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Effect of subinhibitory concentrations of ciprofloxacin on *Mycobacterium fortuitum* mutation rates

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***Objectives*:** Fluoroquinolones have found a place in the management of mycobacterial diseases including tuberculosis. It has been previously shown that subinhibitory concentrations of quinolones increase the mutation rate in *Escherichia coli* and staphylococci. The purpose of this study is to extend this observation to mycobacteria and to quantify mutation rates.

***Methods*:** The mutation rate in *Mycobacterium fortuitum* to ciprofloxacin, levofloxacin, moxifloxacin, rifampicin, erythromycin and gentamicin resistance was determined when grown with and without various sub-MIC concentrations of ciprofloxacin.

***Results*:** *M. fortuitum* exposed to $\frac{1}{2}$ MIC ciprofloxacin had an increase in the mutation rate of between 72- and 120-fold when selected on quinolones or other antimycobacterial antibiotics. Smaller, but significant increases in mutation rate were seen when the organism was exposed to lower concentrations ($\frac{1}{4}$ MIC and $\frac{1}{8}$ MIC).

***Conclusions*:** These data show that sub-MIC concentrations of fluoroquinolone significantly increase mutation rates and these data suggest that care must be taken to ensure that bacteria are not exposed to subinhibitory concentrations when adding quinolones to a regimen used to treat mycobacterial infection.

Keywords: fluoroquinolones, mycobacteria, quinolones, *M. fortuitum*

Introduction

The increasing recognition of antibiotic resistance enhances the threat posed by tuberculosis to public health throughout the world.[1] Antibiotic resistance among mycobacteria arises through mutation in chromosomal genes at a low rate.[2–4]

A number of quinolone antibiotics have been shown to have activity against many mycobacterial species[5–7] and this has been confirmed in animal models of infection.[5] It has also been reported that the bactericidal activity demonstrated *in vitro* and in animal models can also be replicated during short monotherapy clinical trials.[8,9] Some larger scale studies have suggested that regimens containing fluoroquinolone antibiotics are effective.[10,11] Despite this, fluoroquinolones have not established themselves as first line agents in chemotherapy. Rather they are used when patients cannot tolerate the standard regimen of rifampicin, isoniazid, pyrazinamide and ethambutol and in the management of patients with multiple drug resistance.[12,13]

Fluoroquinolones exert their antibacterial effect on mycobacteria by disrupting the action of the DNA gyrase system which results in double stranded DNA breaks.[14] As a result of this action, they trigger the SOS response, a mechanism which enables bacteria to survive in the face of threats to the integrity of their genome.[14,15] The SOS response is usually triggered when the organism is exposed to DNA damaging agents such as fluoroquinolones, ultraviolet light, reactive oxygen intermediates or salicylic acid.[14,16] The SOS response mediates survival of the organism by allowing DNA replication to continue past breaks that would normally block it. In exchange for this survival advantage there is an increased mutation rate as the polymerases that perform the repair are prone to error.[17,18] Recent studies have shown that error prone polymerase activation occurs at the end of stationary phase and in starvation.[19] Previous studies in *Escherichia coli* and methicillin-resistant *Staphylococcus aureus* have suggested that bacteria growing in the presence of sub-lethal concentrations of fluoroquinolones have an increased mutation rate to antibiotic resistance.[14,15,20]

Thus it is important to determine whether fluoroquinolones also exert this effect on mycobacteria and to quantify it as these drugs are used for the management of lower respiratory tract infections and this may expose mycobacteria to these agents.[21]

Materials and methods

Compounds, bacteria and MIC determination

Ciprofloxacin and moxifloxacin were supplied by Bayer, gentamicin, erythromycin and rifampicin were purchased from Sigma Chemical Co. and levofloxacin was supplied by Roussel Laboratories. The drugs were dissolved according to the manufacturers' instructions. A clinical strain of *M. fortuitum* (MF01332) isolated from a specimen submitted to the Department of Medical Microbiology at the Royal Free Hospital was used for these experiments. The MICs of ciprofloxacin, moxifloxacin, levofloxacin, rifampicin, erythromycin and gentamicin for MF01332 were determined by the Etest method (AB Biodisk, Solna, Sweden) using the manufacturer's instructions. These data were used to determine the concentration of antibiotic added to either broth culture or selective plates (see below).

