ANTIMICROBIAL AGENTS

A comparison of the bactericidal activity of quinolone antibiotics in a *Mycobacterium fortuitum* model

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New agents are urgently needed to meet the threat of multiple drug-resistant tuberculosis and to manage infection with the naturally resistant non-tuberculosis mycobacteria. Earlier fluoroquinolones have been shown to have promising in-vitro activity, although mouse infection and clinical studies suggested that they lack sufficient bactericidal activity. Methods were evaluated to measure the bactericidal activity of fluoroquinolones and to compare the new agent moxifloxacin with other fluoroquinolones with *M. fortuitum* as a model system. The optimum bactericidal concentrations (OBC) for the fluoroquinolones were: moxifloxacin, 0.5 mg/L; ciprofloxacin and sparfloxacin, 2 mg/L and ofloxacin, 8 mg/L. The bactericidal indices (BI) for moxifloxacin, ciprofloxacin, sparfloxacin and ofloxacin were 1.8, 0.5, 0.2 and 0.2, respectively. Similar ranking was obtained when the time taken to produce one log₁₀ reduction in viable count was calculated. These data indicate that moxifloxacin was the most bactericidal of the fluoroquinolones tested. Such methods provide a simple in-vitro measure that correlates with in-vivo models.

Introduction

Mycobacterial infection is a significant threat to human health and the total number of cases of tuberculosis is rising throughout the world [1]. Multiple drug resistance is also becoming more common and recent outbreaks of multiple drug-resistant tuberculosis among HIV seropositive individuals have been associated with a high mortality and rapid transmission [2]. The incidence of non-tuberculosis mycobacteria is also rising because of an increase in the number of immunocompromised patients, including HIV seropositive individuals, together with improved diagnostic facilities and better understanding of the pathogenesis of these organisms [3].

The fluoroquinolones are a group of potent bactericidal antibacterial agents that target DNA gyrase [4]. Earlier compounds were mainly active against gram-negative pathogens, but the fluoroquinolones, such as ciprofloxacin, had an increased potency against gram-negative pathogens and a wider spectrum that included staphy-

lococci and mycobacteria. Fluoroquinolones have been shown to have anti-mycobacterial activity *in vitro* [5, 6] and mouse models of infection [6, 7] show that ciprofloxacin has early bactericidal activity, rapidly reducing the sputum viable count [8]. Clinical trials support the idea that they may have a role to play in the treatment of these infections [9, 10].

Developments in the quinolone family have produced agents with enhanced activity against gram-positive organisms and mycobacteria. Studies of substitutions at the C8 position of the quinolone ring suggested that a methoxy group enhanced the anti-mycobacterial activity of quinolones when the position had a cyclopropyl substitution at the N1 position [11, 12]. Moxifloxacin is an 8-methoxyquinolone that is being introduced into clinical practice especially for the treatment of respiratory tract infections. It has enhanced activity against gram-positive pathogens and anaerobes while retaining useful activity against gram-negative organisms [13]. It has been shown to inhibit the growth of the main species of mycobacteria that infect man [14]. Studies of the activity of moxifloxacin in mouse models also suggest that it has important bactericidal activity [7]. Bactericidal activity of antibiotics has been shown to be important early in the course of treatment by ensuring a rapid reduction in the infective load and

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later in killing semi-dormant organisms to sterilise the lesions [9, 15]. In a controlled trial of a regimen containing ciprofloxacin against standard therapy, patients who were HIV seropositive took longer to achieve sterilisation and had a higher relapse rate [8]. This study suggested that ciprofloxacin lacked sterilising activity in comparison with the standard regimen, and that if quinolone antibiotics are to have a role in anti-tuberculosis therapy, agents with enhanced bactericidal activity must be found. In non-tuberculosis mycobacterial infections, bactericidal activity is essential as these infections often arise in patients with reduced cell-mediated immunity, where little help in eradicating the organism can be expected from the immune system [3].

