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Uptake and Intracellular Activity of an Optically Active Ofloxacin Isomer in Human Neutrophils and Tissue Culture Cells

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The penetration of an optically active ofloxacin isomer [(−)-ofloxacin] into human neutrophils and different tissue culture cells (HEp-2, McCoy, MDCK, and Vero) was studied and compared with that of ofloxacin by a fluorometric assay. The cellular-to-extracellular-concentration ratios (*C/E*) of (−)-ofloxacin were always higher than 6, significantly greater than those of ofloxacin at extracellular concentrations of 5 and 10 mg/liter. The penetration of (−)-ofloxacin and ofloxacin was doubled when neutrophils were stimulated by phorbol myristate acetate but not affected after ingestion of opsonized *Staphylococcus aureus*. The *C/E* ratios of (−)-ofloxacin and ofloxacin for different tissue culture epithelial cells and fibroblasts were lower than those of neutrophils but still higher than 2. Both compounds produced a significant reduction in viable intraphagocytic *S. aureus* during 3 h of exposure to antimicrobial agents. We conclude that (−)-ofloxacin appears to reach higher intracellular concentrations than ofloxacin, remaining active inside the neutrophils.

Ofloxacin (OFLX) is a quinolone compound that shows high activity against a variety of gram-positive and gram-negative bacteria (17). By using high-performance liquid chromatography, two optically active OFLX isomers have been prepared: (+)-OFLX and (−)-OFLX (6). The latter isomer inhibited the supercoiling activity of gyrase from *Micrococcus luteus* 7.4 times more effectively than did the (+)-isomer (7). Moreover, the in vitro activity of (−)-OFLX against a variety of gram-positive and gram-negative bacteria was 8 to 128 times higher than that of (+)-OFLX and twice as high as that of racemic OFLX (6).

The intracellular penetration and activity of antimicrobial agents can be an important factor in the treatment of infections caused by intracellular facultative or obligate pathogens. Most studies on the ability of antimicrobial agents to penetrate into cells have utilized phagocytic cells, and only a few studies have evaluated the penetration into other types of cells, such as epithelial cells (1, 2). There is, however, strong evidence that some enteroinvasive bacteria such as enteroinvasive *Escherichia coli* survive in epithelial cells despite high external aminoglycoside concentrations because of the low intracellular penetration rate of these antimicrobial agents (8).

We have previously reported that OFLX penetrates into human polymorphonuclear leukocytes (PMNs) by an active mechanism and remains active intracellularly against *Staphylococcus aureus* (13). Since the antimicrobial activity of OFLX seems to be mediated mainly by (−)-OFLX, it would be important to study whether this isomer can be concentrated into phagocytes and other eucaryotic cells.

The purpose of this study is to evaluate the intracellular penetration of this optically active OFLX isomer, (−)-OFLX, into human PMNs and different tissue culture cells compared with that of OFLX. The intracellular activity in PMNs of both compounds against *S. aureus* is also assessed.

MATERIALS AND METHODS

Isolation of PMNs. PMNs were recovered from heparinized venous blood of healthy donors and were purified by previously described methods (16). PMN preparations were 97% pure. Final cell suspensions were adjusted to 5×10^6 PMNs per ml in Hanks balanced salt solution. PMNs were 95% viable by trypan blue exclusion.

Tissue culture cells. HEp-2, McCoy, MDCK, and Vero cells (Flow Laboratories, Irvine, United Kingdom) were grown in minimal essential medium (Flow) supplemented with 1 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Flow) and containing 10% fetal calf serum (Flow) without antibiotics. For each experiment cells were detached from tissue culture bottles with trypsin-EDTA (Flow) and suspended in Hanks balanced salt solution at a concentration of 5×10^6 cells per ml.

