PMNs and extracellular solution. The PMN pellet formed on the bottom of the microcentrifuge tube and was obtained by cutting off the portion of the tube containing the pellet. It was then placed in 4 ml of 0.1 M glycine-HCl buffer (pH 3.0) and agitated vigorously in a vortex shaker. The samples were then allowed to stand at room temperature for 2 h in order to completely recover the drugs from the cell pellet and were then centrifuged at 5,600 × *g* for 5 min, and the concentration of antimicrobial agent in the supernatant was determined.

Determination of concentration of antimicrobial agents in PMNs. The concentration of antimicrobial agents in PMNs was determined with a spectrofluorometer (FP-770; Japan Spectroscopic Co., Ltd.). The fluorescence excitation and emission maxima of OFLX, LVFX, and DR-3354 in 0.1 M glycine-HCl buffer were 292 and 496 nm, respectively, and those of OPC-17116 were similarly 275 and 447 nm, respectively. Controls without antimicrobial agents were always used to determine the background fluorescence emitted by

* Corresponding author.

TABLE 1. Physicochemical characteristics of four new quinolones[a]

Drug		Partition coefficient	Solubility in water (mg/ml)	Dissociation constant		Protein binding *3 (%)
				pKa_1	pKa_2	
OFLX		4.9 - 5.1*1	24.0	5.7	7.9	32.6
LVFX		5.1*1	24.5	5.5	8.0	46.5
DR-3354		5.1*1	25.8	-	-	51.5
OPC-17116		4.6*2	36.0	7.1	8.8	51.2

[a] * 1, chloroform–0.1 M phosphate buffer (pH 7.4); * 2, *n*-octanol–0.02 M phosphate buffer (pH 7.4); * 3, concentration of tested drug: 1.0 μg/ml.

PMNs. The concentration of antimicrobial agents in PMNs was calculated by dividing the total amount of antimicrobial agent in the sample by the volume of PMN. The intracellular volume of one PMN was estimated to be 0.27 pl, assuming a cell diameter of 8 μm and a true spherical shape. This value for cell volume was close to that found by Klempner et al. (7) and Koga (8) with tritiated water. The ratio of the intracellular concentration to extracellular concentration (I/E ratio) was then calculated.

Mechanisms of accumulation of antimicrobial agents by PMNs. The effects of different incubation conditions and of metabolic inhibitors were investigated to clarify the mechanisms of uptake of new quinolones by PMNs. The PMNs with antimicrobial agents were incubated at both 37 and 4°C for 30 min to study the changes in I/E ratios at low temperature. PMNs were killed by a 30-min treatment with 10% formalin, washed twice with HBSS, and incubated with antimicrobial agents at 37°C, and I/E ratios were determined. In addition, the pH profile of OPC-17116 uptake was measured in media that had been preadjusted to different external pH values (range, 5.0 to 8.0) by the addition of 10 N HCl or 10 N NaOH. Four reagents, potassium fluoride (KF), sodium cyanide (NaCN), 2,4-dinitrophenol (all manufactured by Wako Pure Chemical Industries Ltd., Osaka, Japan) and ouabain (Merck & Co., Inc., Rahway, N.J.) were used as inhibitors of energy metabolism of PMNs. The concentration of inhibitors was 5 mM for KF, NaCN, and ouabain but 0.1 mM for 2,4-dinitrophenol, which was reported as the optimal concentration for inhibition of energy metabolism of PMNs in previous papers (5, 6, 8, 12). PMNs were preincubated with the inhibitors at 37°C for 30 min, and then the antimicrobial agents were added, and the I/E ratios were measured. The following amino acids, nucleoside, and nucleoside analog were investigated for their competitive inhibition of the uptake of antimicrobial agents: L-glycine, L-leucine, L-methionine, and L-serine (all at a concentration of 1 mM, Wako Pure Chemical Industries Ltd.); 1 mM D-fructose-1,6-diphosphate (Hexose, Nakarai Chemicals Ltd.); and adenosine (Tokyo Kasei Kogyo Co., Ltd.) and puromycin (Sigma Chemical Co., St. Louis, Mo.) (both at concentrations of 0.1, 1, and 5 mM). PMNs were incubated with each substance at 37°C for 30 min, and then antimicrobial agents were added, and the I/E ratios were determined. The statistical significance of differences was determined by analysis of variance followed by Student's *t* test.

