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Comparative in vitro and in vivo antimicrobial activities of sitafloxacin, gatifloxacin and moxifloxacin against *Mycobacterium avium*

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ABSTRACT

Moxifloxacin exhibits therapeutic activity against *Mycobacterium avium* infection in mice. Since not only moxifloxacin but also another 8-methoxy quinolone, gatifloxacin, and a C-8-chloro quinolone, sitafloxacin, show favourable antimycobacterial activity in vitro, their anti-*M. avium* activities were compared in vivo. Minimum inhibitory concentrations (MICs), minimum bactericidal concentrations (MBCs) and mutant prevention concentrations (MPCs) of the test quinolones for *M. avium* were determined by microdilution in 7HSF broth. Antimicrobial activity against intracellular bacteria was measured using Mono Mac 6 human macrophages. Therapeutic efficacy of the quinolones when administered subcutaneously with or without clarithromycin plus ethambutol was assessed using mice intravenously infected with *M. avium* in terms of changes in bacterial loads in the lungs and spleen following infection. Based on the MICs, MBCs and MPCs, the in vitro activities of sitafloxacin and moxifloxacin were greater than that of gatifloxacin. Moxifloxacin exhibited the strongest activity against intramacrophage *M. avium*. When each test quinolone was administered alone to infected mice, sitafloxacin and gatifloxacin exhibited greater therapeutic efficacy than moxifloxacin based on intrapulmonary bacterial elimination. However, moxifloxacin exerted greater activity in killing bacteria in the spleen. Moxifloxacin and sitafloxacin exhibited combined effects on intrapulmonary bacterial elimination when administered to mice in combination with clarithromycin plus ethambutol. Sitafloxacin exerted the most marked combined effects in bacterial killing in the spleen. Levofloxacin displayed the lowest in vitro and in vivo activities amongst the tested quinolones. Taken together, these findings indicate that sitafloxacin and moxifloxacin exhibit favourable activities against *M. avium* in vitro and in vivo.

1. Introduction

Clinical management of patients with *Mycobacterium avium* infections is difficult, even with macrolides such as clarithromycin and azithromycin as first-line drugs in multidrug regimens [1,2]. In this context, some regimens including fluoroquinolones have been reported to be efficacious in treating *M. avium* bacteraemia in acquired immune deficiency syndrome (AIDS) patients [1,3]. However, at present the general consensus is that fluoroquinolones are only weakly efficacious against *M. avium* infections, although they are effective as second-line therapeutics against tuberculosis (TB) [1,4]. Bermudez et al. [5–7] reported that moxifloxacin was effective in treating *M. avium*-infected mice and exhibited combined effects in some multidrug regimens, such as moxifloxacin plus ethambutol and moxifloxacin plus mefloquine. It has been reported that another 8-methoxy quinolone, gatifloxacin, as well as sitafloxacin with a chloro substituent at the C-8 position exhibited good antimicrobial activity against extracellular and intramacrophage *M. avium* [8–10]. Therefore, it is of interest to assess both the in vitro and in vivo anti-*M. avium* activities of these quinolones. In the present study, the comparative antimicrobial activities of sitafloxacin, gatifloxacin and moxifloxacin against *M. avium* residing inside macrophages as well as the therapeutic effects of these quinolones on *M. avium* infections induced in mice were examined.

2. Materials and methods

2.1. Antimicrobial drugs

The following drugs were used: sitafloxacin (Daiichi Sankyo Co., Tokyo, Japan); gatifloxacin (Kyorin Pharmaceutical Co. Ltd.,

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Tokyo, Japan); moxifloxacin (Bayer Schering Pharma Co., Berlin, Germany); levofloxacin (Daiichi Sankyo Co.); clarithromycin (Taisho-Toyama Pharmaceutical Co., Tokyo, Japan); and ethambutol (Sigma Chemical Co., St. Louis, MO). Levofloxacin, a fluoroquinolone without a substituent at the C-8 position, which is generally used as a second-line drug against *Mycobacterium tuberculosis* infections in Japan, was used as a control quinolone. These quinolones were initially dissolved in 0.1 N NaOH at a concentration of 2.0 g/L followed by neutralisation to pH 7.2 and were diluted with the prescribed culture media to appropriate concentrations before in vitro use. Using this procedure, all the quinolones were completely dissolved in individual media for minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and mutant prevention concentration (MPC) determination and macrophage cultivation. For in vivo use, all the test drugs except for sitafloxacin and gatifloxacin were dissolved in saline at prescribed concentrations for subcutaneous injection to mice. For sitafloxacin and gatifloxacin, the test agent was finely ground using a mortar and pestle and mixed with a small amount of saline, yielding a fine suspension at 20 g/L. These fine drug suspensions were then mixed again by vigorous vibration using a Vortex mixer immediately before injection. In a separate experiment, the amount of these quinolones in a 0.1 mL suspension was measured, which was practically injected into mice using a hypodermic syringe. When the drug concentration was measured by the usual paper disk method using *Escherichia coli* as an indicator bacterium [11], the drug amounts were estimated as follows: sitafloxacin, 2.02 ± 0.09 mg ($n = 6$); and gatifloxacin, 1.82 ± 0.05 mg ($n = 6$). These values were essentially the same as the amount (2.0 mg) of levofloxacin and moxifloxacin, which were completely dissolved in saline at a concentration of 20 g/L and were each injected into mice in a 0.1 mL volume.