Most studies of anti-mycobacterial activity of new drugs are reported in the form of the minimal inhibitory concentration (MIC). A more relevant measure of potential usefulness would be in-vitro measures of the bactericidal activity [8, 9, 15]. To develop such a method conventional measures of bactericidal action were adapted to mycobacteria in a *Mycobacterium fortuitum* model system. The minimum bactericidal concentration (MBC) and the optimum bactericidal concentration (OBC; the most lethal fluoroquinolone concentration over a range of concentrations) were measured. The bactericidal index (BI) was also calculated to relate bactericidal activity with antibiotic concentrations achievable in serum. With this, the bactericidal activity of the new 8-methoxyquinolone was compared with that of older agents and the utility of different measures of bactericidal activity in mycobacteria was evaluated.

Materials and methods

Drugs and mycobacterial test strain

The antibacterial agents were obtained from the respective manufacturers. Moxifloxacin (Bayer AG, Wuppertal, Germany) and sparfloxacin (Rhône-Poulenc Santé, France) were dissolved in 0.1 M NaOH then diluted in sterile distilled water. Ciprofloxacin (Bayer AG) and ofloxacin (Aventis, Romainville, France) were dissolved in sterile distilled water. The test strain for all experiments was *M. fortuitum* NCTC 10394.

Macrodilution sensitivity testing

The macrodilution method described previously [16] was used to determine the MIC, which was defined as the lowest concentration at which there was no visible growth. The MBC was defined as the lowest concentration that produced a 99.9% reduction in viable count. The method was modified by increasing the recommended incubation period to 48 h and the use of Mueller Hinton Broth (MHB; Mast Diagnostics, Bootle) with Tween 80 (BDH, Poole, Dorset) 0.2%

and albumin dextrose catalase complex (ADC; Becton Dickinson, Oxford) 10%.

Determination of the OBC

Four volumes of 45 ml of MHB with ADC 10% and Tween 80 0.2% were inoculated and incubated for 48 h at 37°C, to give an initial inoculum of 10^7 – 10^8 cfu/ml. The culture was centrifuged at 12 000 *g* for 1 min and the supernate was discarded. Pellets were resuspended in 10 ml of MHB containing ADC and Tween. These were transferred into a 250-ml conical flask and the volume was made up to 180 ml; 9.9-ml volumes of the inoculum were pipetted into 50-ml conical flasks to give two control flasks and 16 test flasks. To the 16 test flasks, 0.1 ml of the various drug concentrations was added. The concentrations tested were: MIC $\times 0.25$, $\times 0.5$, $\times 1$, $\times 2$, $\times 4$, $\times 8$, $\times 16$, $\times 32$. Sterile distilled water (0.1 ml) was added to each of the control flasks. A 0.1-ml sample from the control flasks was serially diluted to 1 in 10^5 to determine the initial viable count ($t = 0$). The flasks were incubated on a rotary shaker (100 rpm) at 37°C for 6 h, when a second viable count was taken.

To reduce the risk of carry over of antibiotic, the broth cultures were centrifuged and the supernate was discarded before serial dilutions were used to perform viable counts by the Miles and Misra method [17] with duplicate spots of 25 μ l for dilutions of 1 in 10^3 to 1 in 10^5 . The plates were then incubated for 48 h at 37°C. The results are expressed as percentage survivors.

BI

The data obtained from viable count studies were re-evaluated by calculating the BI as described previously [18]. Briefly, the $\log_{10}$ reduction in cfu was plotted against the $\log_{10}$ concentration of the fluoroquinolone being used. The area under the curve (AUC) for the bactericidal section of each plot was determined and this was defined as the BI for each drug. The highest concentration used for each calculation was the peak serum concentration ($C_{\max}$) as shown in Table 1 [19, 20].