Mutation induction

Fresh cultures of *M. fortuitum* were grown in Mueller–Hinton (Oxoid, Basingstoke, UK) broth to which ciprofloxacin had been added at concentrations equivalent to $\frac{1}{2}$ MIC (0.06 mg/L), $\frac{1}{4}$ MIC (0.03 mg/L) and $\frac{1}{8}$ MIC (0.015 mg/L). To determine the effect of ciprofloxacin on mutation rate, the organism was grown in a parallel broth with no ciprofloxacin added. Following inoculation of ~10^5 cfu/mL, cultures were incubated at 37°C aerobically for 48 h without shaking. Following incubation, viable counts were estimated by the method of Miles and Misra adapted for *M. tuberculosis*.[22] Briefly, samples were mixed by brief vortexing and log dilutions to 10^{-6} in sterile distilled water were set up. Twenty microlitres of each dilution was spotted onto a blood agar plate and dried. The plates were incubated for 48 h at 37°C then the number of colonies counted. Each determination was made in triplicate and expressed as mean colony forming units (cfu) per mL.

Mutation rate estimation

After incubation for 48 h, mutation rate estimation was performed as follows: bacteria were harvested by centrifugation (3000**g**) for 10 min, supernatants were removed, and pellets were then resuspended in a measured volume. The total volume of the resuspended pellet was noted and recorded for future calculation of mutation rates. The MICs of the various antibiotics used for mutant selection were as follows: ciprofloxacin 0.12 mg/L, moxifloxacin 0.06 mg/L, levofloxacin 0.12 mg/L, erythromycin 24 mg/L, rifampicin 4.0 mg/L, gentamicin 0.75 mg/L for MF 01332. Proportions of each pellet were then spread onto antibiotic-containing Iso-Sensitest (Oxoid, Basingstoke, UK) agar plates at 2× MIC of each antibiotic.[23] Plates were then incubated for 72 h at 37°C and examined on a daily basis and the numbers of colonies were counted and recorded. Mutation rates were estimated using the median mutation method of Drake described by Rosche and Foster.[24] A total of five pairs of median mutation rate estimations was performed for each selection antibiotic consisting of five paired mutation frequency experiments (a total of 25 selection experiments per data point).[23,24] As mutation rate experiments are subject to considerable variation between experiments, this batch to batch variation was controlled by calculating the ratio of the median mutation rate of cultures grown in drug-free medium and the median mutation rate grown in the presence of differing concentrations of ciprofloxacin. Experiments were repeated using rifampicin $\frac{1}{2}$ MIC (8 mg/L) as a control to determine whether the effect was specific to ciprofloxacin.

Statistical assessment

The differences between the mean mutation rate ratios were compared for each concentration of ciprofloxacin and each selecting agent by a one-way analysis of variance (ANOVA) using the Kruskal–Wallis non-parametric method. This was calculated using GraphPad Instat (Graph Pad Software, CA, USA).

Results

Mutation rates

Mutation frequencies take no account of the growth of the organisms nor the possibility of a jackpot mutation.[25] Since culturing in the presence of subinhibitory concentrations significantly affects the growth of the bacteria, it is especially important to calculate a mutation rate by a method which takes account of the reduced growth of the organisms and allows for the possibility of a jackpot mutation.[24] The rate of mutation to resistance to six different antibiotics was tested by selecting against each of these agents after growth in the presence of different subinhibitory concentrations of ciprofloxacin ($\frac{1}{2}$, $\frac{1}{4}$ and $\frac{1}{8}$ MIC). When the bacteria are exposed to $\frac{1}{2}$ MIC ciprofloxacin in the broths, the mutation rate is increased for all of the antibiotics in selection experiments. The mutation rate estimation data for each of the drugs, including drug-free media are listed in Table 1.