Antimicrobial agent uptake by cells. A previously described fluorometric assay to measure quinolone uptake by human PMNs was used (13). The optically active ofloxacin isomer (−)-OFLX was kindly supplied by Daiichi Seiyaku Co., Ltd. (Tokyo, Japan), and OFLX was supplied by Hoescht (Federal Republic of Germany). In these experiments PMNs or tissue culture cells were incubated in Hanks balanced salt solution containing different concentrations of the antimicrobial agent (2, 5, and 10 mg/liter). After different incubation times (1.5, 20, and 30 min) at 37°C, cells were separated from extracellular solution by centrifugation through a water-impermeable silicone-oil barrier in a microcentrifuge tube (9). The entire cell pellet, obtained by cutting off the portion of the microfuge tube containing the pellet, was placed in 2 ml of 0.1 M glycine-HCl buffer (pH 3.0) and agitated vigorously in a vortex shaker. Incubation for 2 h at room temperature was sufficient to release fully intracellular antimicrobial agent. The samples were centrifuged for 5 min at 5,600 × *g*, and the amount of antimicrobial agent was determined by fluorescence emission of the supernatants with a Kontron SFM 25 fluorescence spectrophotometer (Kontron, Switzerland). The fluorescence excitation and emission maxima of OFLX and (−)-OFLX in 0.1 M glycine-HCl (pH 3.0) were 292 and 496 nm, respectively. Controls without antimicrobial agents were always used to determine the background fluorescence.

The intracellular water space was measured by using tritiated water and the extracellular marker [^{14}C]polyethylene glycol (1.4 mCi/g; New England Nuclear Corp., Boston, Mass.). The cells were incubated with these radiolabeled

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compounds for 2 min at 37°C, and then cells were separated from extracellular fluid by velocity gradient centrifugation as described above and counted in a liquid scintillation counter. The total water content of the cell pellet was corrected for trapped extracellular water, i.e., polyethylene glycol space, to obtain the intracellular water space. From the values obtained from this procedure, cell-associated antimicrobial agent concentrations were calculated and expressed as a ratio of the cellular concentration to extracellular concentration (*C/E*) (9).

In a series of experiments, OFLX and (−)-OFLX (extracellular concentration, 5 mg/liter) uptake by human PMNs was measured after stimulation of the cells with 200 nM either phorbol myristate acetate (PMA; Sigma Chemical Co., St. Louis, Mo.) or *S. aureus* ATCC 25923 opsonized in 5% pooled human serum (15 min, 37°C) at a 10/1 ratio of bacteria to PMNs. PMA or opsonized bacteria were added to the PMN suspensions at the same time as the antimicrobial agents, and the uptake was measured as described above. Since the measurement of OFLX and (−)-OFLX is indirect and dependent upon fluorescence, controls were always performed to evaluate the effect of stimuli on the fluorescence of the antimicrobial agents in cell-free systems.

Intracellular activity of antimicrobial agents. To evaluate the intracellular activity of antimicrobial agents, a previously described method was used (15). Briefly, 0.1 ml of opsonized bacterial suspension (5×10^7 CFU/ml) and 0.1 ml of PMNs (5×10^6 PMNs per ml) were combined in a series of polypropylene biovials (Beckman Instruments, Inc., Fullerton, Calif.) and incubated in a shaker (250 rpm) for 60 min at 37°C. After incubation, extracellular bacteria were removed by differential centrifugation. Cells were then suspended in 0.2 ml of RPMI medium (GIBCO, United Kingdom). At this time (designated time zero) the different antimicrobial agents were added, and the vials were incubated in a shaker (250 rpm) at 37°C. Vials were removed at time zero (control) and after 3 h of incubation (control and samples with antimicrobial agents). Cells were lysed in distilled water, and samples were diluted and pour plated in agar. Colonies were counted after 24 h of incubation at 37°C. The data were expressed as percentages of surviving staphylococci compared with controls (without antimicrobial agents) at time zero. In addition to determining bacterial survival, morphologic studies were also routinely performed at time zero and after 3 h of incubation to evaluate the disposition of bacteria (cell associated or extracellular). Samples of 50 μl were removed from biovials and deposited on glass slides. After staining with Wright stain, samples were examined by light microscopy. All assays were performed in duplicate with PMNs from five different donors.