Elution of antimicrobial agents from PMNs. The elution of OPC-17116 and OFLX from the PMNs was measured over time. The PMNs with antimicrobial agents were preincubated at 37°C for 30 min and then centrifuged at 250 × *g* for 10 min. The sample was resuspended in HBSS containing no antimicrobial agent, and the intracellular concentration of antimicrobial agents was determined at uniform intervals. With adjustment of the pH of the HBSS in the resuspended sample to pH 6.0 to 8.5, the intracellular concentration of OPC-17116 and OFLX at 5 min were compared to investigate the effect of extracellular pH on the elution of antimicrobial agents.

RESULTS

Accumulation of antimicrobial agents by PMNs and influence of changed incubation conditions. The uptake of the four antimicrobial agents by PMNs at final concentrations of 1, 2.5, 5, 25, 50, and 100 μg/ml was investigated. The mean values and ranges of the uptake of OFLX, LVFX, DR3354, and OPC-17116 at the six different concentrations were as follows; mean value: 8.5 μg/ml (range, 7.9 to 10.4 μg/ml), 7.5 μg/ml (5.9 to 8.8 μg/ml), 6.6 μg/ml (6.3 to 7.2 μg/ml), and 51.4 μg/ml (40.8 to 61.3 μg/ml), respectively. Since there was no significant difference in the I/E ratio at different extracellular concentrations, a concentration of 5 μg/ml, which was considered to be closest to the in vivo concentration, was used as the optimal concentration. As shown in Table 2, the maximum I/E ratio after incubation at normal temperature

TABLE 2. Influence of cell viability and environmental temperature on accumulation of four new quinolones by human PMNs

Antimicrobial agent (incubation time [min])	I/E ratios[a] (no. of expts)		
	Viable cells		Dead cells (37°C)
	37°C	4°C	
OFLX			
15	7.4 ± 0.6 (3)	1.2 ± 0.1 (3)	4.1 ± 0.4 (4)
30	7.6 ± 1.1 (11)	0.9 ± 0.6 (5)	4.9 ± 0.3 (5)
LVFX			
15	9.8 ± 0.9 (3)	1.8 ± 0.5 (3)	NE[b]
30	7.9 ± 1.1 (6)	2.3 ± 0.4 (3)	4.8 ± 0.2 (5)
DR3354			
15	5.4 ± 1.0 (3)	0.6 ± 0.2 (3)	NE
30	5.8 ± 0.2 (3)	0.9 ± 0.3 (3)	4.6 ± 0.6 (5)
OPC-17116			
15	59.5 ± 18.2 (7)	3.8 ± 1.5 (4)	37.1 ± 2.1 (4)
30	66.2 ± 19.2 (17)	1.3 ± 1.0 (10)	46.4 ± 2.8 (4)

[a] Each value represents the mean ± standard deviation.
[b] NE, not examined.

was the highest for OPC-17116 at 66.2, followed by 9.8 for LVFX, 7.6 for OFLX, and 5.8 for DR-3354. At 4°C, the ratios for all agents decreased to 6 to 24% of those for controls. The killed PMNs also decreased to about 49 to 79%.

The uptake of OPC-17116 by PMNs under various pH conditions is shown in Fig. 1. At pH 5.0, OPC-17116 uptake was markedly inhibited, and the I/E ratio was 3.6. There were significant differences in uptake between pH 5.0 and pH 7.4. OPC-17116 uptake increased gradually with the increasing alkalinity of the medium, reaching a maximum I/E ratio of 51.0 at pH 7.4.

