Introduction

Tuberculosis (TB) remains the leading cause of death in the world from an infectious disease. Once thought to be eradicated in the west, more people died worldwide of TB in 1995 than in any other year in history. Globally the resurgence of TB has been attributed to the HIV/AIDS pandemic and the emergence of multidrug-resistant strains of Mycobacterium tuberculosis (MDR-TB). In addition, increased worldwide travel, increased numbers of immigrants and a decrease in the socio-economic conditions in inner cities have also contributed to the increase in the number of cases of TB. Few antibiotics are effective against M. tuberculosis and because of problems with the dormancy of this bacterium, antibiotic treatment is lengthy. Therefore, new and novel anti-TB agents are required to reduce the treatment time and to combat the spread of MDR-TB.

Rifampicin, a broad-spectrum antibiotic discovered in 1963 and introduced for TB chemotherapy in 1971, is the most potent of current anti-TB drugs in rendering sputum cultures negative for M. tuberculosis during short-course chemotherapy. Rifampicin inhibits bacterial RNA polymerase activity by binding to the β-subunit encoded by rpoB, and forming a stable drug–enzyme complex, preventing the transcription of RNA from the DNA template. Mutation at one of three loci in rpoB has been associated with resistance to rifampicin. Rifampicin is rapidly eliminated from the body but it is rapidly taken up by the tissues in which it is active, such as mycobacteria. Accumulation of rifampicin by wild-type strains of Mycobacterium aurum (A1), Mycobacterium smegmatis (mc2155) and Mycobacterium tuberculosis (H37Rv) were determined. After 10 min exposure M. aurum had accumulated 220 ng rifampicin/mg cells, M. smegmatis had accumulated 120 ng rifampicin/mg cells and M. tuberculosis had accumulated 154 ng rifampicin/mg cells. A steady-state concentration (SSC) of rifampicin was accumulated rapidly by M. aurum and M. tuberculosis within minutes of drug exposure, unlike M. smegmatis, which accumulated rifampicin more slowly. With an increase in the concentration of rifampicin from 0.12 mg/L to 2 mg/L there was an increase in the concentration of rifampicin accumulated by M. tuberculosis, with no detectable loss of viability over the 20 min of the accumulation experiment. With an increase in temperature there was also an increase in the concentration of rifampicin accumulated by M. tuberculosis; between 15 and 30°C the increase was linear. For all three species sub-inhibitory concentrations of ethambutol increased the concentration of rifampicin accumulated. However, both growth and accumulation of rifampicin were lower in the presence of 0.05% Tween 80. Accumulation of rifampicin by M. smegmatis was unaffected by the presence of the proton motive force inhibitor, 2,4-dinitrophenol (1 mM), whether added before or after the addition of rifampicin to the mycobacterial culture. For all three species, the Gram-positive bacterial efflux inhibitor reserpine (20 mg/L) slightly increased the SSC of rifampicin, but the increase was not statistically significant. Addition of glucose to energize a putative efflux pump had little effect on the accumulation of rifampicin in the presence or absence of reserpine for M. tuberculosis; however, for M. aurum and M. smegmatis the reserpine effect was abolished by the addition of glucose. These data suggest that rifampicin may be removed from wild-type mycobacteria by efflux, but that the pump(s) is expressed at low level.

© 2000 The British Society for Antimicrobial Chemotherapy
Materials and methods

Antibiotics and chemicals

[^14C]Rifampicin (specific activity 1.41 μCi/mg) was generously provided by Ciba Geigy Ltd (Basel, Switzerland). Radiochemical purity was determined by Ciba Geigy Ltd and was found to be 98% by HPLC. Biological activity of the radiolabelled drug was determined by measurement of the MIC. All other agents were from Sigma (Poole, UK). Rifampicin was dissolved in methanol; ethambutol was prepared according to manufacturer’s instructions; and 2,4-dinitrophenol (50 mM; DNP) was dissolved in 3 parts 2,4-dinitrophenol (50 mM; DNP) was dissolved in 3 parts distilled water and incubated at 80°C for 1 h to render the cells non-viable. The cell pellets were then dried overnight at 60°C, kept under desiccation and re-weighed until a stable weight was obtained.

