

Uptake and Intracellular Activity of Trovafloxacin in Human Phagocytes and Tissue-Cultured Epithelial Cells

ALVARO PASCUAL,* ISABEL GARCÍA, SOFÍA BALLESTA, AND EVELIO J. PEREA

Department of Microbiology, School of Medicine, University of Seville, Seville 41080, Spain

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The penetration of trovafloxacin into human polymorphonuclear leukocytes (PMNs), human peritoneal macrophages, and tissue-cultured epithelial cells (McCoy cells) was evaluated. The cellular concentration to extracellular concentration (C/E) ratios of trovafloxacin were greater than 9 for extracellular concentrations ranging from 0.5 to 25 µg/ml. The uptake of trovafloxacin by PMNs was rapid, reversible, nonsaturable, not energy dependent, and significantly increased at pH 6. The C/E ratios of trovafloxacin were not affected by cell viability but were significantly increased at 4°C. Ingestion of opsonized zymosan, but not opsonized *Staphylococcus aureus*, significantly increased the amount of PMN-associated trovafloxacin. This agent at concentrations of 0.5 and 1 µg/ml induced a greater reduction in the survival of intracellular *S. aureus* in PMNs than ciprofloxacin and ofloxacin. It was concluded that trovafloxacin reaches concentrations within phagocytic and nonphagocytic cells several times higher than the extracellular ones, while it remains active in PMNs.

Most fluoroquinolones are able to concentrate in phagocytic and nonphagocytic cells, reaching concentrations several times higher than the extracellular ones (9). Moreover, these agents have been shown to remain active intracellularly against different facultative intracellular pathogens.

Most studies on the intracellular pharmacology of antimicrobial agents have used human phagocytes, and only a few studies have evaluated the penetration into other types of cells. Nevertheless, the pathogenic mechanism of several infections involves the survival of bacteria in nonphagocytic cells, as has been described for *Chlamydia trachomatis* and enteroinvasive *Escherichia coli* in epithelial cells.

An important limitation of most commercially available fluoroquinolones is their limited activity against gram-positive pathogens. In recent years, however, new quinolones that offer good activity against these bacteria have been described. Among them, sparfloxacin has been shown to penetrate rapidly into phagocytic cells, remaining active against *Staphylococcus aureus* (4).

Trovafloxacin (CP-99,219) is a new azabicyclo naphthyridone agent with a broad spectrum of antibacterial activity which includes gram-positive bacteria such as *Streptococcus pneumoniae* and *S. aureus* (6). Moreover, this agent showed high in vitro activity against *Chlamydia trachomatis* (2).

The purpose of this study was to evaluate the uptake of trovafloxacin by human polymorphonuclear leukocytes (PMNs), human peritoneal macrophages (PMφs), and tissue-cultured epithelial cells. The mechanism involved in the penetration of this agent into human PMNs and its intracellular activity compared with those of ciprofloxacin and ofloxacin were also evaluated.

MATERIALS AND METHODS

Isolation of phagocytes. PMNs were recovered from the heparinized venous blood of healthy donors and were purified by previously described methods (10). PMN preparations were >97% pure. PMφs were isolated from peritoneal effluents of patients undergoing continuous ambulatory peritoneal dialysis (CAPD) and who were being followed up by the Nephrology Department of the Univer-

sity Hospital of Seville, as described previously (13). Donors were clinically uninfected at the time of the study. Cell preparations from the CAPD donor always contained >75% PMφs and <15% PMNs. Final cell suspensions were adjusted to 2×10^6 PMφs/ml or 2×10^6 PMNs/ml in Hanks balanced salt solution (HBSS) containing 0.1% gelatin. Both populations of cells were $\geq 95\%$ viable by trypan blue exclusion criteria.

Tissue culture cells. McCoy 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, the cells were detached from the tissue culture bottles with trypsin-EDTA (Flow), washed once with minimal essential medium containing fetal calf serum (10%), and suspended in HBSS at a concentration of 5×10^6 cells per ml.

