ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 1996, p. 739–742 Vol. 40, No. 3
0066-4804/96/$04.00+0

Uptake and Intracellular Activity of NM394, a New Quinolone, in Human Polymorphonuclear Leukocytes

MASAKUNI OZAKI,[1]* KINUYO KOMORI,[1] MASATO MATSUDA,[1] REIKO YAMAGUCHI,[1] TAKUYA HONMURA,[1] YOSHIFUMI TOMII,[1] IKUMI NISHIMURA,[1] AND TAKESHI NISHINO[2]

Discovery Research Laboratories II, Nippon Shinyaku Co., Ltd., Nishioji Hachijyo, Minami-ku, Kyoto 601,[1] and Department of Microbiology, Kyoto Pharmaceutical University, Kyoto 607,[2] Japan

Received 6 March 1995/Returned for modification 30 July 1995/Accepted 12 December 1995

The uptake of NM394, a new quinolone, by and its subsequent elution from human polymorphonuclear leukocytes were studied and compared with those of ofloxacin and ciprofloxacin. The kinetics of the uptake of NM394 was similar to that of ciprofloxacin. The maximum intracellular-to-extracellular concentration ratio was 12.3, compared with 8.6 for ciprofloxacin and 4.9 for ofloxacin at the extracellular concentration of 20 μg/ml. The elution of NM394 from human polymorphonuclear leukocytes occurs relatively slowly; 5 min after the removal of extracellular NM394, nearly 100% still remained in polymorphonuclear leukocytes, compared with ofloxacin, which was so rapidly eluted that only 12% remained. The uptake of NM394 was significantly decreased at 4°C and by the presence of NaCN but was not affected by the presence of L-glycine, L-leucine, L-serine, adenosine, or NaF. NM394 showed intracellular activity at a concentration of 0.1 μg/ml that significantly reduced the number of phagocytosed *Pseudomonas aeruginosa* cells with 2 h of incubation. These results suggest that uptake of NM394 by human polymorphonuclear leukocytes occurs via an active transport system differing from that of ofloxacin, whose uptake is affected by the presence of L-glycine and L-leucine, and that once accumulated, NM394 remains intracellularly active and participates in protection against bacterial infection.

NM394 (Fig. 1) is a new thiazetoquinoline carboxylic acid derivative (9) which has a broad spectrum of activity and potent antibacterial activity comparable to those of ciprofloxacin (17). NM394 is a parent compound of a lipophilic prodrug, NM441, which when administered orally is readily absorbed and hydrolyzed to NM394 and which shows antibacterial activity in vivo (10).

The quinolone antibacterial agents are known to penetrate into human polymorphonuclear leukocytes (PMN), and intracellular concentrations are several times higher than extracellular concentrations (2, 11, 12, 16). These agents also show antibacterial activity against phagocytosed bacteria (2, 11).

Since NM441 showed potent protective activity against experimental systemic and local infections including infections caused by *Pseudomonas aeruginosa*, it is of interest to determine the characteristics of uptake and accumulation by PMN and the intracellular activity of NM394.

In this study, uptake of NM394 by PMN and its subsequent elution from PMN were compared with those of ofloxacin (14) and ciprofloxacin. In addition, the influences of pH, amino acids, nucleic acids, and inhibitors of metabolism on penetration of NM394 into PMN were studied. The intracellular activity of NM394 against phagocytosed *P. aeruginosa* was also examined.

MATERIALS AND METHODS

Isolation of PMN. PMN were purified from 30 ml of heparinized blood obtained from healthy volunteers with Mono-Poly resolving medium (M-PRM; ICN Biochemicals, Tokyo, Japan) as described previously (5). Briefly, 5 ml of the blood was overlaid on 3 ml of M-PRM in plastic tubes (100 by 13 mm). After being warmed for 5 min in a 37°C water bath, the tubes were centrifuged at 200 × *g* for 15 min at room temperature. The PMN fraction was then collected and washed with Hanks' balanced salt solution (HBSS; pH 7.4). The remaining erythrocytes were lysed by suspending PMN in 0.2% NaCl solution for 1 min, and then an equal volume of 1.6% NaCl solution was added to make the solution isotonic (this procedure was repeated two to three times). Finally, the PMN were suspended in HBSS at 2.5×10^6 cells per ml. The PMN were more than 99% viable, as shown by the trypan blue exclusion method.