Although the results of the mutation rate experiments on drug-free media are highly reproducible, there was some variation in the growth between experiments. To control for the effect of this variability, the ratio of mutation rates between pairs of cultures one containing antibiotic and the other without was calculated. If ciprofloxacin had no effect on mutability of mycobacteria, the ratio between the mutation rate of bacteria grown in broth containing ciprofloxacin and in drug-free broth would be one, whereas if ciprofloxacin in the broth increased the rate of mutation, then the ratio would be greater than one. For all of the antibiotics tested: gentamicin, rifampicin, erythromycin, ciprofloxacin, levofloxacin and moxifloxacin, the ratio between cells grown in ciprofloxacin containing broth and in drug-free broth was greater than one. The mutation stimulation effect of ciprofloxacin in the broth was greatest when present at $\frac{1}{2}$ MIC. When rifampicin, erythromycin and gentamicin were used as the selecting agents on solid agar, $\frac{1}{2}$ MIC ciprofloxacin caused a 72- to 103-fold increase in the mutation rate (Table 2). Similar results were obtained when each of the quinolones was used as selecting agents on solid medium where rates were found to be between 89- and 121-fold greater (Table 2). The ratio of the mutation rate in drug-containing and drug-free broth was lower when ciprofloxacin at $\frac{1}{4}$ MIC was incorporated into the culture and lower still when $\frac{1}{8}$ MIC ciprofloxacin was in the broth suggesting that the effect is dose-dependent. At each of the mutation stimulating concentrations, the increase in the ratio of mutation rates was similar for all of the antibiotics used in the selection step. The differences between the mutation rate ratios were assessed by a one-way analysis of variance and the differences between the mutation ratios were statistically significant for all of the selecting agents (Table 2). To confirm that this effect was specific to ciprofloxacin, the experiment was repeated using $\frac{1}{2}$ MIC rifampicin in

Effect of ciprofloxacin on *M. fortuitum* mutation rate

Table 1. Summary of the mean mutation rates for *M. fortuitum* (MF01332) grown in differing concentrations of ciprofloxacin and selected on various antibiotics by the median mutation method

Selective agent	Median mutation rate in the drug-free broth (mutations/cell division)	$\frac{1}{2}$ MIC median mutation rate (mutations/cell division)	$\frac{1}{4}$ MIC mean mutation rate (mutations/cell division)	$\frac{1}{8}$ MIC mean mutation rate (mutations/cell division)
Ciprofloxacin	5.1×10^{-9}	2.6×10^{-7}	2.2×10^{-8}	1.6×10^{-8}
Levofloxacin	3.8×10^{-9}	2.0×10^{-7}	1.4×10^{-8}	9.6×10^{-9}
Moxifloxacin	4.2×10^{-9}	3.6×10^{-7}	1.5×10^{-8}	1.3×10^{-8}
Erythromycin	1.3×10^{-8}	4.9×10^{-7}	3.3×10^{-7}	3.6×10^{-8}
Rifampicin	2.6×10^{-9}	3.4×10^{-7}	5.3×10^{-8}	7.0×10^{-9}
Gentamicin	7.8×10^{-9}	3.5×10^{-7}	2.3×10^{-7}	3.3×10^{-8}

Table 2. Ratio (mean and standard error of mean for five median mutation estimates) between the mutation rates for *M. fortuitum* (MF01332) grown with and without ciprofloxacin in the broth for six antibiotics