KF and 2,4-dinitrophenol slightly decreased the I/E ratios

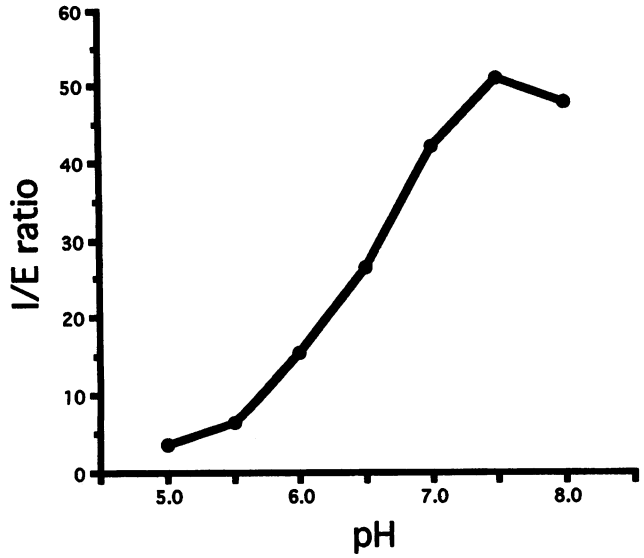


FIG. 1. Effects of pH on OPC-17116 uptake by human PMNs after a 30-min incubation period. Data represent the means of three experiments.

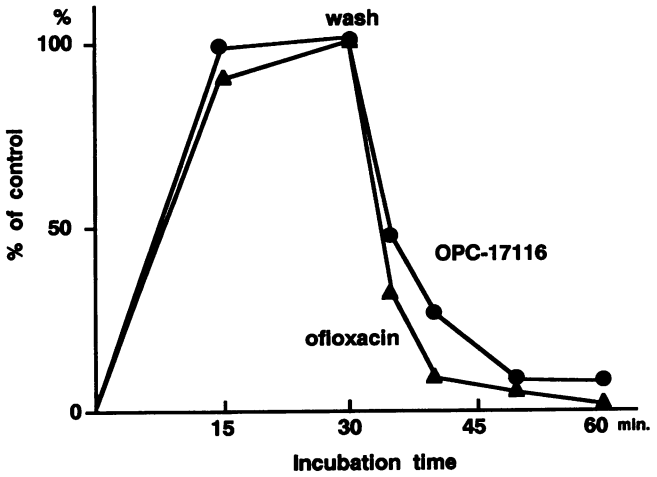


FIG. 2. Elution of OPC-17116 and OFLX from human PMNs. Data represent the means of three experiments.

of OFLX and LVFX to 78 to 82% without significant difference. NaCN, however, strongly decreased the I/E ratios of all quinolones with significant difference to 67, 80, 51, and 38% for OFLX, LVFX, DR3354, and OPC-17116, respectively. Ouabain had little effect on the I/E ratio of any antimicrobial agent. Four amino acids and hexose had little effect on the I/E ratios, showing an 87 to 129% change for the four agents (data not shown). Adenosine slightly decreased the I/E ratio of only OPC-17116 to 92% at the concentration of 5 mM with significant difference, but 0.1 and 1 mM had no influence on any agent. Puromycin, however, at a concentration of 5 mM, markedly decreased the ratios for all quinolones with significant difference to 70, 73, 61, and 27% for OFLX, LVFX, DR3354, and OPC-17116, respectively, and even at the concentration of 1 mM effected a decrease to 57% for OPC-17116.

Elution of antimicrobial agents from PMNs. OPC-17116 and OFLX in the PMNs showed elution from cells or reversibility after removal of the extracellular drug. The elution of OFLX was rapid, and the intracellular concentration decreased to 30% at 5 min, 7% at 10 min, and 3% at 20 min. However, the elution of OPC-17116 was slightly slower at 46% at 5 min, 24% at 10 min, and 7% at 20 min (Fig. 2). A pH range of 6.0 to 8.5 in the extracellular HBSS caused a difference in the elution of OPC-17116 and OFLX (Fig. 3). In the alkaline HBSS, OPC-17116 showed high intracellular concentration. Particularly, at pH 7.7 to 8.0, the intracellular concentrations were 86 to 89% of the controls before washing, but the concentrations were low in the acidic HBSS. On the other hand, OFLX was slightly effected by pH, and almost complete elution was observed except at pH levels centered around 7.4.