Uptake of antimicrobial agents by PMN. NM394 (synthesized in the Chemistry Department II of Nippon Shinyaku Co., Ltd., Kyoto, Japan), ofloxacin (Daiichi Seiyaku Co., Ltd., Tokyo, Japan), and ciprofloxacin (Bayer Yakuhin Co., Ltd., Osaka, Japan) were dissolved in HBSS (pH was adjusted to 7.4) and mixed with PMN suspensions to a final concentration of 20 μg/ml. The mixtures were incubated in a shaker (130 rpm) at 37°C, and after 5, 15, 30, and 60 min the PMN were separated (1 ml of each mixture was used) by centrifugation (1,200 × *g*, 3 min) through silicone oil in a microcentrifugation tube. The tubes were kept in a freezer (−80°C), and after the extracellular solution was frozen, the PMN pellet was recovered by cutting off the tube. The pellets were suspended in 1.8 ml of 0.1 M glycine-HCl buffer (pH 3.0) and were agitated in a Vortex mixer. The suspensions were kept at room temperature for 2 h to extract the antibacterial agent from the PMN. The extracts were centrifuged at 1,200 × *g* for 5 min, and concentrations of the drugs in the supernatant were measured.

The influence of pH (pH 6.5, 7.0, 7.5, and 8.0) at 37°C and of temperature (at 4 and 37°C) on the uptake of NM394, ofloxacin, and ciprofloxacin were studied.

Competitive inhibition by 1 mM L-glycine, L-leucine, and L-serine (nacalai tesque, Kyoto, Japan), adenosine (Kohjin Co., Ltd., Tokyo, Japan), and D-fructose-1,6-diphosphate (nacalai) and the influence of metabolic inhibitors (5 mM NaF, NaCN, and ouabain and 0.1 mM 2,4-dinitrophenol [DNP; nacalai]) on the uptake of NM394 by PMN were also studied.

Measurement of concentrations of antibacterial agents in the PMN. The concentrations of the antibacterial agents in the PMN were measured by fluorometric assaying (12) with a fluorescence spectrophotometer (F-2000; Hitachi). The fluorescence excitation and emission maxima of NM394, ofloxacin, and ciprofloxacin in 0.1 M glycine-HCl buffer were 275 and 429 nm, 293 and 494 nm, and 277 and 443 nm, respectively. Controls without drugs were always used to determine the background fluorescence emitted by PMN. Intracellular water volume was estimated to be 2.15 μl per 10^7 PMN as reported by Koga (5), and the ratio of the intracellular concentration to extracellular concentration (I/E ratio) was calculated.

Elution of antimicrobial agents from PMN. After PMN were incubated with 20 μg of NM394, ofloxacin, or ciprofloxacin per ml for 30 min at 37°C, then were collected by centrifugation at 1,200 × *g* for 5 min and the pellets were suspended in drug-free HBSS. After removal of the drug, the PMN were incubated at 37°C, and at 5, 10, 20, and 60 min, the concentrations of the drugs remaining in the PMN were measured.

* Corresponding author. Mailing address: Discovery Research Laboratories II, Nippon Shinyaku Co., Ltd., Nishioji Hachijyo, Minami-ku, Kyoto 601, Japan. Phone: 75-321-1111 (ext. 6226). Fax: 75-321-9038.

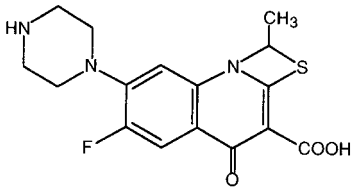

FIG. 1. Chemical structure of NM394.

