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Intracellular Penetration and Activity of BAY Y 3118 in Human Polymorphonuclear Leukocytes

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The penetration of a new quinolone (BAY Y 3118) into human polymorphonuclear leukocytes (PMNs) was evaluated by a fluorometric assay. The cellular concentration-to-extracellular concentration (C/E) ratio was higher than 6.3 at extracellular concentrations ranging from 2 to 100 mg/liter. The uptake of BAY Y 3118 was rapid, reversible and nonsaturable. The intracellular penetration of BAY Y 3118 was significantly affected by environmental temperature (C/E ratio at 4°C, 5.4 ± 0.5; control, 7.5 ± 0.9; $P < 0.05$) and cell viability (C/E ratio in dead PMNs, 5.5 ± 0.8; control, 7.5 ± 0.9; $P < 0.05$), but it was not affected by metabolic inhibitors. The ingestion of opsonized zymosan or opsonized *Staphylococcus aureus* significantly decreased the levels of PMN-associated BAY Y 3118. Cell stimulation by a membrane activator, however, significantly increased the intracellular concentration of this quinolone. At therapeutic extracellular concentrations (0.5, 2, and 5 mg/liter), BAY Y 3118 showed intracellular activity greater than that of ciprofloxacin against *S. aureus* in human PMNs. It was concluded that BAY Y 3118 reaches high intracellular concentrations within human PMNs and remains active intracellularly.

The entry of antimicrobial agents into phagocytic cells is essential for activity against intracellular microorganisms. The use of antimicrobial agents that reach high intracellular concentrations and remain active within phagocytes could be important in the therapy of infections caused by facultative intracellular bacteria (2).

Most of the new quinolones are able to penetrate and reach high intracellular concentrations in different phagocytic cells. Among them, norfloxacin, ciprofloxacin, ofloxacin, and levofloxacin have been shown to be effective against typical facultative intracellular pathogens, such as mycobacteria or *Legionella* spp., and against other bacteria, such as *Staphylococcus aureus*, that in certain circumstances are able to survive within phagocytic cells (4, 5, 10).

BAY Y 3118 is a new chlorofluoroquinolone with antibacterial activity against an expanded spectrum, including gram-positive cocci and anaerobic bacteria (14). It was found to be more active than ciprofloxacin against *S. aureus*, including ciprofloxacin-resistant and methicillin-resistant species (1).

The purpose of this study was to evaluate the uptake of BAY Y 3118 by human polymorphonuclear leukocytes (PMNs). The mechanism involved in the penetration of these agents into human PMNs and its intracellular activity against *S. aureus* compared with that of ciprofloxacin were also evaluated.

MATERIALS AND METHODS

Isolation of PMNs. PMNs were recovered from heparinized venous blood of healthy donors and purified by previously described methods (13). 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.

BAY Y 3118 uptake by PMNs. A previously described fluorometric assay was used to measure quinolone uptake by human PMNs (9). BAY Y 3118 and ciprofloxacin were kindly supplied by Bayer A.G., Leverkusen, Germany. In these experiments, PMNs were incubated in Hanks balanced salt solution containing different concentrations of antimicrobial agent (2 to 100 mg/liter). After different incubation times at 37°C, cells were separated from extracellular solution by centrifugation through a water-impermeable silicone-oil barrier in a microcentrifuge tube. The entire cell pellet, obtained by cutting off the portion of the microcentrifuge 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 the intracellular antimicrobial agent fully (9). Samples were centrifuged for 5 min at 5,600 × *g*, and the amount of antimicrobial agent was determined by fluorescence emission of supernatants with an F 2000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). The fluorescence excitation and emission maxima in 0.1 M glycine-HCl (pH 3.0) were 285 and 459 nm, respectively. Controls without antimicrobial agents were always used to determine the background fluorescence.

Intracellular water space was measured by using triated water and the extracellular marker [^{14}C]polyethylene glycol (1.4 mCi/g; New England Nuclear Corp., Boston, Mass.). Cells were incubated with these radiolabeled compounds for 2 min at 37°C, separated from extracellular fluid by velocity gradient centrifugation as described above, and counted in a liquid scintillation counter. 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 by this procedure, cell-associated antimicrobial agent concentrations were calculated and expressed as ratios of the cellular concentration to extracellular concentration (C/E ratios) (7).