DISCUSSION

OPC-17116, newly developed by Otsuka Pharmaceutical Co., is a promising new quinolone and has potent antibacterial activity (4). LVFX and DR-3354 are optically active isomers of racemic OFLX. LVFX, which is considered to be the main active isomer, has an antibacterial activity twice as high as that of OFLX (3, 16). Since these new quinolones show a high tissue and intracellular distribution (8), they have been used aggressively for the treatment of infections

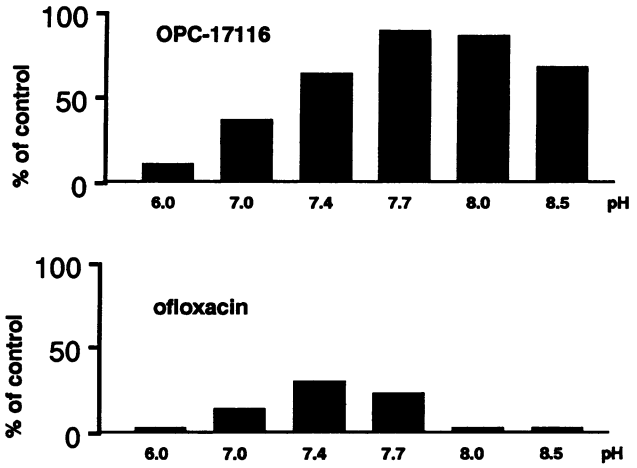


FIG. 3. Elution of OPC-17116 and OFLX from human PMNs at various extracellular pH levels. Data represent the means of three experiments.

caused by *Legionella* spp. (10, 14, 15) and mycobacteria (9) in recent years. This underlines the need for evaluation of the accumulation of a newly developed compound by phagocytes. We chose the fluorescence spectrophotometer assay reported by Pascual et al. (11) to determine the intracellular concentration, as the method can avoid the restrictions and risks inherent with the radioisotope method (5–7, 12) and has the advantage of a sensitivity about 100 times that of HPLC.

The accumulation of various antimicrobial agents by phagocytes has been the topic of many reports. Relatively high I/E ratios of between 2 to 8 have been demonstrated for the new quinolones (2, 8), with OFLX having the highest ratio at about 8. This study showed the I/E ratios of LVFX and DR-3354 to be similar to that of OFLX, although the ratio of LVFX was slightly higher than those of DR-3354 and OFLX. In comparison with these ratios, OPC-17116 showed an extremely high ratio of greater than 60. This ratio was higher than those of clindamycin (10 to 40 times) (6, 7, 12) and macrolides (10 to 30 times) (5), which are known to be high.

The mechanisms of intracellular uptake of antimicrobial agents by PMNs, reported in previous papers, are broadly classified into the two categories: passive and active transport systems. Clindamycin and the macrolides are typical of drugs which are classified into the latter category, and OFLX was recently reported to have the same mechanism (11). Although the antimicrobial agents examined in this report indicated low I/E ratios at low temperature, the ratios were partially decreased by the killing of PMNs, metabolic inhibitors, adenosine, and puromycin. This suggests that the accumulation of these agents may involve, in part, the active transport via a nucleoside transport system, particularly with OPC-17116.

The determination of the elution or reversibility of PMN-associated antimicrobial agents is also an important consideration in evaluating their pharmacokinetic behavior. Although the elution of OPC-17116 was slightly slower than that of OFLX, OPC-17116 showed reversible elution with little accumulation. However, at various extracellular pH levels, the intracellular concentration of OPC-17116 was high with alkaline HBSS and low with acidic HBSS. These phenomena suggest the possibility that the elution of antimicrobial agents is greatly affected by the pH value of the inflammatory site in the body. For example, the pH value of sputum usually ranges between 7 and 8, but purulent sputum tends to be more alkaline because of the large number of PMNs (1, 13), and sites infected with anaerobes, such as lung abscess, tend to be acidic. Therefore, we considered it necessary to determine the difference in pH values in lesion tissues to clarify the degree of penetration of OPC-17116.

For an antimicrobial agent to act most effectively in the body, it is necessary to achieve a high concentration at the inflammatory site and then maintain the concentration for a long time. This means that the tissue and intracellular distribution, and the efficient elution from cells to the body fluid containing microorganisms, are important, and a well-balanced antimicrobial agent having both these characteristics would be an attractive candidate for development. OPC-17116 substantially meets the former of these requirements; however, further investigation into improvement of the latter is necessary.

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