Trovafloxacin uptake by cells. Uptake of radiolabeled trovafloxacin by phagocytic and epithelial cells was determined by a velocity gradient centrifugation technique described by Klemperer and Styrt (7). [14 C]trovafloxacin (21.53 µCi/mg) and trovafloxacin were kindly supplied by Pfizer Central Research (Groton, Conn.). In these experiments, phagocytes or tissue cells were incubated in HBSS containing different concentrations of trovafloxacin (0.5, 1, 2, 5, 10, and 25 µg/ml). After different incubation periods at 37°C, the cells were separated from the extracellular solution by centrifugation through a water-impermeable silicone oil barrier in a microcentrifuge tube. A 10-µl aliquot of the extracellular medium and the entire cell pellet, obtained by cutting off the portion of the microcentrifuge tube containing the pellet, were placed in 3 ml of scintillation fluid (Ready Micro; Beckman Instruments, Inc., Fullerton, Calif.), and the radioactivity was counted in a liquid scintillation counter (model LS 1801; Beckman). After determination of the cell volume with radiolabeled polyethylene glycol and water (New England Nuclear), the rate of accumulation of the antimicrobial agent in PMNs, PMφs, or tissue cells was calculated and expressed as cellular concentration to extracellular concentration (C/E) ratio (13).

Characterization of trovafloxacin uptake. Further studies to elucidate the mechanism of trovafloxacin uptake by PMNs were performed as described previously (10). The importance of cell viability was studied by using PMNs killed by exposure to 10% formalin for 30 min. These cells were then washed and suspended in fresh medium. Moreover, the influence of environmental temperature, pH, and metabolic inhibitors was evaluated. The influence of temperature was examined by comparing antimicrobial uptake at 4 and 37°C. The pH profiles of trovafloxacin uptake in media preadjusted to different external pHs (pH 6, 7, and 8) by the addition of 10 N HCl or 10 N NaOH were measured. An inhibitor of glycolysis (sodium fluoride, 1.5×10^{-3} M; Sigma Chemical Co., St. Louis, Mo.), an inhibitor of mitochondrial oxidative metabolism (sodium cyanide, 1.5×10^{-3} M; Sigma), a blocker of the proton gradient (carbonyl cyanide *m*-chlorophenylhydrazone; 1.5×10^{-5} M; Sigma), and an uncoupler of oxidative phosphorylation (2,4-dinitrophenol; 1×10^{-4} M; Sigma) were used as metabolic inhibitors. PMNs in HBSS with and without metabolic inhibitors were incubated for 30 min at 37°C, trovafloxacin (final concentration, 2 µg/ml) was then added, and the uptake was measured, as described above.

In a series of experiments, trovafloxacin (extracellular concentration, 2 µg/ml) uptake by human PMNs was measured after the stimulation of cells with 200 nM phorbol myristate acetate (PMA; Sigma) and after the phagocytosis of either opsonized zymosan (0.9 mg/liter; Sigma) or *S. aureus* ATCC 25923, opsonized in 5% pooled human serum (15 min at 37°C) at a 10/1 ratio of bacteria to PMNs.

* Corresponding author. Mailing address: Department of Microbiology, School of Medicine, University of Seville, Apdo. 914, Seville 41080, Spain. Phone: 34.5.4557448. Fax: 34.5.4377413.

PMA or opsonized particles were added to PMN suspensions at the same time that the antimicrobial agent was added, and the uptake was measured as described above.

The efflux or reversibility of the binding of cell-associated trovafloxacin was also studied. PMNs, PM ϕ s, or McCoy cells were incubated for 10 min at 37°C with trovafloxacin (extracellular concentration, 2 μ g/ml), collected by centrifugation, and rapidly suspended in quinolone-free medium. Cell-associated trovafloxacin was quantified at various time intervals (5, 10, and 20 min) after the removal of the extracellular antimicrobial agent. All assays were performed in duplicate with PMNs from five different donors.

Partition coefficient of quinolones. The partition coefficients of quinolones were determined by the modified method of Nikaido (8). Solutions (10 μ g/ml) of quinolones were made in 0.1 M phosphate buffer (pH 7.2). After being shaken with an equal volume of *n*-octanol at 25°C for 48 h and centrifuged at $1,870 \times g$ for phase separation, the concentrations of quinolones in the aqueous phase were determined by a spectrophotometric assay measuring the A_{260} for trovafloxacin and the A_{286} for ofloxacin (Hoechst AG, Barcelona, Spain). The partition coefficients were expressed as the ratio of the amount of the compound in the *n*-octanol to that in the aqueous phase.

Organisms and susceptibility testing. *S. aureus* ATCC 25923 was used for the killing assays. Susceptibility studies were determined by dilution assay. The MICs and minimum bactericidal (MBCs) of ciprofloxacin (Bayer AG, Leverkusen, Germany), ofloxacin (Hoechst AG), concentrations and trovafloxacin for this strain were 0.25, 0.25, and 0.03 μ g/ml, respectively.