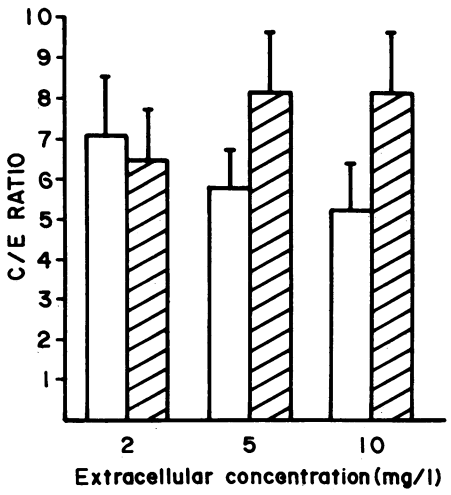

FIG. 1. OFLX (□) and (−)-OFLX (▨) uptake by human PMNs at different extracellular concentrations ($n = 6$). Incubations were done for 20 min. For the data from concentrations of 5 and 10 mg/liter, $P < 0.05$.

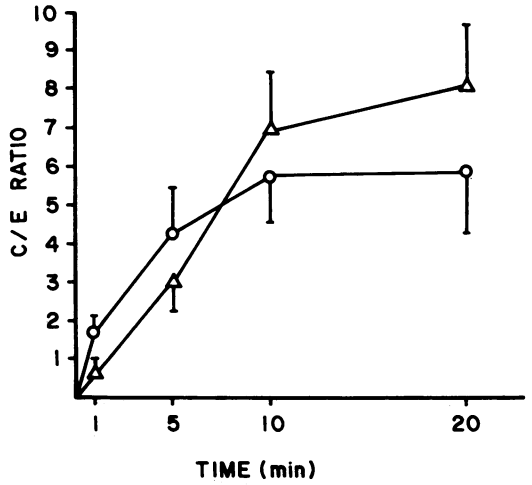

FIG. 2. OFLX (○) and (−)-OFLX (△) uptake by human PMNs at different times ($n = 4$). The extracellular concentration was 5 mg/liter.

Statistical analysis of data. Data were expressed as the means ± standard deviations. Differences among groups were compared by analysis of variance with the Bonferroni method (18), used to assess statistical significance at $P \leq 0.05$.

RESULTS

Uptake of antimicrobial agents by PMNS. Figure 1 shows the *C/E* ratios of OFLX and (−)-OFLX incubated with human PMNs at different extracellular concentrations. Both OFLX and (−)-OFLX showed high *C/E* ratios, being significantly higher for the latter at extracellular concentrations of 5 and 10 mg/liter. The penetration or cell association of OFLX occurred slightly more rapidly than did that of (−)-OFLX (Fig. 2).

The stimulation of PMNs by a membrane activator (PMA) enhanced the intracellular penetration of OFLX and (−)-OFLX 2.4 and 1.5 times, respectively (Table 1). The incubation of PMNs with opsonized *S. aureus* ATCC 25923, however, did not modify the uptake of either compound.

TABLE 1. Effect of the incubation of human PMNs with either opsonized *S. aureus* ATCC 25923 or PMA on the uptake of OFLX and (−)-OFLX by these cells ($n = 4$)[a]

Stimulus	*C/E* ratio	
	OFLX	(−)-OFLX
None (control)	6.0 ± 1.3	8.3 ± 1.7
Opsonized *S. aureus*	5.2 ± 1.1	7.0 ± 1.6
PMA (200 nM)	14.4 ± 2.3[b]	13.0 ± 2.3[b]

[a] Incubations were done for 20 min. The extracellular concentration of OFLX or (−)-OFLX was 5 mg/liter.
[b] $P < 0.05$ compared with the control.

TABLE 2. OFLX and (−)-OFLX uptake by human PMNs and different tissue culture cells ($n = 4$)[a]

Antimicrobial agent	C/E ratio				
	PMN	HEp-2	McCoy	MDCK	Vero
OFLX	5.8 ± 1.2	2.1 ± 0.2	3.9 ± 1.1	2.1 ± 0.9	3.4 ± 1.2
(−)-OFLX	8.2 ± 1.7[b]	2.8 ± 0.6	4.7 ± 1.3	1.9 ± 0.9	2.4 ± 0.6

[a] Incubations were done for 20 min. The extracellular concentration of OFLX or (−)-OFLX was 5 mg/liter.
[b] $P < 0.05$ compared with value for OFLX.