Intracellular activity of antimicrobial agents. An overnight culture of *P. aeruginosa* E-2 (resistant to intracellular killing by PMN, maintained in the Department of Microbiology, Kyoto Pharmaceutical University, Kyoto, Japan) at 37°C in brain heart infusion broth (Nissui, Tokyo, Japan) was centrifuged (1,600 × *g*, 10 min) and washed with HBSS containing 25 mM 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HBSS-HEPES). The PMN (5×10^6 cells) and *P. aeruginosa* (2.5×10^7 CFU) were suspended in 2 ml of HBSS-HEPES supplemented with normal human serum at a final concentration of 10% in polystyrene tubes (12 by 75 mm; Falcon) and were incubated with a rotating cultivator (Rotater; Taitec, Koshigaya, Saitama Pref., Japan), which is a method that has been previously described (8), at 37°C for 30 min (6 rpm). After incubation, extracellular bacteria were removed by differential centrifugation (130 × *g*, 5 min) as described previously (6, 11). The PMN were then washed and suspended in HBSS-HEPES–10% serum in the polystyrene tubes. To the PMN suspensions were added NM394, ofloxacin, and ciprofloxacin to final concentrations including MICs and 1/2 MICs (the MICs of NM394, ofloxacin, and ciprofloxacin against *P. aeruginosa* E-2 were 0.2, 0.78, and 0.2 μg/ml, respectively), and the tubes were rotated at 37°C. After 0, 0.5, 1.0, and 2.0 h of incubation, samples (100 μl each) were taken from the tubes, the PMN were placed in 900 μl of distilled water, and the numbers of bacteria in PMN were counted by plating on heart infusion agar (Nissui) after 18 h of incubation at 37°C.

Statistical analysis. The data were expressed as means and standard deviations. Differences between groups were analyzed by Tukey's method or Student's *t* test, and a probability of less than 5% was considered statistically significant.

RESULTS

Uptake of antimicrobial agents by PMN. The rates of uptake of NM394, ofloxacin, and ciprofloxacin by PMN are shown in Fig. 2. The kinetics of the uptake of NM394 by PMN was similar to that of ciprofloxacin. The maximum I/E ratios for ofloxacin, ciprofloxacin, and NM394 were 4.9 ± 0.6, 8.6 ± 2.8, and 12.3 ± 3.5 ($P < 0.05$, compared with ofloxacin), respectively. Uptake of NM394 by PMN did not quite reach steady state even after 60 min of incubation. Although there is no significant difference compared with values at 30 min, further incubation is probably necessary to reach the plateau phase. Ciprofloxacin reached maximum ratio after 30 min while ofloxacin reached maximum ratio after 5 min of incubation. No significant difference between NM394 and ciprofloxacin was observed at each point.

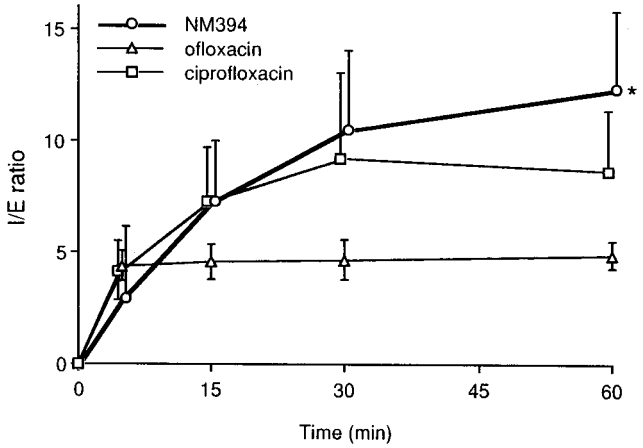

FIG. 2. Uptake of NM394, ofloxacin, and ciprofloxacin by human PMN. Data are the means ± standard deviations for three experiments. *, $P < 0.05$ compared with ofloxacin.