Intracellular activities of antimicrobial agents. To evaluate the intracellular activities of antimicrobial agents, a previously described method was used (13). 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), and the vials were incubated in a shaker (250 rpm) for 60 min at 37°C. After incubation, the mixtures were washed three times with 2.5 ml of ice-cold phosphate-buffered saline by using differential centrifugation ($160 \times g$; 5 min at 4°C) to remove the extracellular bacteria. The cells were then suspended in 0.2 ml of RPMI medium (Sigma). At this time, different concentrations of the different fluoroquinolones were added, and the vials were incubated in a shaker (50 rpm) at 37°C. The vials were removed at time zero 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 the percentages of staphylococci surviving compared with the levels in the controls (without antimicrobial agents) at 3 h. 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 the bacteria (cell associated or extracellular). Samples (50 μ l) were removed from the biovials and were deposited on glass slides. After being stained with Wright stain, the samples were examined by light microscopy. All assays were performed in duplicate with PMNs from five different donors.

Statistical analysis of data. The data were expressed as the means \pm standard deviations. Differences among groups were compared by analysis of variance, used to assess statistical significance at $P \leq 0.05$.

RESULTS

Uptake of trovafloxacin by PMNs, PM ϕ s, and McCoy cells.

Figure 1 shows the kinetics of uptake of trovafloxacin by human PMNs. Trovafloxacin uptake by these cells was rapid and high. With extracellular concentrations of 2 μ g/ml, the C/E ratios were greater than 10 after 1 min of incubation. The effect of the extracellular concentrations of trovafloxacin on uptake by PMNs is presented in Fig. 2. Cell-associated trovafloxacin was not saturable at extracellular concentrations ranging from 0.5 to 25 μ g/ml. The uptake of trovafloxacin by human PM ϕ s and tissue-cultured epithelial cells was similar to that obtained with PMNs. At extracellular concentrations of 2 μ g/ml (30 min; 37°C), the C/E ratios were 10.6 ± 2.0 for PMNs, 10.2 ± 2.0 for PM ϕ s, and 9.6 ± 1.9 for McCoy cells.

The effects of pH, environmental temperature, cell viability, and metabolic inhibitors on the uptake of trovafloxacin by human PMNs are indicated in Table 1. Trovafloxacin uptake by PMNs was significantly higher at pH 6 and when viable PMNs at 4°C were used. None of the metabolic inhibitors used affected the intracellular penetration of this quinolone.

The stimulation of PMNs by a membrane activator (PMA) and the phagocytosis of opsonized *S. aureus* cells did not affect the intracellular penetration of trovafloxacin (C/E ratios, 9.0 ± 1.7 and 8.2 ± 1.6 , respectively). The phagocytosis of opsonized

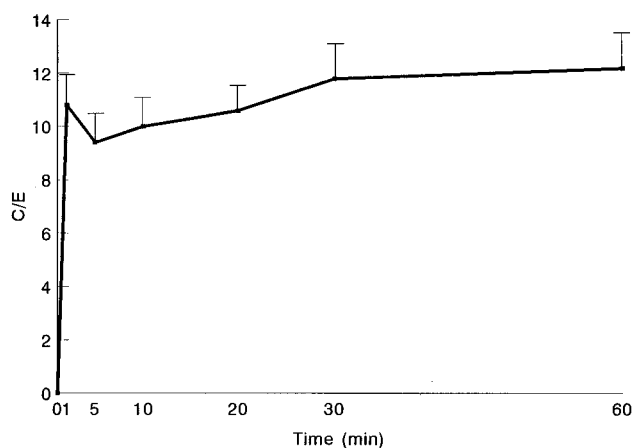


FIG. 1. Kinetics of trovafloxacin uptake by human PMNs ($n = 5$). The extracellular concentration was 2 μ g/ml. Error bars indicate standard deviations.

zymosan, however, significantly increased the uptake of trovafloxacin by PMNs (C/E ratio, 13.9 ± 1.7 ; $P < 0.05$).

To evaluate whether trovafloxacin that had been taken up by human PMNs, PM ϕ s, and tissue-cultured epithelial cells was tightly bound to cellular components, we evaluated the kinetics of efflux (Fig. 3). The reversibility of binding of trovafloxacin was rapid, with 80, 65, and 72% of the cell-associated drug being lost by 5 min in PMNs, PM ϕ s, and McCoy cells, respectively. The kinetics of the efflux of trovafloxacin from PMNs at 37°C was similar to that observed in PMNs at 4°C or dead PMNs at 37°C (data not shown).

