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Comparative antimycobacterial activities of ofloxacin, ciprofloxacin and grepafloxacin

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Infections caused by non-tuberculous mycobacteria and multidrug-resistant *Mycobacterium tuberculosis* are difficult to treat. New compounds potentially active against these bacteria are therefore constantly being sought. Among them is grepafloxacin, a new C5 fluoroquinolone. A panel of 130 isolates of mycobacteria including 33 *M. tuberculosis* isolates and 97 isolates of different species of atypical mycobacteria were analysed for susceptibility to grepafloxacin, ofloxacin and ciprofloxacin. The MICs of these fluoroquinolones were determined using the agar-dilution method. Different mycobacterial species showed different degrees of susceptibility to grepafloxacin, ofloxacin and ciprofloxacin but little difference was observed between the MICs of the three antibiotics against strains of the same mycobacterial species. In addition, to evaluate the intracellular activity of these drugs, six strains of mycobacteria were studied using a human-macrophage infection model. Preliminary results of macrophage experiments showed that grepafloxacin was more active than ofloxacin and ciprofloxacin, particularly against *Mycobacterium kansasii* and, to a lesser degree, against *Mycobacterium avium* complex and *Mycobacterium marinum*. However, the three fluoroquinolones had comparable activities against *M. tuberculosis*.

Introduction

Over the last few years, an important problem has arisen in the treatment of infections caused by mycobacteria. Relatively few drugs are effective against these microorganisms, especially non-tuberculous (or atypical) mycobacteria and multidrug-resistant (MDR) *Mycobacterium tuberculosis*. Thus, new drugs potentially active against these bacteria are needed.

Some fluoroquinolones are currently used to treat mycobacterial infections.[1] The mechanism of action of the quinolone antibacterial agents is thought to result from the combination of their abilities to penetrate into bacterial cells and inhibit DNA gyrase, an essential bacterial enzyme that maintains the superhelical twists of DNA.[2]

The aim of the present study was to determine the activity of grepafloxacin (OPC-17116), a new C5 methyl fluoroquinolone, and to compare it with those of ciprofloxacin and ofloxacin against different strains of mycobacteria. First, the MICs of these three fluoroquinolones against 130 isolates and laboratory strains of mycobacteria, including 33 isolates of *M. tuberculosis*, were determined. Because of the intracellular multiplication of most mycobacteria, MIC determination is not sufficient to predict the in-vivo efficacy of the tested agents. Therefore, to evaluate the intracellular activity of the three fluoroquinolones, six strains were studied using a human-macrophage infection model.

Materials and methods

Bacteria

All mycobacteria were isolated from the clinical samples of patients with or without AIDS admitted to the Centre Hospitalier Régional of Bordeaux. Isolates were identified by classical culture and biochemical characteristics or DNA probes (Gen-Probe, San Diego, CA, USA). A total of 130 isolates and laboratory strains from seven species were studied: 33 isolates of *M. tuberculosis* (one reference strain, H37 Rv and 32 respiratory isolates; one of them was an MDR strain), 17 isolates of *Mycobacterium kansasii* (12 respiratory isolates and five blood culture isolates), 41 isolates of *Mycobacterium avium* complex (four respiratory isolates and 37 blood culture isolates), 10 isolates and strains of *Mycobacterium marinum* (nine clinical isolates and one reference strain, CIP 6423), 12 isolates and strains

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of *Mycobacterium chelonae* (11 clinical isolates and one reference strain, ATCC 19236), 10 isolates of *Mycobacterium fortuitum* (nine clinical isolates and one reference strain, CIP 6843) and seven isolates of *Mycobacterium scrofulaceum*.

Among them, three isolates of *M. avium* complex, one of *M. marinum*, one of *M. kansasii* and one of *M. tuberculosis* were selected for evaluation of antibiotic activity in human monocyte-derived macrophages.

Escherichia coli CIP 7624 was tested simultaneously as a control under standard culture conditions (Mueller–Hinton broth) and under the same conditions used for mycobacteria (7H11 agar medium; Difco Laboratories, Detroit, MI, USA).

Antimicrobial agents

The following fluoroquinolones were used: ofloxacin (Roussel–Diamant, Paris, France), ciprofloxacin (Bayer Pharma, Puteaux, France) and grepafloxacin (this molecule is under licence to Glaxo–Wellcome, Marly-le-Roi, France, from Otsuka Pharmaceutical Co., Ltd, Japan). Stock solutions of each drug were prepared in accordance with its manufacturer's instructions to obtain a final concentration of 1280 mg/L.

