Introduction

Tuberculosis (TB) is one of the major causes of death worldwide. The number of individuals succumbing to TB has increased vastly due to the HIV/AIDS pandemic, and global travel has increased the transfer of virulent and multidrug resistant (MDR) TB. Compared with other infections there are relatively few antimicrobial agents that are clinically active against *Mycobacterium tuberculosis*. Prolonged antibiotic treatment is also required due to the ability of the bacterium to enter a dormant, antibiotic-resistant phase.

Some fluoroquinolones, including ofloxacin, levofloxacin, moxifloxacin and ciprofloxacin show *in vitro* activity against *M. tuberculosis*. As *M. tuberculosis* possesses only the genes *gyrA* and *gyrB*, which encode the topoisomerase II enzyme DNA gyrase, it is assumed that this is the single target of this class of drugs as there are no *parC/parE* genes that encode the second target enzyme (topoisomerase IV) found in other bacterial species. This hypothesis is supported by the finding that only *gyrA* mutations have been reported in fluoroquinolone-resistant *M. tuberculosis* clinical isolates. Although the major form of quinolone resistance is altered DNA gyrase, energy-dependent quinolone efflux has been shown to be a contributory factor in other bacteria. Active efflux of norfloxacin has been reported in a quinolone-resistant strain of *Mycobacterium smegmatis mc²155*, and the gene encoding this system, *lfrA*, has been cloned. The *lfrA* efflux pump is homologous to QacA from *Staphylococcus aureus*, but not to NorA. QacA confers resistance to ethidium and other organic cations, such as chlorhexidine, via PMF-dependent efflux. However, as for NorA, LfrA preferentially pumps out hydrophilic quinolones.

Owing to moderate *in vivo* activity and increasing fluoroquinolone resistance, these agents are generally considered second-line agents against tuberculosis. However, with the increasing incidence of MDR TB, and the increased use of fluoroquinolones in combination with other agents to treat TB, much attention has focused on the therapeutic value of the fluoroquinolones for the treatment of TB and other mycobacterial diseases. As DNA gyrase is intracellular, in order to exert their antibacterial effect the fluoroquinolones must cross the bacterial cell wall. However, there have only been a few studies measuring the accumulation/transport of antituberculous agents by *M. tuberculosis*, and hence the role permeability plays, if any, in this species’ drug resistance has not been determined.

We have previously used the modified fluorescence method to measure accumulation of norfloxacin by mycobacteria and shown for *Mycobacterium aurum* and *M. smegmatis* that similar kinetics of accumulation were obtained to those found for fluoroquinolones and other bacteria. Of interest, there was no effect of Tween-80 or subinhibitory concentrations of ethambutol or of the proton motive force (efflux pump) inhibitor 2,4-dinitrophenol (DNP), whether added before or after norfloxacin, on the accumulation of each agent.

Accumulation of five fluoroquinolones by *Mycobacterium tuberculosis* H37Rv

Laura J. V. Piddock* and Vito Ricci

Antimicrobial Agents Research Group, Division of Immunity and Infection, The Medical School, University of Birmingham, Vincent Drive, Edgbaston, Birmingham B15 2TT, UK

The accumulation of ciprofloxacin, norfloxacin, moxifloxacin, levofloxacin and ofloxacin by *Mycobacterium tuberculosis* H37Rv was determined with a modified fluorescence method. The time to achieve a steady-state concentration (SSC) of each agent in *M. tuberculosis* was 60–240 s. Moxifloxacin was accumulated to the lowest concentration and ciprofloxacin to the highest. However, ciprofloxacin took longer to achieve an SSC than the other four agents; levofloxacin reached steady state in the shortest time. Larger fluoroquinolones accumulated to the lowest concentration and more slowly. Although all five agents had low hydrophobicity values (*P* < 0.11), those with the lowest values accumulated to the higher concentrations.