Partition coefficient of quinolones. The partition coefficients of trovafloxacin and ofloxacin were 0.646 and 0.168, respectively. This means that trovafloxacin is approximately four times more hydrophobic than ofloxacin.

Intracellular activity of trovafloxacin. The intracellular activity of trovafloxacin against *S. aureus* compared with those of ciprofloxacin and ofloxacin was evaluated by a 3-h assay (Fig. 4). At extracellular concentrations of 0.5 and 1 μ g/ml, the intracellular activity of trovafloxacin was significantly greater than those of ciprofloxacin and ofloxacin.

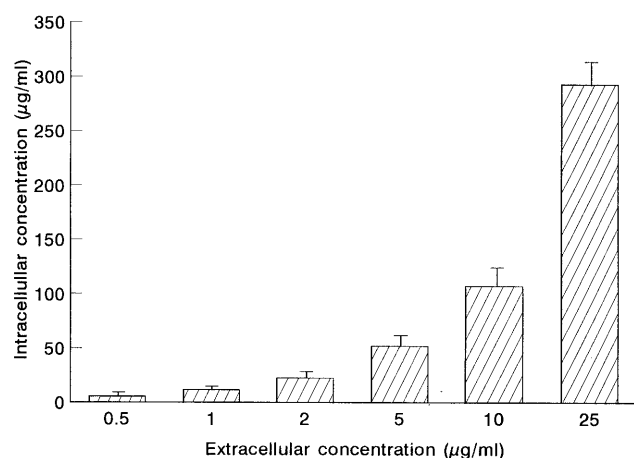


FIG. 2. Trovafloxacin uptake by human PMNs at different extracellular concentrations ($n = 5$). Incubations were carried out for 20 min. Error bars indicate standard deviations.

TABLE 1. Effects of pH, cell viability, environmental temperature, and metabolic inhibitors on the intracellular penetration of trovafloxacin in human PMNs

Condition ^a	C/E ratio
pH 7.2 (control).....	11.3 ± 0.8
pH 6.....	16.5 ± 0.4 ^b
pH 8.....	10.3 ± 1.0
Viable cells, 4°C.....	62.0 ± 14.0 ^b
Dead cells, 37°C.....	14.4 ± 4.3
Sodium fluoride.....	11.1 ± 1.7
Sodium cyanide.....	9.3 ± 1.8
Carbonyl cyanide <i>m</i> -chlorophenyldrazone.....	9.7 ± 1.7
2,4-Dinitrophenol.....	13.4 ± 3.5

^a Experiments were carried out for 20 min at an extracellular concentration of 2 µg/ml with PMNs from five subjects.

^b *P* < 0.05 compared with the control.

DISCUSSION

The uptake of radiolabeled trovafloxacin by phagocytic cells (PMNs and PMφs) and nonphagocytic cells has been evaluated. At therapeutic extracellular concentrations, trovafloxacin reached concentrations in PMNs 10 or more times higher than the extracellular concentrations. These values were slightly higher than those observed with other fluoroquinolones such as ofloxacin, lomefloxacin, sparfloxacin, and BAY-Y-3118 (4, 5, 11, 12). This could be related to the fact that trovafloxacin is more hydrophobic than those quinolones and theoretically could pass more easily through the cell membrane.

The uptake of trovafloxacin by PMφs was similar to that observed for PMNs. This finding could be interesting since intraleukocytic survival of bacteria has been postulated as an important cause of persistent peritonitis in patients undergoing CAPD (1) and little information on the penetration of antimicrobial agents in human PMφs is available. At higher extracellular concentrations (10 µg/ml) and under different experimental conditions, Edelstein et al. (3) reported C/E ratios for trovafloxacin of greater than 20 for guinea pig alveolar macrophages.