M. tuberculosis growth kinetics

7H9 broth (100 mL) was inoculated with a 3-4 day old culture (10 mL) of M. tuberculosis (A550 0.4-0.5) followed by incubation at 37°C with aeration at 120 rpm. Growth was evaluated by measuring the viable count and optical density (OD) of the culture at 550 nm. Prior to measurement the culture was placed in a sonicating water bath (Jencons Scientific, Leighton Buzzard, UK) for 2 min to gently disrupt the mycobacterial clumps and obtain a consistent OD reading. Cell dry weight for M. tuberculosis was determined by removing 20 mL aliquots of the growing culture at selected OD readings, and centrifugation at 1698 g for 15 min at 4°C. The cell pellets were washed to remove growth medium, with 10 mL sterile distilled water and centrifuged as before. The cell pellets were resuspended in 1 mL sterile distilled water and incubated at 80°C for 1 h to render the cells non-viable. The cell pellets were then dried overnight at 60°C, kept under desiccation and re-weighed until a stable weight was obtained.

Measurement of rifampicin accumulation by mycobacteria

Mycobacterial cells were grown to mid-logarithmic phase (A550 0.1-0.12 for M. aurum; A550 0.5-0.6 for M. smegmatis; A550 1-1.2 for M. tuberculosis) and harvested at 3003 g for 15 min at 4°C (M. tuberculosis was harvested in a Sigma centrifuge at 1698 g). The cells were washed in 10 mL 50 mM sodium phosphate buffer pH 7, resuspended in the same buffer to give an OD550 of 8 and placed in a 37°C water bath for 10 min to equilibrate. After the addition of[^14C]Rifampicin to the required concentration, 500 μL samples were removed and added to 1 mL 50 mM sodium phosphate buffer pH 7 on ice at various time intervals and centrifuged at 12,000g at 4°C for 3 min. The cell pellets were resuspended in 1 mL ice-cold 50 mM sodium phosphate buffer pH 7, recentrifuged and the pellet thoroughly mixed with 5 mL Hisafe scintillation fluid (Fisher Chemicals, Liecester, UK). The cell-associated radioactivity was determined by liquid scintillation counting after correction for background radiation. Each sample was counted for 20 min (TC2500, Packard, Pangbourne, UK). Using a calibration curve of the radioactivity counts (dpm) versus[^14C]Rifampicin concentration (mg/L) and a curve of mycobacterial OD versus cell dry weight (mg/mL), the data were
Mycobacterial accumulation of rifampicin

expressed as ng rifampicin/mg cells (dry weight). A desorption of rifampicin to the cell wall was estimated by performing the experiments at 0°C.

To investigate the effect of ethambutol on the accumulation of rifampicin by mycobacteria, sub-inhibitory concentrations (¼ × MIC) of ethambutol (0.5 mg/L for M. aurum and M. smegmatis, 0.25 mg/L for M. tuberculosis) were added to the mycobacterial cultures 24 h (48 h for M. tuberculosis) before the cell harvesting step. The accumulation of rifampicin by mycobacteria was then determined as described above.

The effect of Tween 80 on the accumulation of rifampicin by mycobacteria was determined by replacing the glycerol with 0.05% Tween 80 in the growth and uptake medium.

The effect of DNP (1 mM) on the accumulation of rifampicin by M. smegmatis was determined by adding the inhibitor either 5 min before, or 5 min after, the addition of rifampicin to the mycobacterial culture. The effect of reserpine (20 mg/L) on the concentration of rifampicin accumulated was determined by adding the inhibitor after 5 min exposure to [14C]rifampicin. Samples were withdrawn after 5, 10 and 20 min exposure to both agents. A accumulation of [14C]rifampicin alone was monitored in parallel up to 20 min. In addition, the effect of 11 mM glucose upon the concentration of [14C]rifampicin accumulated in the presence or absence of reserpine was also determined.