---

*Corresponding author. Tel: +44-121-414-6966; Fax: +44-121-414-3454; E-mail: l.j.v.piddock@bham.ac.uk

© 2001 The British Society for Antimicrobial Chemotherapy
Materials and methods

Antibiotics and chemicals

Fluoroquinolones (ciprofloxacin and moxifloxacin, Bayer AG, Wuppertal, Germany; norfloxacin, Sigma, Poole, UK; levofloxacin and ofloxacin, Aventis, Romainville, France), ethambutol, Tween-80, CCCP (carbonyl cyanide m-chlorophenyl-hydrazone) and reserpine were prepared according to the manufacturers’ instructions. All other agents were from Sigma.

Bacterial strains, growth conditions and antibiotic susceptibility testing

All experiments were performed in a class I cabinet within a category III facility. *M. tuberculosis* H37Rv was maintained on Lowenstein–Jensen slopes and cultured on Middlebrook 7H11 agar (Difco, West Molsey, UK) supplemented with 10% v/v OADC (oleic acid, albumin fraction V, dextrose and catalase) or Middlebrook 7H9 broth (Difco) supplemented with 10% v/v ADC (albumin fraction V, dextrose and catalase) and grown exactly as described previously.16,17 The MIC of each agent was also determined as described previously.16,17 The plates were incubated for 21 days and the MIC was defined as the lowest concentration of drug at which no visible growth was observed. The effect of ethambutol, Tween-80, CCCP and reserpine on the susceptibility of *M. tuberculosis* was also determined. The growth kinetics and estimation of cell dry weight were also as described previously.17

Measurement of fluoroquinolone accumulation

A modified fluorometric method, adapted to accommodate the growth characteristics of mycobacteria, was used.15 Cells were grown to mid-exponential phase, OD550 of 1–1.2, and harvested by centrifugation in a Sigma 6K10 centrifuge (supplied by Phillip Harris) at 3000g for 20 min at 15°C. The cells were washed in 10 mL of 50 mM sodium phosphate buffer (pH 7) and concentrated with the same buffer to give the suspension of *M. tuberculosis* an OD2590 of 8. This suspension was then incubated at 37°C water bath and left for 10 min to equilibrate. Fluoroquinolone was added to a final concentration of 10 mg/L and 1 mL samples were removed at timed intervals. The cells were centrifuged immediately at 12 000g (Sigma 6K10) for 3 min at 4°C and the cell pellets washed once with ice-cold sodium phosphate buffer (50 mM, pH 7) and resuspended in 1 mL of 0.1 M glycine hydrochloride (pH 3). The samples were left overnight, at room temperature with agitation, to lyse. The following day the samples were centrifuged at 12 000g (Eppendorf 5403) and the fluorescence of the supernatants determined at the appropriate excitation and emission wavelengths for each agent, and the data expressed as ng fluoroquinolone/mg dry weight cells. All experiments were performed at least three times and mean values ± S.D. are shown. The MICs values were plotted against the molecular size of the free form of each agent and the partition coefficient (Papp). The Papp was calculated by determining the concentration of agent in the aqueous phase (0.1 M sodium phosphate buffer pH 7) and organic phase (n-octanol) of each agent as described previously.18

Results

Activity of fluoroquinolones for *M. tuberculosis* H37Rv

The most active fluoroquinolones were moxifloxacin and sparfloxacin with MICs of 0.5 mg/L, ciprofloxacin, clinafloxin and grepafloxacin all had the same MIC, 1 mg/L, and gatifloxin and norfloxacin each had an MIC of 2 mg/L. The MICs of various inhibitors were: ethambutol >0.5 mg/L, 0.25% Tween-80, reserpine 128 mg/L, 64 μM DNP and 32 μM CCCP. Addition of ethambutol (0.25 mg/L), Tween-80 (0.05%), CCCP (32 μM) and reserpine (20 mg/L) had no effect upon the MIC of any agent.

Accumulation of fluoroquinolones

A method to determine accumulation of norfloxacin was established for mycobacteria in a previous study.15 Despite the MIC of norfloxacin for *M. tuberculosis* being 2 mg/L, due to the fluorescence of the fluoroquinolones studied, the optimum concentration for accumulation studies was 10 mg/L, and in the time frame of the accumulation experiment (20 min) had no deleterious effect upon cell viability or growth (data not shown).