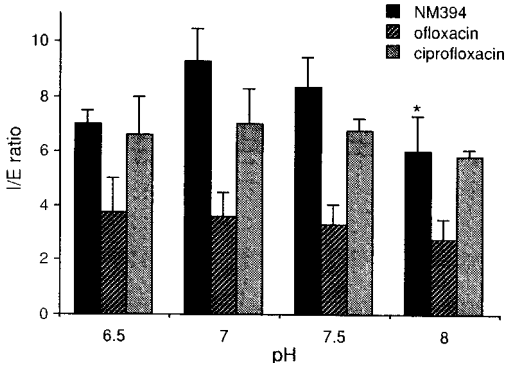

FIG. 3. Influence of environmental pH on uptake of NM394, ofloxacin, and ciprofloxacin by human PMN. Data are the means ± standard deviations for three experiments. *, $P < 0.05$ compared with I/E ratio at pH 7.0.

The influence of pH on uptake of antimicrobial agents by PMN is shown in Fig. 3. Uptake of NM394 was at its maximum at pH 7.0 and significantly decreased ($P < 0.05$) at pH 8.0, compared with pH 7.0 (no significant difference between pH 6.5 and 7.5). Uptake by ciprofloxacin and ofloxacin was not affected by pH values between 6.5 and 8.0. Uptake of NM394, ofloxacin, and ciprofloxacin by PMN was significantly decreased ($P < 0.05$) at 4°C to 11.9, 10.6, and 15.1%, respectively, of uptake values at 37°C (Fig. 4).

Other factors affecting the uptake of NM394 by PMN. The uptake of NM394 was not affected by the presence of 1 mM L-glycine, L-leucine, L-serine, adenosine, or D-fructose-1,6-diphosphate (Fig. 5). The presence of 5 mM NaCN significantly decreased ($P < 0.05$) the uptake of NM394 to 50.4%, but other metabolic inhibitors (5 mM NaF and ouabain and 0.1 mM 2,4-dinitrophenol) had no influence on its uptake (Fig. 6).

Elution of antibacterial agents from PMN. As shown in Fig. 7, after removal of the drug, the elution of NM394 from the PMN occurred rather slowly compared with those of ofloxacin and ciprofloxacin. After 5 min, almost 100% remained and after 10 min, 68% of the initial intracellular concentration of NM394 remained, with 57% still present at 20 min. Ciprofloxacin showed 70, 50, and 27% retention levels at 5, 10, and 20 min, respectively, while the ofloxacin level decreased to 12% at

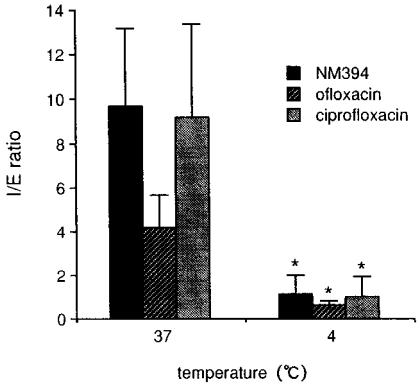

FIG. 4. Influence of environmental temperature on uptake of NM394, ofloxacin, and ciprofloxacin by human PMN. Data are the means ± standard deviations for four experiments. *, $P < 0.05$ compared with I/E ratio at 37°C.

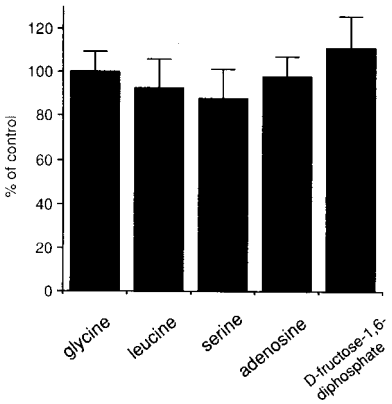

FIG. 5. Influence of amino acids, adenosine, and D-fructose-1,6-diphosphate on uptake of NM394 by human PMN. Data are the means ± standard deviations for three experiments.