2.2. Microorganisms

Mycobacterium avium N-444 strain (serovar 8) was used throughout the experiments. This strain was identified by a DNA probe test. Organisms were grown in 7H9 medium.

2.3. Antimicrobial activity against extracellular Mycobacterium avium

MICs of the test drugs were determined by the broth dilution method using 7HSF medium (a broth medium with the same composition as 7H11 agar without malachite green). The bacterial suspension in 7HSF medium (0.1 mL) containing 1×10^5 colony-forming units (CFU) was added to 7HSF medium (0.1 mL) containing test drugs in a microculture well. Following cultivation at 37 °C for 14 days, MICs were read as the minimum concentration of drug completely inhibiting visible growth of the organism. Following MIC determination, MBCs were determined by inoculating 10 μL samples from the wells in which test agents allowed no visible growth of the organism onto a 7H11 agar plate, followed by 14 days cultivation at 37 °C. MBCs were read as the minimum concentration of drug causing >99.9% killing of the inoculated organism. MPCs of test drugs were determined as follows. A bacterial suspension (0.1 mL) of *M. avium* (2×10^9 CFU) was spread onto five 7H11 agar plates containing test drugs and was cultured at 37 °C for 4 weeks. MPCs were read as the minimum concentration of drug that allowed the recovery of no colonies.

2.4. Antimicrobial activity of test drugs against intramacrophage Mycobacterium avium

The following methods were used [12,13]. First, a monolayer culture of Zymosan A-induced mouse peritoneal macrophages (3×10^5 cells) prepared on a microculture well was infected with *M. avium* (3×10^6 CFU) in 0.1 mL of 5% (v/v) foetal bovine serum (FBS)–RPMI 1640 medium at 37 °C for 2 h. The infected macrophage monolayer was washed with 2% FBS–Hanks' balanced salt solution (HBSS) and was cultured in 0.2 mL of 5% FBS–RPMI 1640 medium in microculture wells in the presence or absence of test antimicrobials when added at the maximum drug concentration in serum (C_{max}) (see Section 2.5) for up to 7 days. Alternatively, 4×10^6 cells of Mono Mac 6 (MM6) human macrophages (a monocytic cell line with characteristics of matured macrophages obtained from the German Collection of Microorganisms and Cell Cultures; this clone expresses NaF-sensitive non-specific esterase, produces reactive oxygen, exhibits phagocytic ability and stains with a panel of monoclonal antibodies that are specific for mature monocytes) were infected with *M. avium* (1.2×10^8 CFU) in 5 mL of 5% FBS–RPMI 1640 medium at 37 °C for 3 h and was then washed with 2% FBS–HBSS by centrifugation. Infected MM6 macrophages were cultured in 0.2 mL of 1% FBS–RPMI 1640 medium in the presence or absence of test drugs for 7 days when added at the C_{max} of each drug when administered to humans (healthy volunteers) at a clinical dosage of 2–10 mg/kg as follows: sitafloxacin, 2.0 mg/L; gatifloxacin, 4.3 mg/L; moxifloxacin, 3.4 mg/L; and levofloxacin, 4.4 mg/L [14,15]. At intervals, cells were lysed with 0.07% (w/v) sodium dodecyl sulphate (SDS) followed by subsequent neutralisation with 6% (w/v) bovine serum albumin and were washed with distilled water by centrifugation. The number of CFU of recovered organisms was counted on Middlebrook 7H11 agar plates. In separate experiments using MM6 macrophages, the numbers of intracellular and extracellular *M. avium* on Day 0 and Day 7 of cultivation of infected macrophages were estimated as follows (log CFU/culture well): intracellular CFU on Day 0, 3.72 ± 0.05; extracellular CFU on Day 0, <1.30; intracellular CFU on Day 7, 5.52 ± 0.03; extracellular CFU on Day 7, 3.26 ± 0.03. Thus, in the experimental system used in the present study, growth of extracellular *M. avium* was more than 100-fold slower and the increase was much smaller than that of intramacrophage *M. avium*. Therefore, the influence on the growth of extracellular *M. avium* was negligible. In addition, another experiment showed that MM6 macrophages with or without *M. avium* infection essentially retained their viability during cultivation for 7 days. On Day 7, the ratios of viable cells measured by nigrosin dye exclusion test of uninfected and infected macrophages were $86.1 \pm 1.1\%$ and $85.9 \pm 0.6\%$, respectively. Moreover, the test quinolones did not cause any cytotoxicity to MM6 macrophages. The viability of macrophages treated with these quinolones at 100 mg/L for 24 h ranged from 98.0% to 98.5%. These values are in the range of 98.5% estimated for the untreated control macrophages.

2.5. Determination of maximum drug concentration in serum (C_{max})

Serum samples were obtained at intervals from mice injected subcutaneously with the test drugs at 100 mg/kg. Drug concentrations were determined from the diameter of zones of growth inhibition around disks saturated with sample solutions, which were placed onto Mueller–Hinton agar plates inoculated with *Bacillus subtilis* as an indicator bacterium [11].

2.6. Experimental infection

Six-week-old C3H/HeN mice infected intravenously with 1×10^7 or 2×10^7 CFU of *M. avium* N-444 were subcutaneously given or not given test quinolones (100 mg/kg) with or without combined administration of clarithromycin (20 mg/kg) plus ethambutol (15 mg/kg) via the subcutaneous route, once daily, six times per week, for 8 weeks from Week 1 to Week 9 post infection. At