The time to achieve an SSC of each agent in *M. tuberculosis* was 60–240 s (Table and Figure). Of all five agents examined, moxifloxacin accumulated the lowest concentration, 31.5 ± 1.9 ng/mg dry weight cells, which was followed by levofloxacin, norfloxacin, ofloxacin and ciprofloxacin (97.7 ± 7.5 ng/mg dry weight cells) (Table and Figure). However, ciprofloxacin took longer to achieve an SSC than the other four agents; levofloxacin reached steady state in the least time (60 s). There was only a weak correlation between molecular size and MIC (r = −0.59). However, a stronger correlation was found between molecular size and SSC (r = −0.76), and initial rate (r = −0.86); larger molecules accumulated to the lowest concentration and more slowly. However, these molecules (moxifloxacin and levofloxacin) were also the most active. Although all five
Mycobacterial accumulation of fluoroquinolones

Table. Properties of five fluoroquinolones compared with their activity and concentration accumulated by *M. tuberculosis* H37Rv

<table>
<thead>
<tr>
<th>Fluoroquinolone</th>
<th>Free form (mol. wt)</th>
<th>MIC (mg/L)</th>
<th>SSC (ng antibiotic/mg dry cells)</th>
<th>Time to reach SSC (s)</th>
<th>Initial rate (ng antibiotic/mg dry cells/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofloxacin</td>
<td>294.9</td>
<td>0.031</td>
<td>1</td>
<td>97.8 ± 7.5</td>
<td>240</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>436.8</td>
<td>0.089</td>
<td>0.5</td>
<td>31.5 ± 1.9</td>
<td>180</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>319.3</td>
<td>0.022</td>
<td>2</td>
<td>53.2 ± 1.8</td>
<td>180</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>361.4</td>
<td>0.088</td>
<td>0.5</td>
<td>65.1 ± 2.3</td>
<td>210</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>361.3</td>
<td>0.11</td>
<td>0.5</td>
<td>31.9 ± 2.1</td>
<td>60</td>
</tr>
</tbody>
</table>

Figure. Accumulation of 10 mg/L (a) ofloxacin, (b) levofloxacin, (c) ciprofloxacin, (d) norfloxacin and (e) moxifloxacin over 20 min.

agents had low hydrophobicity values (P_{app} ≤ 0.11), those with the lowest values accumulated to the higher concentrations. As for molecular size, there was a correlation between the P_{app} values and MIC (r = −0.86), SSC (r = −0.64) and initial rate (r = −0.68). There was no correlation between MIC and either SSC (r = 0.24) or initial rate (r = 0.41).

As ethambutol, Tween-80, CCCP and reserpine had no effect upon the activity of any of the agents for *M. tuberculosis* H37Rv, and in the previous study with *M. aurum* DNP had no effect upon norfloxacin accumulation, no accumulation experiments were performed in the presence of these compounds.

Discussion

In the previous study with *M. aurum* and norfloxacin, the silicon oil method was shown to be inferior to a modified fluorescence method as it consistently gave higher, and more variable, SSCs than the modified fluorescence method. These higher values were shown to be due to binding of the fluoroquinolone to the cell surface. Therefore, the accumulation of fluoroquinolones with activity for *M. tuberculosis* was determined with the latter method. *M. tuberculosis* H37Rv had similar kinetics of accumulation to norfloxacin to *M. aurum* and *M. smegmatis* examined in the previous study and to those of other bacteria. However, *M. tuber-
coulis H37Rv accumulated lower concentrations of norfloxacin than did M. aurum and M. smegmatis, presumably reflecting the lower permeability of the cell envelope of M. tuberculosis. Kocagoz et al. also measured the concentration of norfloxacin accumulated by M. tuberculosis, but with the attenuated strain H37Ra; this strain accumulated approximately 35 ng norfloxacin/mg dry cells, which was lower than that accumulated by H37Rv. This may reflect differences between the two strains, but is more likely to be due to the lower temperature (22°C) at which Kocagoz et al. measured accumulation.