Characterization of BAY Y 3118 uptake. Further studies to elucidate the mechanism of BAY Y 3118 uptake of PMNs were performed as described previously (9). The importance of cell viability was studied by using PMNs killed by exposure to 10% Formalin for 30 min. These cells were washed and then suspended in fresh medium. Moreover, the influences of environmental temperature, pH, and metabolic inhibitors were evaluated. The influence of temperature was examined by

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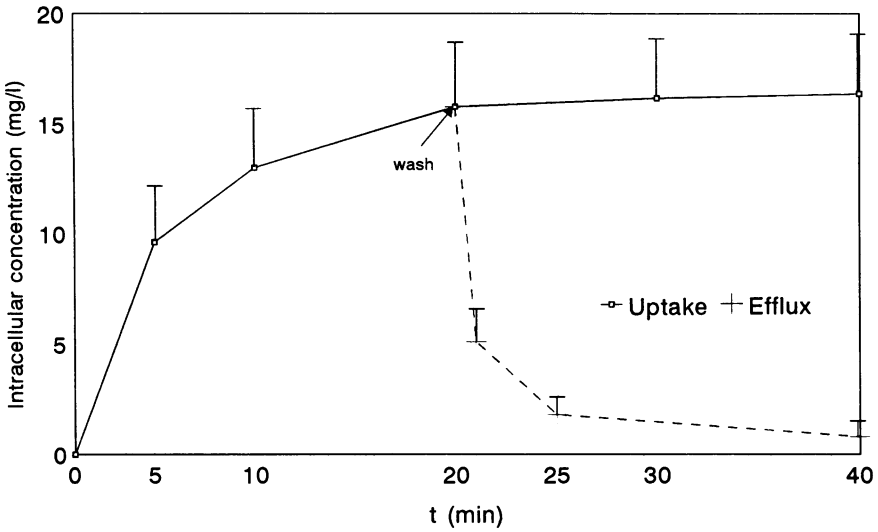

FIG. 1. BAY Y 3118 uptake by human PMNs and efflux of PMN-associated BAY Y 31118 after the removal of the extracellular drug (n = 5). The extracellular concentration was 2 mg/liter. Error bars indicate standard deviations.

comparing antimicrobial uptake at 4 and 37°C. The pH profiles of BAY Y 3118 uptake in media preadjusted to different external pHs (pH 5, 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 Hanks balanced salt solution with and without metabolic inhibitors were incubated for 30 min at 37°C. BAY Y 3118 (final concentration, 2 mg/liter) was then added, and the uptake was measured as described above.

In a series of experiments, BAY Y 3118 (extracellular concentration, 2 mg/liter) 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, 37°C) at a 10/1 ratio of bacteria to PMNs. PMA or opsonized particles were added to PMN suspensions at the same time as the antimicrobial agent, and the uptake was measured as described above.

The efflux or reversibility of the binding of PMN-associated BAY Y 3118 was also studied. PMNs were incubated for 10 min at 37°C with BAY Y 3118 (extracellular concentration, 2 mg/liter), collected by centrifugation, and rapidly suspended in quinolone-free medium. PMN-associated BAY Y 3118 was quantitated at various time intervals (5, 10, and 20 min) after the removal of the extracellular antimicrobial agent. Controls were always used to evaluate the effects of inhibitors and substrates on the fluorescence of BAY Y 3118 in cell-free systems.

Organisms and susceptibility testing. *S. aureus* ATCC 25923 was used for killing assays. Susceptibility studies were determined by dilution assay. The MICs and MBCs of ciprofloxacin and BAY Y 3118 against this strain were 0.25 and 0.5 mg/liter and 0.03 and 0.06 mg/liter, respectively.

Intracellular activity of quinolones. To evaluate the intracellular activities of antimicrobial agents, a previously described method was used (12). 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) in Hanks balanced salt solution were combined in a series of polypropylene biovials (Beckman Instruments, Inc., Fullerton, Calif.) and incubated in a shaker (50 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, Middlesex, United Kingdom). At that time (designated time zero), different antimicrobial agents were added and vials were incubated in a shaker (50 rpm) at 37°C. Vials were removed at time zero (control) and after 3 h of incubation (control and samples with antimicrobial agents). Extracellular antimicrobial agents were removed by washing cells with ice-cold phosphate-buffered saline. Cells were lysed in distilled water, and samples were diluted and pour plated on agar. Colonies were counted after 24 h of incubation at 37°C. Data were expressed as percentages of surviving staphylococci compared with controls (without antimicrobial agents) at 3 h. In addition to determin-