Death rates

The data were also re-calculated to give figures on time taken to achieve one $\log_{10}$ death of *M. fortuitum* against each fluoroquinolone, for each concentration

Table 1. Maximum serum concentration achieved after a single dose for various fluoroquinolones

Drug	Single oral dose (mg)	$C_{\max}$ (mg/L)
Moxifloxacin	800	4.73
Ciprofloxacin	750	3.4
Sparfloxacin	400	2
Ofloxacin	400	7.17

used. This was calculated by a method published previously [21]. Briefly, the formula used to calculate the death rate was $D = t / \log_{10} N_0 - \log_{10} N_t$; where D = the death rate, t = the relevant time, N_0 is the initial viable count at $t = 0$ and N_t is the viable count at t .

Results

Macrodilution MIC and MBC

The susceptibility of *M. fortuitum* to the fluoroquinolones as defined by MIC and MBC is shown in Table 2. The modal values were determined from six experiments.

OBC

The results expressed graphically in Fig. 1 are averages of six experiments for each fluoroquinolone after incubation for 6 h. The OBCs were calculated from the plots as follows: moxifloxacin 0.5 mg/L, ciprofloxacin and sparflaxacin 2 mg/L and ofloxacin 8 mg/L. Results from 16-h investigations (data not shown) gave no clearly defined OBC as all concentrations exhibited a >99% drop in cfu/ml.

Table 2. Comparison of the MIC and MBC of fluoroquinolones against *M. fortuitum*

Drug	Modal MIC (mg/L)	Modal MBC (mg/L)	MIC/MBC ratio
Moxifloxacin	0.12	0.25	2
Ciprofloxacin	0.25	0.5	2
Sparfloxacin	0.25	0.5	2
Ofloxacin	2	4	2

Fig. 1. The OBC plots for moxifloxacin (○), sparflaxacin (●), ciprofloxacin (□) and ofloxacin (■) over 6 h. Data points represent the mean of six observations.

BI

Fig. 2 shows plots of the $\log_{10}$ reduction in viability against the $\log_{10}$ of the drug concentration after 6 h. The $\log_{10}$ reduction values for C_{max} were taken from Table 1. This was the cut-off point for AUC calculations as shown in Fig. 2. The BIs were calculated to be 1.8 for moxifloxacin, 0.5 for ciprofloxacin, 0.2 for sparflaxacin and 0.2 for ofloxacin.

Death rates

The results are presented in Table 3 and these correspond to the time the concentrations would take to produce one $\log_{10}$ death at differing concentrations.

Discussion

The results clearly show that the fluoroquinolones used in this investigation were all bactericidal with MIC/MBC ratios of 2. MIC and MBC results for three of the fluoroquinolones were similar to those published previously [22]. With this method of evaluation, the activity of moxifloxacin is similar to the older compounds.

The biphasic nature of quinolone action described in other organisms was less marked in these experiments with the exception of ciprofloxacin, which gave the most clearly defined OBC. It has been proposed that the biphasic nature of fluoroquinolone bactericidal profiles is a consequence of inhibition of RNA synthesis at high fluoroquinolone concentration [23, 24]. This is thought to occur because fluoroquinolones require RNA synthesis for full bactericidal activity; therefore, this biphasic phenomenon is due to self-antagonism. Fluoroquinolones vary in their reliance on RNA synthesis for killing. Older quinolones such as nalidixic acid are devoid of bactericidal activity if RNA synthesis is inhibited, because they can kill only by the mechanism classified as type A [24]. On the other hand, newer quinolones can still exert bactericidal activity under RNA synthesis inhibition if they possess the type B and B₁ bactericidal mechanism [24]. However, the action of ciprofloxacin against *M. smegmatis* and *M. bovis* BCG could be partially blocked by pre-treatment with chloramphenicol, a potent inhibitor of protein synthesis [25]. It was also blocked by very high concentrations of ciprofloxacin, as in the present study. Recently, it has been shown that with increasing concentrations of ciprofloxacin, there is a shift from a chloramphenicol-inhibited to chloramphenicol-insensitive mode of killing [26] and it is thought unlikely that inhibition of RNA synthesis would affect a process independently of protein synthesis. The data presented in Fig. 1 shows a weak biphasic pattern, suggesting that moxifloxacin, sparflaxacin and ofloxacin exhibit type B killing against *M. fortuitum*. Ciprofloxacin may exert its bactericidal

Fig. 2. Plots of the log₁₀ reduction in cfu against the log₁₀ of drug concentration for moxifloxacin (a), ciprofloxacin (b), sparfloxacin (c) and ofloxacin (d). The data points represent the mean of six observations. AUCs were determined for the region of each plot dissected by the vertical and horizontal dotted lines, which are the log₁₀C_{max} and bacteriostatic point, respectively.