Statistical analysis

The differences in the accumulation data obtained for each species were compared and the mean steady-state concentration (SSC) values were analysed by Student’s t test. P < 0.05 was considered significant. Analysis of variance was performed to compare data over a range of time points.

Results

Growth of M. tuberculosis

A suspension of mycobacteria with little or no clumping was required for accumulation experiments, hence the water sonication step. This did not inhibit growth (data not shown).

Accumulation of rifampicin

A method to determine accumulation of [14C]rifampicin had been established for Staphylococcus aureus in a previous study.14 Despite the MIC of rifampicin for S. aureus being 0.002 mg/L, 2 mg/L [14C]rifampicin was the optimum concentration for accumulation studies and, in the time frame of the accumulation experiment (maximum of 20 min), had no deleterious effect upon cell viability or growth.14 Therefore, this concentration was used to study accumulation of rifampicin by mycobacteria. For M. aurum an SSC of rifampicin of ~220 ng/mg cells was obtained 10 min after the addition of rifampicin (Figure 1). The concentration of rifampicin accumulated ‘intracellularly’ was determined by measuring accumulation at 0°C (= adsorption to the cell surface) and subtracting these values from those obtained at 37°C. At 0°C, 20–25 ng rifampicin/mg cells was adsorbed, and the calculated ‘intracellular’ SSC of rifampicin for M. aurum was ~200 ng/mg cells (Figure 1).

For M. smegmatis an SSC of rifampicin of ~120 ng/mg cells was obtained after 20 min exposure, although the concentration of rifampicin accumulated increased slightly between 20 and 30 min. At 0°C, 45–50 ng rifampicin/mg cells was adsorbed, and the calculated ‘intracellular’ SSC of rifampicin was 60–70 ng/mg cells (Figure 1).

Figure 1. A accumulation of rifampicin (2 mg/L) by M. tuberculosis (left), M. aurum (centre) and M. smegmatis (right). Symbols: ■, 37°C; ◆, 0°C; ▲, intracellular.
For M. tuberculosis an SSC of rifampicin of 154 ng/mg cells was obtained after 1–2 min exposure (Figure 1), and remained constant over the next 20 min. At 0°C ~20 ng rifampicin/mg cells was adsorbed, and the calculated ‘intracellular’ concentration of rifampicin was 134 ng/mg cells (Figure 1). To determine the effect of the concentration of rifampicin upon growth and concentration accumulated by M. tuberculosis, a range of concentrations from 0.12 to 2 mg/L was investigated. Over the time course of the experiment there was no decrease in the viable count, even at concentrations above the MIC (data not shown). With an increase in the concentration of rifampicin there was an increase in the concentration accumulated (Figure 1). The effect of temperature upon rifampicin accumulation was also investigated. As the temperature increased from 1 to 15°C the concentration accumulated increased by 15 ng/mg cells (Figure 3). Between 15 and 30°C, there was a linear relationship between concentration and temperature, while between 30 and 37°C there was only a small increase in the concentration accumulated, of 10 ng/mg cells.

Effect of ethambutol on rifampicin accumulation

The MIC of rifampicin was reduced four-fold for M. tuberculosis and eight-fold for M. aurum and M. smegmatis in the presence of sub-inhibitory concentrations (0.5 mg/L) of ethambutol (Table). The concentration of rifampicin accumulated by all three species was increased in the presence of sub-inhibitory concentrations (four-fold less than the MIC) of ethambutol (Figure 4). This effect was most marked for M. aurum, with more than double the concentration of rifampicin accumulated in the presence of ethambutol. For M. smegmatis, although a similar effect upon susceptibility was observed for rifampicin plus ethambutol as for M. aurum, a similar increase in the concentration of rifampicin accumulated was not seen.