MIC determination using the agar-macrodilution method

Several colonies of mycobacteria were harvested from Lowenstein–Jensen or Coletsos slants, suspended in Dubos medium (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France) and thoroughly homogenized by shaking. A 7-day-old culture in this medium was adjusted to 1 mg/L (*c.* 10^7 cfu/mL) and diluted $1:10^3$ and $1:10^5$ in fresh medium. For each strain, 50 μL of the $1:10^3$ dilution were inoculated on to Petri dishes coated with 7H11 agar medium supplemented with 10% oleic acid–albumin–dextrose complex (OADC) (Difco Laboratories) containing serial two-fold dilutions of the different antibiotics (final concentration ranging from 0.12 to 64 mg/L). In addition, 50 μL of the 10^{-3} and 10^{-5} dilutions were plated on control dishes without antibiotic.

After 7 (rapid growers) or 14–21 days (slow growers) of culture at 37°C in a CO_2 incubator, bacterial growth was measured. The MIC was considered to be the lowest concentration of the drug that inhibited more than 99% of bacterial proliferation.

Testing antimicrobial activity against mycobacteria in macrophages

Human monocyte-derived macrophages from healthy donors were prepared. Briefly, 50 mL of peripheral blood was mixed in the same volume of RPMI 1640 tissue culture medium (GibcoBRL, Life Technologies, Cergy Pontoise, France) and centrifuged for 20 min at 2500 rpm with Lymphoprep (Nycomed Pharma AS, Oslo, Norway) to obtain purified mononuclear cells. The cells were washed twice in phosphate-buffered saline then suspended at 10^7 cells/mL in RPMI 1640 medium. They were incubated in Lab-Tek wells (Nalge Nunc International, Naperville, IL, USA), 1 mL/well, for 3 h at 37°C in a 5% CO_2 atmosphere. After sedimentation, non-adherent cells were washed off and adherent cells, corresponding to monocytes, were incubated in RPMI 1640 medium containing 10% human AB serum for 7 days at 37°C in a CO_2 incubator. This nutrient medium was changed on days 1 and 3. On day 6, a confluent monolayer of macrophages was obtained. Mycobacterial suspensions were prepared as for MIC determination. After several days of incubation, these suspensions were diluted in RPMI 1640 medium supplemented with 10% human AB serum.

The medium covering each well containing a macrophage monolayer was removed and replaced with 1 mL of the bacterial suspension. Macrophage phagocytosis of the mycobacteria was then allowed to proceed for 3 h at 37°C (30°C for *M. marinum*) in a 5% CO_2 atmosphere. Extracellular bacteria were removed by two washes with phosphate-buffered saline and RPMI 1640 containing 10% human AB serum and different antibiotic concentrations were added to every well containing infected macrophages, except the well serving as an untreated control. It has previously been shown that, in this system, mycobacteria do not multiply in the extracellular medium.[3,4]

The antibiotic-containing wells were then cultured for 7 days, at which time their supernatants were harvested by aspiration. The macrophage monolayers were detached by scraping and lysed with 1 mL of distilled water, frozen at –20°C for 10 min, and after thawing, pooled with their corresponding supernatants. Ten-fold serial dilutions of these supernatant–cell lysate mixtures were plated on to 7H11 agar medium supplemented with 10% OADC. Plates were cultured at 37°C (30°C for *M. marinum*) and cfu were counted 1 or 2 weeks later. The cfu counts of the antibiotic-containing wells were compared with that of the D0 control. The control well was processed in the same manner but immediately following the 3 h of incubation with the mycobacterial strains (D0).

In the macrophage model, a drug was considered to be intracellularly bactericidal when it effectively reduced the viable cfu counts in the test samples by more than one $\log_{10}$ compared with that of the D0 control.[5] A bacteriostatic effect was defined as a reduction of the viable cfu counts by less than one $\log_{10}$.

Results

Effect of mycobacterial culture conditions on antibiotic activity

Comparative cultures of the control strain of *E. coli* performed in Mueller–Hinton medium and 7H11 agar

medium supplemented with 10% OADC showed that the conditions used for mycobacteria did not affect the activities of the fluoroquinolones tested. In addition, regardless of the medium used, no difference in the MICs was observed after 24 h or 14 days of incubation. For this *E. coli* strain, the MICs were 0.015 mg/L for ofloxacin, 0.003 mg/L for ciprofloxacin and ⩽0.006 mg/L for grepafloxacin.

In-vitro activity

Table I presents MIC_{50}, MIC_{90} and the MIC range of the tested fluoroquinolones for 130 mycobacterial isolates.