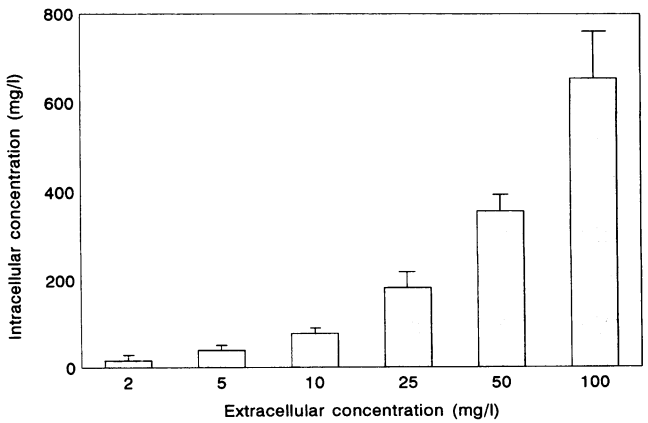

FIG. 2. BAY Y 3118 uptake by human PMNs at different extracellular concentrations (n = 5). Incubations were carried out for 20 min. Error bars indicate standard deviations.

TABLE 1. Effects of cell viability, environment temperature, and pH on the intracellular penetration of BAY Y 3118 in human PMNs (n = 6)

Exptl condition(s)[a]	C/E ratio
Viable cells at 37°C	7.5 ± 0.9
Viable cells at 4°C	5.4 ± 0.5 ($P = 1.30 \times 10^{-2}$)[b]
Dead cells at 37°C	5.5 ± 0.8 ($P = 2.18 \times 10^{-2}$)[b]
pH 5	8.2 ± 1.9
pH 6	7.6 ± 1.9
pH 7	7.5 ± 1.6
pH 8	7.5 ± 1.9

[a] Experiments were carried out for 20 min at extracellular concentrations of 2 mg/liter.
[b] Significant compared with viable cells at 37°C.

ing 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 being stained with Wright stain, samples were examined by light microscopy. All assays were performed in duplicate with PMNs from five different donors.

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

RESULTS

Uptake of BAY Y 3118 by human PMNs. The uptake and efflux kinetics of BAY Y 3118 in human PMNs are shown in Fig. 1. BAY Y 3118 penetrates into human PMNs rapidly, reaching intracellular concentrations six to seven times greater than the extracellular ones. The efflux of BAY Y 3118 was also rapid, with 86% of the cell-associated drug being lost within 5 min (compare values at 20 and 25 min in Fig. 1).

The effects of different extracellular concentrations on the uptake of BAY Y 3118 by PMNs are shown in Fig. 2. The levels of the cell-associated drug ranged from 15.8 ± 3.2 mg/liter at an extracellular concentration of 2 mg/liter (C/E ratio, 7.9 ± 1.6) to 655 ± 75 mg/liter at an extracellular concentration of 100 mg/liter (C/E ratio, 6.7 ± 1.4).

The effects of cell viability, environmental temperature, and pH on BAY Y 3118 uptake by human PMNs are shown in Table 1. Intracellular penetration of BAY Y 3118 into viable PMNs was significantly reduced at 4°C, as was the case with dead cells, but still reached C/E ratios of greater than 5.

The effects of different metabolic inhibitors on BAY Y 3118 uptake by PMNs are shown in Table 2. Among the inhibitors evaluated, only sodium fluoride and sodium cyanide significantly impaired intracellular penetration of this quinolone.

TABLE 2. Effects of metabolic inhibitors on the intracellular penetration of BAY Y 3118 in human PMNs (n = 6)[a]

Inhibitor	C/E ratio
Control	7.5 ± 0.9
Sodium fluoride	4.3 ± 2.1 ($P = 4.8 \times 10^{-2}$)[b]
Sodium cyanide	2.6 ± 2.0 ($P = 7.9 \times 10^{-3}$)[b]
Carbonyl cyanide m-chlorophenylhydrazone	6.3 ± 1.8
2,4-Dinitriphenol	5.3 ± 1.9

[a] Experiments were carried out for 20 min at extracellular concentrations of 2 mg/liter.
[b] Significant compared with control.

TABLE 3. Effects of cell membrane-stimulating agents on the intracellular penetration of BAY Y 3118 in human PMNs (n = 6)[a]

Stimulus	C/E ratio
Control	6.9 ± 2.3
Opsonized *S. aureus*	4.4 ± 1.3 ($P = 0.025$)[b]
Opsonized zymosan	3.6 ± 1.4 ($P = 1.91 \times 10^{-1}$)[b]
PMA	16.6 ± 8.2 ($P = 1.22 \times 10^{-2}$)[b]

[a] Experiments were carried out for 20 min at extracellular concentrations of 2 mg/liter.
[b] Significant compared with control.

Finally, we evaluated the influence of phagocytosis and cell membrane stimuli on the uptake of BAY Y 3118. We found that the ingestion of opsonized particles such as zymosan or bacteria significantly reduced the uptake of BAY Y 3118 PMNs (Table 3). Stimulation with PMA, however, significantly increased intracellular concentrations of this drug.