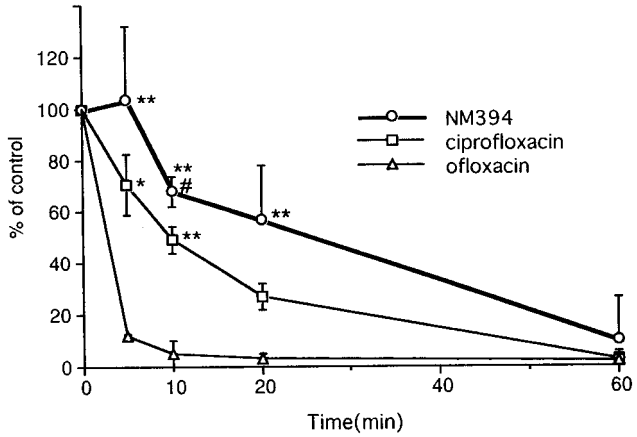

FIG. 7. Elution of intracellular NM394, ofloxacin, and ciprofloxacin from human PMN after removal of extracellular drugs. Data are means ± standard deviations for three experiments. I/E ratios (100% values) for NM394, ciprofloxacin, and ofloxacin are 6.8 ± 1.17, 8.64 ± 1.06, and 4.11 ± 0.34, respectively. * and **, $P < 0.01$ and $P < 0.05$ compared with ofloxacin, respectively; #, $P < 0.05$ compared with ciprofloxacin.

5 min. Significant differences between NM394 and ofloxacin at 5, 10, and 20 min ($P < 0.01$) and ciprofloxacin at 10 min ($P < 0.05$) were observed. In addition, significant differences between ciprofloxacin and ofloxacin at 5 and 10 min ($P < 0.05$ and $P < 0.01$, respectively) were found.

Intracellular activities of antimicrobial agents. The intracellular activities at MICs and 1/2 MICs of NM394, ofloxacin, and ciprofloxacin against *P. aeruginosa* E-2 are plotted in Fig. 8. The numbers of viable cells in control PMN (without antimicrobial agents) were not significant but slightly decreased from 5.75 to 5.61 × 10^5 CFU per 2.5 × 10^5 PMN with 2 h of incubation. On the other hand, at the extracellular MICs of NM394, ofloxacin, and ciprofloxacin (0.2, 0.78, and 0.2 μg/ml, respectively) and the 1/2 MIC of NM394, significant reductions ($P < 0.01$) in the numbers of CFU in the PMN were observed after 2 h of incubation.

DISCUSSION

Determination of the concentration of antimicrobial agents in PMN has been performed by bioassays (1, 16), radiometry (2, 4, 13), high-performance liquid chromatography (5), and fluorometry (12). Since the fluorometric assay was reported to be as sensitive as radiometry and is a simple method, in the present study the uptake and elution of NM394 were measured by fluorometry. In previous reports, extracellular concentrations of 2 to 5 μg/ml, corresponding to clinically relevant concentrations, were used (11, 12, 15). However, at the fluorescence excitation and emission maxima of NM394 (275 and 429 nm, respectively), the background fluorescence emitted by PMN, which should be subtracted, was relatively large. Therefore, measurement of intracellular values at lower extracellular concentrations, especially those near 2 μg/ml, corresponding to the level of NM394 in plasma reported in a previous clinical study (7), was inefficient. In the present study, we used 20 μg/ml as an extracellular concentration, a value which was also used by Easmon and Crane (1) in studies of the uptake of ciprofloxacin.

NM394 was well taken up by PMN, and a maximum I/E ratio of 12.3 was obtained after 60 min of incubation. Elution of NM394 from PMN occurred slowly, such that 5 and 10 min after removal of the extracellular NM394, 100 and 68% remained, respectively. These results compare favorably with those obtained for ofloxacin uptake, which reached plateau phase after 5 min of incubation, obtained an I/E ratio of 4.9, and eluted rapidly from the PMN. The I/E ratio of 8.6 for ciprofloxacin found here agrees with the result reported by Easmon and Crane (1). For ofloxacin, our result (I/E ratio of 4.9) was slightly lower than those reported by Pascual et al. (11, 12) and Taira et al. (15) at extracellular concentrations of 2 to