According to these results, the susceptibilities of the different mycobacteria to fluoroquinolones varied widely. If the fluoroquinolone breakpoints given for non-fastidious organisms can be applied to mycobacteria, these drugs seemed, for the most part, not to be very active. However, in order of decreasing effectiveness, they appeared relatively active against *M. fortuitum*, *M. tuberculosis*, *M. marinum* and *M. kansasii* (Table I). *Mycobacterium avium* complex and *M. chelonae* can be considered resistant, while *M. scrofulaceum* had intermediate susceptibility.

Table I. In-vitro activities of ofloxacin, ciprofloxacin and grepafloxacin against different species of mycobacteria determined using the agar-dilution method

Bacterial species (fluoroquinolone)	MIC (mg/L)		
	MIC_{50}	MIC_{90}	range
M. tuberculosis (n = 33)			
ofloxacin	0.5	1	<0.12–2
ciprofloxacin	0.5	1	0.12–2
grepafloxacin	0.5	1	0.12–2
M. kansasii (n = 17)			
ofloxacin	1	2	0.25–4
ciprofloxacin	2	4	0.25–4
grepafloxacin	1	2	0.25–4
M. avium complex (n = 41)			
ofloxacin	8	32	4–32
ciprofloxacin	8	16	2–32
grepafloxacin	4	16	2–32
M. scrofulaceum (n = 7)			
ofloxacin	4	4	4–4
ciprofloxacin	2	16	2–16
grepafloxacin	2	4	2–4
M. marinum (n = 10)			
ofloxacin	2	4	2–4
ciprofloxacin	0.5	1	0.5–1
grepafloxacin	0.5	1	0.5–2
M. chelonae (n = 12)			
ofloxacin	16	32	4–32
ciprofloxacin	4	16	2–16
grepafloxacin	8	32	2–32
M. fortuitum (n = 10)			
ofloxacin	0.25	0.5	<0.12–1
ciprofloxacin	<0.12	0.25	<0.12–0.25
grepafloxacin	<0.12	0.25	<0.12–0.5

Macrophage model of infection

The efficacies of the different antibiotics, as assessed by cfu counts, are shown in the Figure. At a concentration of 16 mg/L, all three fluoroquinolones effectively slowed the intracellular replication of all three *M. avium* complex strains. However, against *M. kansasii*, bactericidal activity was observed at 0.5 mg/L for grepafloxacin, 2 mg/L for ofloxacin and 4 mg/L for ciprofloxacin. Only grepafloxacin was bactericidal against *M. marinum (*2 mg/L), while the other drugs were merely bacteriostatic at this concentration. For *M. avium* complex and *M. marinum*, grepafloxacin's activity increased at the higher concentrations, unlike those of the other fluoroquinolones. These differences in activity were not seen at lower concentrations. For *M. tuberculosis*, no surviving intracellular microorganisms were seen with the three drugs at 0.25 mg/L, while only ciprofloxacin was as effective at 0.12 mg/L.

Comparison of MICs and antibiotic activities in the macrophage infection model

When the activities of the three fluoroquinolones assessed in human macrophages were compared with those determined using the agar-dilution method (MICs in Table II), ofloxacin seemed to be less effective than grepafloxacin and ciprofloxacin for *M. avium* complex and *M. marinum*. For *M. kansasii* and *M. tuberculosis*, the activities of the three fluoroquinolones were comparable in both systems.

Discussion

The in-vitro activities of fluoroquinolones, especially ofloxacin, ciprofloxacin[6–10] and sparfloxacin,[11] against mycobacteria have been studied previously. However, little is known about the antimycobacterial activity of grepafloxacin.[12]

In agreement with the majority of authors, we found that the different mycobacterial species exhibited different degrees of susceptibility to ofloxacin, ciprofloxacin[6–10] and grepafloxacin.[12] Against rapidly growing mycobacteria, such as *M. fortuitum*, the activities of these antibiotics were excellent, while *M. chelonae* was resistant. Against slowly growing mycobacteria, ofloxacin, ciprofloxacin and grepafloxacin activities varied. While they exhibited good activities against *M. kansasii* and *M. marinum*, they were less effective against *M. avium* complex. Finally *M. tuberculosis* was susceptible to ofloxacin, ciprofloxacin and grepafloxacin.