![Figure 2.](image2.png)  
Figure 2. Effect of concentration of rifampicin upon accumulation by M. tuberculosis.

![Figure 3.](image3.png)  
Figure 3. Effect of temperature upon accumulation of rifampicin by M. tuberculosis.

**Table.** Effect of ethambutol and Tween 80 upon accumulation and susceptibility of mycobacteria to rifampicin

<table>
<thead>
<tr>
<th>Drug</th>
<th>M. aurum</th>
<th>M. smegmatis</th>
<th>M. tuberculosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>SSC&lt;sup&gt;b&lt;/sup&gt;</td>
<td>MIC&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>2</td>
<td>220</td>
<td>8</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>2</td>
<td>ND</td>
<td>2</td>
</tr>
<tr>
<td>Tween 80</td>
<td>0.2%</td>
<td>ND</td>
<td>0.2%</td>
</tr>
<tr>
<td>Rifampicin + 0.25 mg/L ethambutol</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Rifampicin + 0.5 mg/L ethambutol</td>
<td>0.25</td>
<td>500</td>
<td>1</td>
</tr>
<tr>
<td>Rifampicin + 1 mg/L ethambutol</td>
<td>0.12</td>
<td>ND</td>
<td>0.12</td>
</tr>
<tr>
<td>Rifampicin + 0.05% Tween 80</td>
<td>0.5</td>
<td>130</td>
<td>4</td>
</tr>
</tbody>
</table>

ND, not determined.
<sup>a</sup>in mg/L except for Tween 80.
<sup>b</sup>SSC, steady-state concentration of rifampicin expressed as ng/mg cells after 10 min exposure to rifampicin (mean value of at least three experiments).

Values not adjusted for cell adsorption.
Mycobacterial accumulation of rifampicin

both M. smegmatis and M. tuberculosis in the presence of ethambutol the increase in the SSC of rifampicin was 16–20% more than in the absence of ethambutol. This small increase was statistically significant.

Effect of Tween 80 on the accumulation of rifampicin

The MIC of rifampicin was reduced for all three species in the presence of sub-inhibitory concentrations (four-fold less than the MIC) of Tween 80 (Table). For M. aurum 0.05% Tween 80 reduced the MIC of rifampicin four-fold; for M. tuberculosis and M. smegmatis the MIC of rifampicin was reduced two-fold. Despite Tween 80 acting synergistically with rifampicin, the concentration of rifampicin accumulated by all three species was reduced in the presence of 0.05% Tween 80 (Figure 4). For M. aurum and M. smegmatis approximately half the concentration was accumulated compared with cultures grown with glycerol in the growth medium. One explanation for these data is that growth in 0.05% Tween 80 gives rise to lower numbers of cells than growth in glycerol (despite using a suspension of equivalent optical density in the accumulation experiments), and that it is this lower number that gives rise to the lower SSC values. To examine this, the viable count of all three species was determined after growth in 0.05% Tween 80 compared with parallel cultures grown with glycerol. For all three species the viable count was lower for the cultures grown in 0.05% Tween 80 than those grown with glycerol. The effect was most marked for M. aurum (Figure 5).

Figure 4. Effect of ethambutol and Tween 80 on the SSC of rifampicin by M. aurum, M. smegmatis and M. tuberculosis; 5 min, antibiotic alone ■; 5 min, + ethambutol □; 10 min, antibiotic alone ■; 10 min, + ethambutol □; 5 min, + Tween 80 ▓; 10 min, antibiotic alone ■; 10 min, + Tween 80 ▓.

Figure 5. Viable count of M. aurum, M. smegmatis and M. tuberculosis after growth in the absence (■) and presence (□) of 0.05% Tween 80. M. aurum and M. smegmatis were grown for 72 h, and M. tuberculosis for 14 days. Data shown are typical of a single experiment.