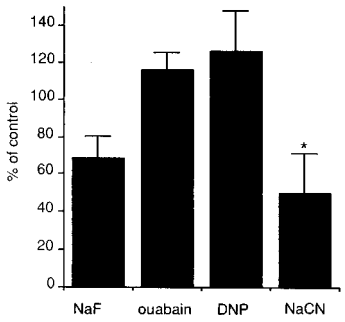

FIG. 6. Influence of metabolic inhibitors on uptake of NM394 by human PMN. Data are the means ± standard deviations for three experiments. DNP, 2,4-dinitrophenol; *, $P < 0.05$ compared with the control.

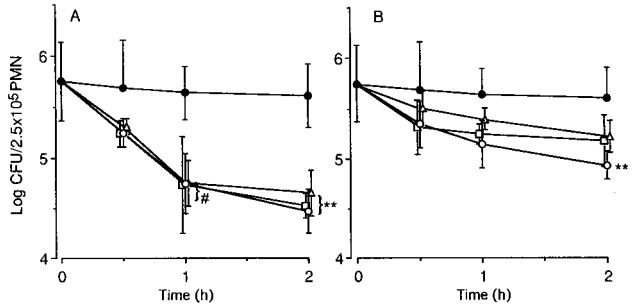

FIG. 8. Intracellular activities of NM394, ofloxacin, and ciprofloxacin against phagocytosed *P. aeruginosa* E-2. ●, control; ○, NM394; △, ofloxacin; □, ciprofloxacin. Extracellular concentrations at MICs (A) and at 1/2 MICs (B) are given. MICs of NM394 and ciprofloxacin, 0.2 μg/ml; MIC of ofloxacin, 0.78 μg/ml. Data are the means ± standard deviations for four experiments. #, $P < 0.01$ compared with 1-h control; **, $P < 0.01$ compared with 2-h control.

5 μg/ml. However, Pascual et al. (11) also reported that the I/E ratio of ofloxacin decreased as the extracellular concentration increased and that the I/E ratio at 10 μg/ml was close to 5.

The uptake of NM394 was not affected by the presence of L-glycine, L-leucine, L-serine, adenosine, and NaF but significantly decreased in the presence of NaCN. In addition, uptake significantly decreased at 4°C in a manner similar to results for ofloxacin and ciprofloxacin. These findings suggest that NM394 penetrates into the PMN via an active transport system. Clindamycin and OPC-17116 have been reported to penetrate PMN via a nucleoside transport system (3, 15) and ofloxacin has been reported to penetrate PMN in part via an amino acid transport system (12). NM394 uptake was not affected by the presence of adenosine and thus occurred via a transport system different from that for OPC-17116. Since NM394 uptake was not affected by L-glycine or L-leucine, both of which affect ofloxacin uptake (12), the uptake of NM394 occurred in an active transport system different from that which mediates ofloxacin uptake.

NM394 showed intracellular activity against phagocytosed *P. aeruginosa* cells comparable to that of ciprofloxacin at an extracellular concentration of 0.2 μg/ml (MIC). However, only NM394 significantly reduced the number of CFU in PMN at an extracellular concentration of 0.1 μg/ml (1/2 MIC).

This means that even such low extracellular concentrations of NM394 penetrated well into PMN, where it accumulated, and shows that intracellular activity plays a role in host defense against bacterial infection.

In the present study, we found that the uptake and elution characteristics of NM394 from PMN compared favorably with those of ofloxacin: NM394 penetrated into PMN well, showed an I/E ratio higher than that of ofloxacin, and remained active intracellularly. The mechanism of uptake of NM394 by PMN was considered to be mediated by an active-transport system, although it differed from that through which ofloxacin penetration is achieved. Further investigation of the mechanisms of NM394 uptake and retention by PMN should be conducted.