Table 3. Comparison of the rate of killing (death rate) for four fluoroquinolones against *M. fortuitum*

Antibiotic concentration (mg/L)	Time (h) to produce one log ₁₀ reduction in viable count with			
	moxifloxacin	ciprofloxacin	sparfloxacin	ofloxacin
0.12	200	ND	ND	ND
0.25	2.9	300	ND	ND
0.5	3.9	5.7	46.2	ND
1.0	4.1	5.3	11.5	ND
2.0	4.4	7.9	7.9	66.7
4.0	5.6	12	9.4	15.4
8.0	ND	7.5	8.7	9.5
16.0	ND	ND	ND	10.9
32.0	ND	ND	ND	13.4
64.0	ND	ND	ND	13.6

ND, not determined.

activity by a type A mechanism because the biphasic nature of the response is more pronounced. Furthermore, as the OBCs became less pronounced at 16 h compared with 6 h, this suggests that mechanisms B or B1 may act more slowly than mechanism A against *M. fortuitum*. Further studies are required to evaluate this more fully.

It has been suggested previously that the potency of the fluoroquinolones can be compared by reference to the OBC value measured after 6 h [23]. This method was adapted for use in mycobacteria. The data indicate that moxifloxacin has the lowest OBC value (0.5 mg/L), suggesting that it is more bactericidal than the comparitors sparflaxacin (2 mg/L), ciprofloxacin (2 mg/L) and ofloxacin (8 mg/L). After 16 h, >99% of the bacteria were killed by all of the fluoroquinolones with no discernable difference in the OBC. This difference may be important when the plasma half-lives of the drugs are taken into consideration. If the fluoroquinolone possesses a short half-life, the OBC is an important factor because the optimum kill rate would be desirable, if time were limited. However, if the fluoroquinolone possessed a long half-life the OBC would be less relevant. The drug would persist for longer, thereby allowing more time for bactericidal killing to occur. This could have a direct relevance to once-daily dosing in tuberculosis regimens, thereby allowing for a more manageable drug course to be administered, which would aid patient compliance.

The results show that moxifloxacin was approximately twice as bactericidal as ciprofloxacin and almost 10 times more bactericidal than both sparflaxacin and ofloxacin as defined by BI. Ciprofloxacin and sparflaxacin were more active than ofloxacin and ciprofloxacin was more bactericidal than sparflaxacin. The low AUC value and correspondingly low bactericidal area displayed by sparflaxacin (0.3) may be due to the fact that this fluoroquinolone is widely distributed into the tissues of the patient, rather than remaining in the serum [27]. The analysis of killing rates concurred with the BI data indicating that moxifloxacin is more bactericidal than the other fluoroquinolones tested.

This study adapted previously described methods of determining OBC, death rates and BI to use with mycobacteria. The results suggest that this approach is valuable and correlates well with in-vivo studies that show that moxifloxacin is more bactericidal than sparflaxacin [7]. If a conventional MIC/MBC approach had been used, the superior bactericidal activity of moxifloxacin over sparflaxacin would not have been demonstrated.

In conclusion, with the incidence of drug-sensitive and drug-resistant tuberculosis steadily increasing on a global scale, moxifloxacin shows a great deal of promise in the future management of mycobacterial infections. New quinolones with potential activity

against mycobacteria should be tested *in vitro* by the methods described in this paper, as this will provide a more accurate measure of bactericidal activity. Further study of the bactericidal activity of fluoroquinolones are required in *M. tuberculosis* and other slow-growing species and this will allow the most promising new agents to be selected for clinical studies.

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