Selective agent	Concentration of ciprofloxacin in test broth			
	$\frac{1}{2}$ MIC (SEM)	$\frac{1}{4}$ MIC (SEM)	$\frac{1}{8}$ MIC (SEM)	*P* (ANOVA)
Ciprofloxacin	88.8 (36.6)	5.0 (1.2)	3.8 (0.6)	0.02
Levofloxacin	94.9 (35.7)	5.2 (1.3)	3.1 (0.5)	0.01
Moxifloxacin	121.1 (32.9)	5.6 (1.1)	3.9 (0.8)	0.0006
Rifampicin	81.7 (36.9)	21.2 (11.6)	4.2 (1.8)	0.003
Erythromycin	72.1 (29.4)	21.8 (10.8)	9.6 (4.9)	0.04
Gentamicin	102.5 (41.6)	29.7 (15.3)	6.8 (3.3)	0.007
	Growth in $\frac{1}{2}$ MIC rifampicin			
Rifampicin	1.8	(mean of two median mutation experiments)		

the broth medium. The ratio between bacteria grown in $\frac{1}{2}$ MIC (8 mg/L) and without was 1.8 (mean of two median mutation experiments, a total of 10 mutation frequency experiments).

Discussion

To facilitate this study, we have used *M. fortuitum* as a model system as the faster growth rate permits experiments to be performed and repeated more quickly. *Mycobacterium smegmatis* cannot be used as this organism is intrinsically resistant to fluoroquinolones as are *Mycobacterium chelonae* and some other rapid growers. In contrast, *M. fortuitum* is usually susceptible to most drugs used to treat tuberculosis.[26,27] Other workers have shown that *M. fortuitum* is a useful surrogate for *M. tuberculosis* in studies of mycobacterial susceptibility testing and the results of susceptibility in rapidly growing organisms correlate well with the results in *M. tuberculosis* and it has been used extensively for developing background data in structure activity analysis and computer modelling.[6,28–32] For this reason, we believe that the results we have obtained are likely to predict the behaviour of other mycobacteria including *M. tuberculosis*.

Our experiments show that when *M. fortuitum* is grown in the presence of a fluoroquinolone, in this instance ciprofloxacin, there is an increase in the rate at which mutations occur. The greatest increase in the mutation rate, in comparison with organisms grown

in the absence of antibiotic was up to 120-fold when the antibiotic concentration was the equivalent of $\frac{1}{2}$ MIC. The effect also appeared to be dose-dependent as smaller, but significant increases in the mutation rates were also seen at lower ciprofloxacin concentrations. Previous authors have used a difference of 10-fold to distinguish hypermutators from normal bacteria by differences in mutation rates of as little as sevenfold, indicating that the increase in mutation rate that we have demonstrated must be considered both statistically and biologically significant.[33–35]

The increases in mutation rate were found irrespective of selecting agent (the antibiotic incorporated into the plates): ciprofloxacin, levofloxacin, moxifloxacin, erythromycin, gentamicin and rifampicin. This is an important finding as it suggests that the effect of the fluoroquinolone affects the whole genome since, to become resistant to the antibiotics tested, mutations must occur in a wide range of different genes.[4]

The concentrations of quinolone that are responsible for the increase in mutation rate demonstrated in this paper are clinically relevant and likely to occur between doses of antibiotics. Fluoroquinolones are often used in the management of non-tuberculosis mycobacteria[26,27] and multiple drug-resistant *M. tuberculosis* disease and are combined with other second line agents.[36,37] Mutation to resistance occurs at a higher rate for second line anti-tuberculosis agents than isoniazid and rifampicin. For example, the mutation rate for rifampicin is between 10^{-8} and 10^{-9}/cell division but 10^{-6}/cell division for ethambutol.[2]

This means that any increase in mutation rate may have a significant effect on the speed at which resistance may emerge to other second line agents in a regimen. This throws into question the common practice of adding a fluoroquinolone to a mycobacterial treatment regimen when a resistant strain is isolated. In such circumstances, the capacity of isoniazid and rifampicin to prevent the emergence of resistant mutants is lost. This is especially likely in those patients with cavitatory disease, who have intestinal malabsorption or do not adhere closely to the prescribed regimen.[38,39] This suggests that when fluoroquinolones are used, care must be taken to ensure that a regimen is prescribed that minimizes the risk of exposing bacteria to subinhibitory concentrations of quinolone. The data presented in this paper require to be confirmed in *M. tuberculosis* and these experiments, coupled with DNA array analysis are currently under way in our laboratory.

Acknowledgements

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