Trovafloxacin also reached high intracellular concentrations (C/E ratios, ≥9) in tissue-cultured epithelial cells. These values are higher than those observed for ofloxacin and sparfloxacin (4, 11). Since trovafloxacin yielded high intrinsic activity

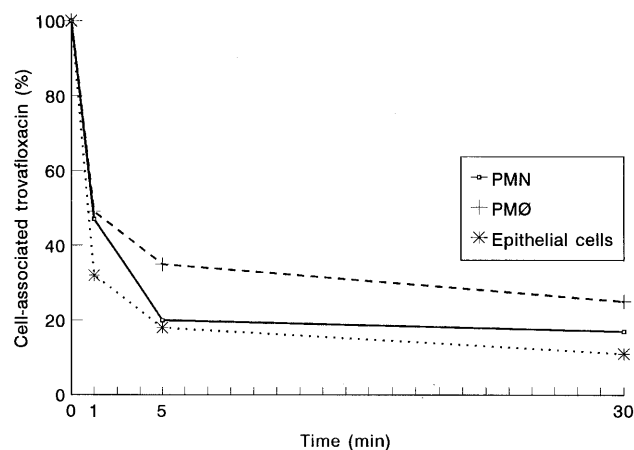


FIG. 3. Efflux of trovafloxacin from human PMNs, PMφs, and McCoy cells (*n* = 3). After incubation with 2 µg of trovafloxacin per ml for 20 min, the cells were washed and resuspended in antimicrobial agent-free medium. Cell-associated trovafloxacin was then measured at different times.

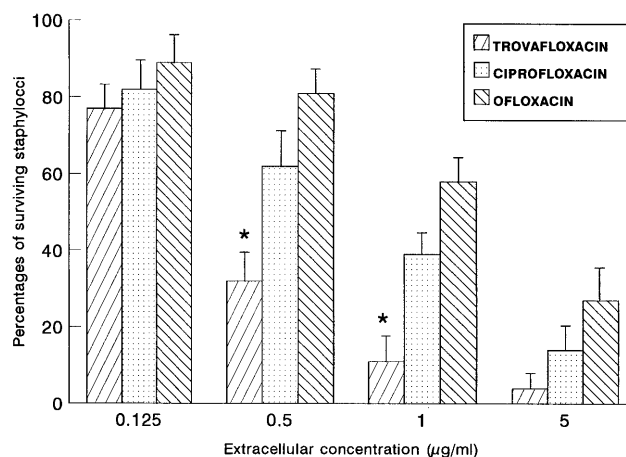


FIG. 4. Activity of trovafloxacin compared to those of ciprofloxacin and ofloxacin against intracellular *S. aureus* in human PMNs (*n* = 5). Data are expressed as percentages of surviving bacteria compared to that for the control. Error bars indicate standard deviations. *, *P*, 0.05 compared to ciprofloxacin and ofloxacin.

against *C. trachomatis* and since these microorganisms multiply within epithelial cells, these results could reinforce the potential use of this agent against chlamydial infections.

The uptake of trovafloxacin by human PMNs was rapid, nonsaturable (extracellular concentrations ranged from 0.5 to 25 µg/ml), reversible (as was the case for PMφs and McCoy cells), and not energy dependent. It was significantly increased at acidic pH, as was reported elsewhere for other quinolones (4, 5), but to a lesser extent. This phenomenon could be related to the fact that such quinolones display a free carboxyl group that becomes protonated when the pH changes from alkaline to acidic. It has been observed that the ionized forms of weak organic acids diffuse through biological membranes much more slowly than their un-ionized forms (14). The uptake of trovafloxacin by human PMNs was significantly increased at 4°C and was not affected by cell viability. We have previously observed that the uptake of some quinolones such as temafloxacin and lomefloxacin significantly decreased at 4°C and was affected by cell viability (12), while the uptake of other quinolones such as sparfloxacin was not affected by these parameters (4). We have not previously observed such a remarkable increase at 4°C with other quinolones. These data could indicate that more than one mechanism may mediate the intracellular penetration of different quinolones. For trovafloxacin, most data show that a passive mechanism, such as that described for sparfloxacin (4), is involved.

The effects of phagocytosis and stimulation of the cell membrane on the uptake of trovafloxacin by human PMNs was similar to those previously observed with sparfloxacin (4). The ingestion of opsonized *S. aureus* cells did not affect the intracellular penetration of trovafloxacin, but the ingestion of opsonized zymosan significantly increased the C/E ratio for trovafloxacin. Nevertheless, the uptake of trovafloxacin was still high under both conditions.

At extracellular concentrations of 0.5 and 1 µg/ml, trovafloxacin showed activity against *S. aureus* greater than those of ciprofloxacin and ofloxacin in human PMNs. This effect was primarily due to a higher intrinsic activity of trovafloxacin against the strain that we used.

In summary, trovafloxacin penetrates into phagocytic and nonphagocytic cells, reaching intracellular concentrations several times greater than the extracellular ones, while it remains

active intracellularly in human PMNs. These properties, besides its broad spectrum of activity, enhance the potential clinical usefulness of this fluoroquinolone.

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