Intracellular activity of BAY Y 3118. The intracellular activity of BAY Y 3118 against *S. aureus* compared with that of ciprofloxacin was evaluated by a 3-h assay (Fig. 3). At each of three extracellular concentrations, the intracellular activity of BAY Y 3118 in human PMNs was significantly greater than that of ciprofloxacin.

DISCUSSION

In this study, the uptake of BAY Y 3118 by human PMNs was evaluated. Using a fluorometric assay, we observed that this antimicrobial agent was highly concentrated in these phagocytic cells even at therapeutical concentrations. The C/E ratios of BAY Y 3118 were similar to those observed for ciprofloxacin, ofloxacin, sparfloxacin, and other quinolones (3, 9–11).

The uptake of BAY Y 3118 of PMNs was rapid, nonsaturable (at extracellular concentrations of between 2 and 200 mg/liter), and reversible. The intracellular penetration of BAY Y 3118 was affected by cell viability, environmental temperature, and some metabolic inhibitors (sodium fluoride and sodium cyanide). It is interesting that even an inhibitor of mitochondrial oxidative metabolism, such as sodium cyanide, decreased the uptake of ofloxacin by PMNs since these cells have few mitochondria and do not depend upon oxidative metabolism for energy (9). This phenomenon could be related

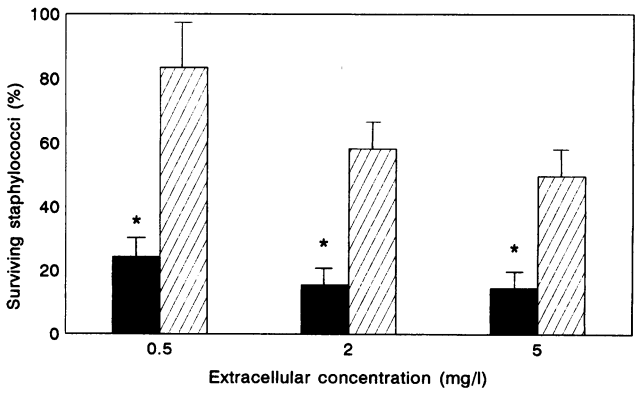

FIG. 3. Effects of BAY Y 3118 (solid bars) and ciprofloxacin (hatched bars) on the killing of *S. aureus* ATCC 25923 ingested by human PMNs. Data are expressed as percentages of the levels in controls (without antimicrobial agents) (n = 5). *, $P < 0.05$ when compared with ciprofloxacin. Error bars indicate standard deviations.

to a secondary or minor activity of this inhibitor in other metabolic functions of phagocytes. Similar results have previously been described for ofloxacin and levofloxacin (10). The uptake of other quinolones, such as sparfloxacin, by human PMNs was not affected by any of these parameters (3). Lomefloxacin penetration and temafloxacin penetration into human PMNs, however, were affected by environmental temperature (11). These data indicate that more than one mechanism may mediate the intracellular penetration of different quinolones.

The penetration of BAY Y 3118 decreased slightly when PMNs were incubated with opsonized zymosan or *S. aureus*. The stimulation of PMN membranes by PMA, an activator of the NADPH oxidase system via protein kinase C (8), significantly increased the uptake of this quinolone by human PMNs. This divergent effect could be related to the fact that the activation of NADPH oxidase by opsonized particles differs from that of PMA, the former being mediated by phospholipase A_2 (8). The mechanism whereby BAY Y 3118 accumulates in cells is not yet known, and no simple model can be presented in view of the findings presented above. Although certain data point to a passive mechanism, as has been postulated for sparfloxacin (3), many others are typical of an active mechanism, as has been described for ofloxacin and levofloxacin (10). Our previous experiences show that although most quinolones reach similar intracellular concentrations in phagocytic cells, different mechanisms may be involved. These mechanisms do not seem to be related either to the degree of fluorination of the molecule (11) or to the partition coefficients (3), as has been previously demonstrated.

A contrast between phagocyte antimicrobial agent uptake and intracellular activity has been described for a few antimicrobial agents (6). In our study, BAY Y 3118 showed intracellular activity against *S. aureus*. In fact, this activity was significantly greater than those of ciprofloxacin and other quinolones previously evaluated (10, 11); this effect was partially due to the higher bactericidal activity of this antimicrobial agent against the strain evaluated.

In summary, BAY Y 3118 penetrates into human PMNs, reaching intracellular concentrations several times greater than the extracellular ones while remaining active intracellularly. The high antimicrobial activity of this quinolone in addition to the properties observed in this study enhances the potential uses of BAY Y 3118.

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