In the present study, accumulation of fluoroquinolones with activity for M. tuberculosis was investigated to determine whether the concentration accumulated reflected activity. Sparfloxacin was not available in radiolabelled form for this study and as it fluoresces poorly it was not studied. Likewise we have found that clinafloxacin and grepafloxacin also fluoresce poorly, so these agents were not examined further. Moxifloxacin, levofloxacin and ofloxacin had the same MIC for this strain of M. tuberculosis H37Rv; moxifloxacin and levofloxacin also accumulated to the same concentration. However, levofloxacin accumulated at a greater rate than moxifloxacin, and ofloxacin accumulated slowly. It has been postulated previously that the rates of influx of rifampicin and KRM-1648 were associated with activity and not the SSC. It was further postulated that as the concentration accumulated mirrored the IC50 value for inhibition of the target enzyme, the SSC values reflected binding of the drug to its target. The same may also be true for fluoroquinolones, and the rate of influx is sufficiently rapid to ensure that moxifloxacin reaches its target protein(s) to give rise to an MIC similar to that of levofloxacin. For strains of M. tuberculosis with greater susceptibility to fluoroquinolones than those investigated in the present study, it is likely that the target enzyme(s) is more susceptible to the drug.

A well-established effect of ethambutol is to lower the MICs of several antmycobacterial agents. We have previously shown that the concentrations of rifampicin and KRM-1648 accumulated in the presence of ethambutol increased, supporting the hypothesis that ethambutol interacts with components of the mycobacterial cell wall increasing cell wall permeability. However, as ethambutol had no effect on the MIC of any agent in this study, no accumulation experiments were performed. It may be that as most fluoroquinolones are zwitterions and enter the cell rapidly, any permeabilizing effect of ethambutol is irrelevant.

TWEEN-80 is a non-ionic surface-active detergent often added to liquid media to obtain homogeneous cell suspensions of mycobacteria. It has been proposed that Tween-80 acts directly on the mycobacterial cell wall and subsequently alters its permeability. Despite this, in studies in this laboratory with rifampicin and KRM-1648, Tween-80 had no effect on the concentration of these norfloxacin accumulated by M. aurum. There was also no antimicrobial synergy between Tween-80 and any fluoroquinolone tested against M. tuberculosis H37Rv and so no accumulation experiments were performed.

As there was no effect upon the activity of each agent in the presence of the efflux pump inhibitors CCCP, DNP and reserpine, and our studies with M. smegmatis and M. aurum demonstrated no effect upon the concentration of norfloxacin accumulated, no accumulation experiments in the presence of these inhibitors were performed with M. tuberculosis.

In conclusion, the antimycobacterial activities of fluoroquinolones are probably due to the rapid influx of these agents combined with good affinity for the target enzyme.

Acknowledgements

We are grateful to Dr T. E. Elliott, Mr T. Johnson and Mr M. Palmer for allowing us to perform much of this work in the category III facility of the Queen Elizabeth University Hospital Trust. This study was supported by Bayer AG.

References

Mycobacterial accumulation of fluoroquinolones


    *Mycobacterium smegmatis* mediated by LfrA, a multidrug efflux pump. 


    fluoroquinolones for the treatment of mycobacterial diseases. 


    *Mycobacterium aurum* and *Mycobacterium smegmatis*. 

    *Mycobacterium aurum*, *Mycobacterium smegmatis* and 
    *Mycobacterium tuberculosis*. 

    by *Mycobacterium aurum* and *Mycobacterium tuberculosis*. 

    accumulation of quinolones by Enterobacteriaceae, *Pseudomonas aeruginosa* and 
    *Staphylococcus aureus*. 

    Quinolone accumulation by *Pseudomonas aeruginosa*, *Staphylococcus aureus* and 
    *Escherichia coli*. 

    Gyrase mutations in laboratory-selected, fluoroquinolone-resistant mutants of 
    *Mycobacterium tuberculosis* H37Ra. 

    Recognition of multiple effects of ethambutol on metabolism of mycobacterial cell envelope. 

    *In vitro* and *in vivo* susceptibility of atypical mycobacteria to various drugs. 

    Effect of Tween 80 on the growth of *Mycobacterium avium* complex. 
    *Microbiology and Immunology* **34**, 653–63.

Received 14 May 2001; returned 11 August 2001; revised 10 September 2001; accepted 17 September 2001