When comparing the activities of different fluoro-

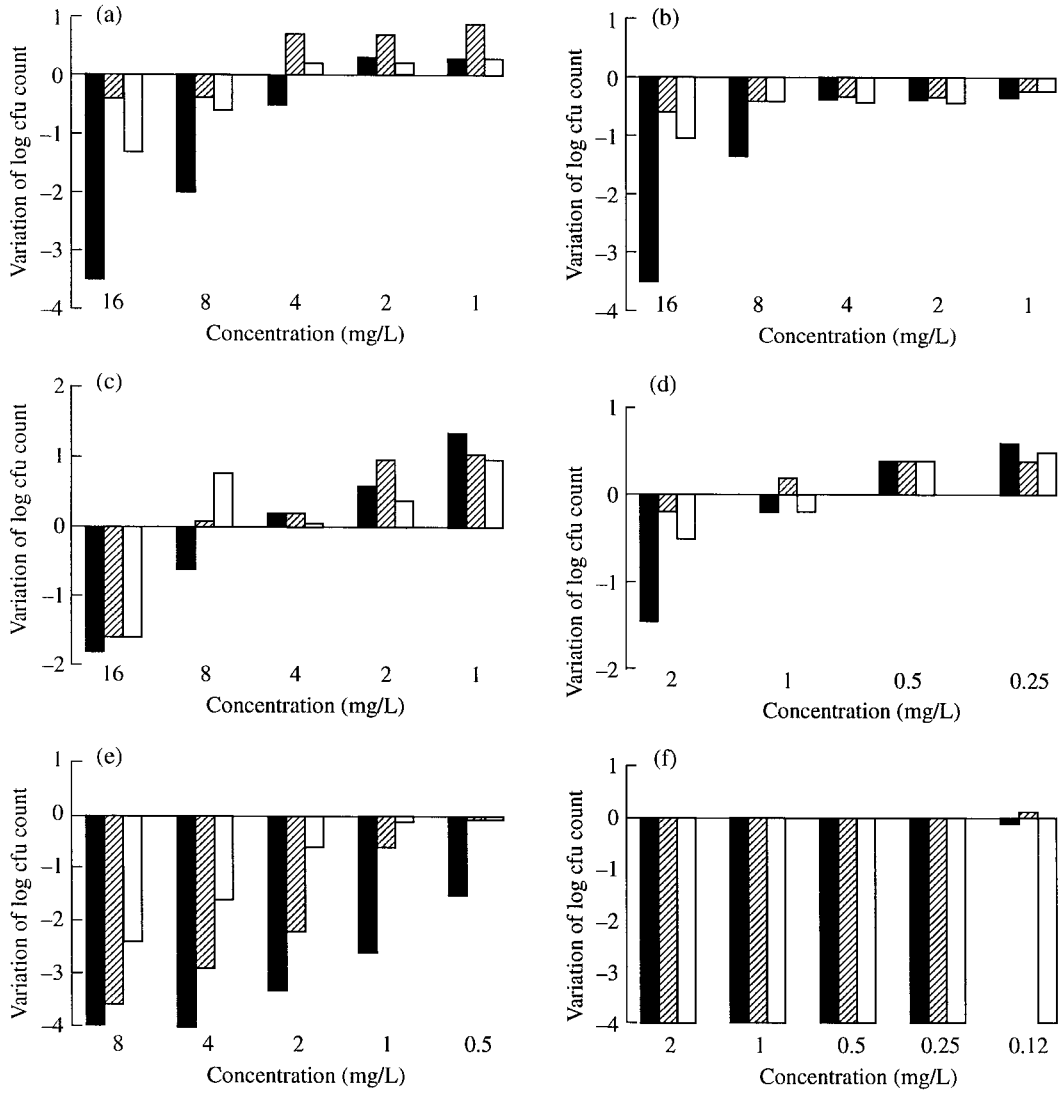

Figure. Activities of grepafloxacin (■), ofloxacin(▨) and ciprofloxacin (□) against six strains of mycobacteria in cultured human macrophages. The activities on day 7 against each strain are expressed as the variations of the $\log_{10}$ cfu counts observed on day 7 compared with day 0.

Table II. The ofloxacin, ciprofloxacin and grepafloxacin MICs determined by the agar-dilution method, against the six strains tested in human macrophages

	MIC (mg/L)		
Mycobacterial strain	ofloxacin	ciprofloxacin	grepafloxacin
M. avium complex 1	32	16	8
M. avium complex 2	16	8	8
M. avium complex 3	8	8	4
M. marinum 4	2	1	0.5
M. kansasii 5	2	2	2
M. tuberculosis 6	0.5	0.5	0.5

quinolones against different strains of the same species of mycobacteria, Saito *et al.*[12] noted that grepafloxacin had in-vitro antimicrobial activities against *M. tuberculosis* and *M. avium* complex similar to those of ofloxacin and ciprofloxacin. But they found grepafloxacin to be considerably less effective against other atypical mycobacteria species, such as *M. kansasii*, *M. scrofulaceum*, *M. marinum*, *M. chelonae* and *M. fortuitum*, than ofloxacin.[12] Our results are in partial agreement with their observations. We also noted the similar activities of grepafloxacin, ofloxacin and ciprofloxacin against *M. tuberculosis* (highly susceptible) and *M. avium* complex (resistant). In contrast, we observed little difference between the MICs of these molecules against *M. kansasii* (good activity), *M. marinum* (good; ofloxacin poorer activity), *M. scrofulaceum* (intermediate; ciprofloxacin less active), *M. chelonae* (resistant) and *M. fortuitum* (highly susceptible).