Uptake of antimicrobial agents by tissue culture cells. The *C/E* ratios of OFLX and (−)-OFLX incubated with three tissue culture epithelial cells from different origins (HEp-2, McCoy, and MDCK) and one tissue culture fibroblast cell line (Vero) for 20 min at 37°C are shown in Table 2. The *C/E* ratios of OFLX and (−)-OFLX were 2 to 3 times lower than those observed with PMNs, and there were no significant differences between the two compounds.

Intracellular activities of antimicrobial agents. The intracellular activities of different concentrations of OFLX and (−)-OFLX against *S. aureus* ATCC 25923 were evaluated in a 3-h assay (Table 3). At all of the concentrations evaluated, (−)-OFLX produced a significant reduction of the survival of *S. aureus* in PMNs, similar to that produced by OFLX.

DISCUSSION

(−)-OFLX is an optically active OFLX isomer that shows higher in vitro activity than (+)-OFLX and the racemic compound OFLX against different microorganisms (6, 7). The purpose of this study was to evaluate whether this increased activity was accompanied by a higher intracellular penetration and activity than those of OFLX. The (−)-OFLX uptake by human PMNs was significantly higher than that of OFLX at extracellular concentrations of 5 and 10 mg/liter. This difference was not observed at a lower concentration (2 mg/liter). Nevertheless, both compounds reached high intracellular concentrations in these phagocytes, as has been described for other quinolones with different methodology (3, 10).

The penetration of (−)-OFLX and OFLX was not affected when PMNs were incubated with opsonized *S. aureus*. This has been described for other antimicrobial agents such as erythromycin, rifampin, and gentamicin (5). However, the penetration of other antimicrobial agents, such as clindamycin, has been shown to increase after phagocytosis of *S. aureus* (5).

TABLE 3. Effect of OFLX and (−)-OFLX on the survival of *S. aureus* ATCC 25923 within human PMNs ($n = 5$)

Antimicrobial agent and concn (mg/liter)	% Survival of *S. aureus* at 3 h
No agent	45.5 ± 5.2
OFLX	
0.5	20.9 ± 3.1[a]
2	17.6 ± 3.0[a]
5	15.0 ± 2.7[a]
10	5.8 ± 1.4[a]
(−)-OFLX	
0.5	17.8 ± 3.2[a]
2	15.5 ± 3.1[a]
5	12.2 ± 2.6[a]
10	9.1 ± 2.1[a]

[a] $P < 0.05$ compared with the control.

The stimulation of PMN membranes by PMA, however, doubled the uptake of both compounds by human PMNs, pointing out that the penetration of (−)-OFLX into PMNs could be mediated by an active mechanism, as has been demonstrated for ofloxacin (13), since PMA activates the NADPH oxidase system via protein kinase C (11). To study the penetration of these antimicrobial agents into other eucaryotic cells, we used a tissue culture cell model. The penetration of both compounds into tissue culture epithelial cells and fibroblasts was significantly lower than that observed in PMNs but still reached intracellular concentrations that were at least double the extracellular concentrations.

The use of this model offers an interesting insight into the penetration of antibacterial substances into cells and may have relevance to human infections produced by microorganisms that are able to survive and even multiply within epithelial cells.

Contrasts between phagocyte antimicrobial agent uptake and intracellular activity have been described for a few antimicrobial agents (5, 15). In our study, (−)-OFLX showed a good intracellular penetration into and activity in PMNs against *S. aureus*. The intracellular antimicrobial activity of this isomer was similar to that observed for the racemic compound and similar to those described for other quinolones against different bacteria (4, 12, 14).

In summary, (−)-OFLX, an optically active OFLX isomer, penetrated into human PMN, reaching concentrations higher than those reached by OFLX and remaining active intracellularly. This compound also showed a high penetration into tissue culture epithelial cells and fibroblasts. The high antimicrobial activity of this compound, higher even than that of the racemic compound OFLX, added to the properties observed in this study, could justify additional studies to evaluate the possible clinical relevance of these findings.

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