REFERENCES

1. **Easmon, C. S. F., and J. P. Crane.** 1985. Uptake of ciprofloxacin by human neutrophils. J. Antimicrob. Chemother. **18:**251–260.
2. **Garcia, I., A. Pascual, M. C. Guzman, and E. J. Perea.** 1992. Uptake and intracellular activity of sparfloxacin in human polymorphonuclear leukocytes. Antimicrob. Agents Chemother. **36:**1053–1056.
3. **Hand, W. L., and N. L. King-Thompson.** 1982. Membrane transport of clindamycin in alveolar macrophages. Antimicrob. Agents Chemother. **21:** 241–247.
4. **Klempner, M. S., and B. Styrt.** 1981. Clindamycin uptake by human neutrophils. J. Infect. Dis. **144:**472–479.
5. **Koga, H.** 1987. High-performance liquid chromatography measurement of antimicrobial concentration in polymorphonuclear leukocytes. Antimicrob. Agents Chemother. **31:**1904–1908.
6. **Mandell, G. L., and K. Vest.** 1972. Killing of intraleukocytic *Staphylococcus aureus* by rifampin: in-vitro and in-vivo studies. J. Infect. Dis. **125:**486–490.
7. **Nakashima, M., T. Uematsu, K. Kosuge, Y. Okuyama, A. Morino, M. Ozaki, and Y. Takebe.** 1994. Pharmacokinetics and safety of NM441, a new quinolone, in healthy male volunteers. J. Clin. Pharmacol. **34:**930–937.
8. **Nielsen, B., and H. Nielsen.** 1987. Bactericidal effect of polymorphonuclear leukocytes on *Pseudomonas aeruginosa* pre-incubated in ofloxacin. Acta Pathol. Microbiol. Immunol. Scand. Sect. B **95:**227–232.
9. **Ozaki, M., M. Matsuda, Y. Tomii, K. Kimura, K. Kazuno, M. Kitano, M. Kise, K. Shibata, M. Otsuki, and T. Nishino.** 1991. In vitro antibacterial activity of a new quinolone, NM394. Antimicrob. Agents Chemother. **35:** 2490–2495.
10. **Ozaki, M., M. Matsuda, Y. Tomii, K. Kimura, J. Segawa, M. Kitano, M. Kise, K. Shibata, M. Otsuki, and T. Nishino.** 1991. In vivo evaluation of NM441, a new thiazetoquinoline derivative. Antimicrob. Agents Chemother. **35:**2496–2499.
11. **Pascual, A., I. Garcia, and E. J. Perea.** 1990. Uptake and intracellular activity of an optically active ofloxacin isomer in human neutrophils and tissue culture cells. Antimicrob. Agents Chemother. **34:**277–280.
12. **Pascual, A., L. Martinez, and E. J. Perea.** 1989. Fluorometric measurement of ofloxacin uptake by human polymorphonuclear leukocytes. Antimicrob. Agents Chemother. **33:**653–656.
13. **Pascual, A., D. Tsukayama, J. Kovarik, G. Gekker, and P. Peterson.** 1987. Uptake and activity of rifapentine in human peritoneal macrophage and polymorphonuclear leukocytes. Eur. J. Clin. Microbiol. **6:**152–157.
14. **Sato, K., Y. Matsuura, M. Inoue, T. Une, Y. Osada, H. Ogawa, and S. Mitsuhashi.** 1982. In vitro and in vivo activity of DL-8280, a new oxazine derivative. Antimicrob. Agents Chemother. **22:**548–553.
15. **Taira, K., H. Koga, and S. Kohno.** 1993. Accumulation of a newly developed fluoroquinolone, OPC-17116, by human polymorphonuclear leukocytes. Antimicrob. Agents Chemother. **37:**1877–1881.
16. **Van Der Auwera, P., T. Matsumoto, and M. Husson.** 1988. Intraphagocytic penetration of antibiotics. J. Antimicrob. Chemother. **22:**185–192.
17. **Wise, R., J. M. Andrews, and L. J. Edwards.** 1983. In vitro activity of Bay 09867, a new quinolone derivative, compared with those of other antimicrobial agents. Antimicrob. Agents Chemother. **23:**559–564.