In an attempt to overcome the shortcomings of in-vitro studies, the macrophage model, which more closely approximates to an infectious process, was used to test the activities of the three antibiotics. Indeed, it takes into consideration the pathophysiology of mycobacterial infections and the penetration of antibiotics into macrophages. Furthermore, it has been reported previously that fluoroquinolones tend to concentrate in macrophages.[13]

In our study, when comparing the efficacies of the different fluoroquinolones tested against *M. avium* complex, grepafloxacin was more effective than ciprofloxacin, which was more effective than ofloxacin. These results concur with those of Saito *et al.*[12] who observed a better activity of grepafloxacin than ofloxacin against *M. intracellulare* phagocytosed in macrophages; however, the three fluoroquinolones were merely bacteriostatic. In the macrophage model, grepafloxacin exhibited the best bactericidal activities against all three *M. avium* complex strains at the highest doses (8–16 mg/L).

We found all three fluoroquinolones to be bactericidal against *M. tuberculosis* at a concentration of 0.25 mg/L, whereas Saito *et al.*[12] reported a better bactericidal efficacy of grepafloxacin than ofloxacin. The similar high susceptibility of *M. kansasii* and *M. tuberculosis* to fluoroquinolones has been observed previously *in vivo*.[1] Indeed, *M. kansasii* is one of the rare atypical mycobacterial species to respond to a classical antituberculous treatment. Moreover, ofloxacin and sparfloxacin are used to treat MDR strains of *M. tuberculosis* with good clinical results.[14]

Generally, the potential activity of an antibiotic is predicted by comparing the MIC and the serum concentration of the drug. Since mycobacteria grow intracellularly, assessment of bactericidal activity is not sufficient and the intramacrophage drug concentrations should be evaluated pharmacokinetically in our macrophage model, for example.

The peak serum concentrations (C_{max}s) of grepafloxacin, ofloxacin and ciprofloxacin were reported to be 1.8 mg/L, 5.6 mg/L and 2.3 mg/L, respectively, when given orally to humans at a dosage of 400 mg for grepafloxacin and ofloxacin, and a dosage of 500 mg for ciprofloxacin.[12] The MIC_{90}s of grepafloxacin, ofloxacin and ciprofloxacin against *M. tuberculosis* are inferior to their C_{max}s, suggesting the potential therapeutic efficacy of these drugs in the treatment of tuberculous infections. This possibility is supported by the observation that these drugs, when added at concentrations lower than their C_{max}s, were bactericidal against *M. tuberculosis* phagocytosed by macrophages. For *M. kansasii*, the MIC_{90} of ofloxacin was lower than its C_{max} and that of grepafloxacin approximately equal to its C_{max}. In light of our data obtained with the macrophage model, which showed bactericidal activities of grepafloxacin and ofloxacin at concentrations lower than their C_{max}, therapeutic efficacy of these two molecules would be expected against infections caused by *M. kansasii*. For *M. marinum*, despite the MIC_{90}s of the three fluoroquinolones being below their C_{max}s, we did not observe bactericidal activity in the macrophage model. However, it is difficult to extrapolate our results to an eventual therapeutic use because the concentrations tested in the macrophage model did not reach the C_{max}.

The antibiotic concentrations necessary to obtain a killing effect against *M. avium* complex are considerably higher than the C_{max}, suggesting a weak therapeutic efficacy of fluoroquinolones *in vivo* against *M. avium* complex infections. However, fluoroquinolones might potentiate other drugs given in combination.[1,15]

In conclusion, this study revealed that grepafloxacin has relatively potent in-vitro activity against *M. tuberculosis* and some species of atypical mycobacteria, as compared with other quinolones. In the macrophage model, grepafloxacin activity against *M. tuberculosis* was similar to those of ofloxacin and ciprofloxacin, was more potent than ofloxacin and ciprofloxacin against *M. kansasii* and, to a lesser degree, against *M. marinum* and *M. avium* complex. Thus grepafloxacin seems to merit further investigation in animals to evaluate its real therapeutic efficacy in the treatment of mycobacterial infections.

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