Effect of efflux inhibitors on the concentration of rifampicin accumulated

There was no effect of 1 mM DNP on the final concentration of rifampicin accumulated by M. smegmatis. A n SSC of rifampicin of c. 70 ng/mg cells was obtained whether the DNP was added before or after the addition of rifampicin to the culture medium (data not shown). For all three species, reserpine (20 mg/L) had a minimal effect, increasing the concentration of rifampicin accumulated by M. aurum by no more than 6 ng/mg dry cells, M. smegmatis by 5 ng/mg dry cells and M. tuberculosis by 5 ng/mg dry cells.
These increases were statistically insignificant (P values of 0.129, 0.278 and 0.1, respectively). A dilution of glucose (to energize an efflux pump) had little effect on the accumulation of rifampicin in the presence or absence of reserpine for M. tuberculosis; however, for M. aurum and M. smegmatis the small reserpine effect was abolished by the addition of glucose.

Discussion

It was generally thought that, with the introduction of rifampicin and isoniazid to treat TB and BCG vaccination to prevent TB, the disease was in retreat, and research into TB between the 1970s and the 1980s was minimal. As a result, many aspects of TB infection and chemotherapy have not been determined. For example, the mode of action of, and mechanisms of resistance to, some of the first line agents, e.g. ethambutol and isoniazid, were not fully understood for 30 years after their introduction. Similarly, few publications describe the permeation of drugs across the mycobacterial cell wall. This is the first investigation into the accumulation of rifampicin by mycobacteria.

In the present study, higher concentrations of rifampicin were accumulated by M. aurum than the other two mycobacterial species. The time taken for the SSCs to be reached varied between the three species, with both M. aurum and M. tuberculosis achieving an equilibrium concentration within 10 min of exposure. Although the concentrations of rifampicin accumulated did not appear to correlate with the MIC of rifampicin for each species, there was a correlation between the time taken for the SSC to be reached and the MIC value: with an increase in the MIC a lower rate of drug influx was observed. It has been widely proposed that a major factor in the intrinsic resistance of M. smegmatis and atypical mycobacteria to rifampicin is a cell wall permeability barrier. Based on the data from this study, it may be that the intrinsic resistance of M. smegmatis is due to a reduced rate of drug influx. The effect of temperature and external concentration of rifampicin upon accumulation by M. tuberculosis suggests that uptake is by passive diffusion.

There have been several reports of the effects of ethambutol on the mycobacterial cell wall structure and the effect of ethambutol on increasing anti-mycobacterial activity of other therapeutic drugs. Ethambutol has been proposed to act upon several cell wall components including the transfer of mycolic acids into the cell wall, and inhibition of the incorporation of arabinogalactan and arabinan into lipopolysaccharide. In order to gain further insight into the mechanism of synergy between rifampicin and ethambutol, the concentration of rifampicin accumulated in the absence and presence of ½ × MIC of ethambutol was determined. Ethambutol increased the concentration of rifampicin accumulated by all three mycobacterial species, with M. aurum most affected. These data confirm the proposals that the synergy between rifampicin and ethambutol is a direct consequence of increased cell wall permeability to the drug.

Tween 80 belongs to a class of non-ionic surface-active detergents often added to liquid media to reduce cell clumping and obtain homogeneous cell suspensions of mycobacteria. Mycobacterial clumping can cause problems when OD and viable count measurements are required. It has been proposed that Tween 80 acts directly on the mycobacterial cell wall and subsequently alters its permeability. Tween 80 has also been shown to alter colony morphology of M. avium complex and M.ycobacterium paratuberculosis and acts synergically with those drugs with an intracellular target. Mycobacteria may also be less virulent after growth in the presence of Tween 80, again suggesting an effect on the bacterial cell surface. Despite Tween 80 acting synergically with rifampicin, lower concentrations were accumulated after growth in this detergent than by cells grown in glycerol. This is counter-intuitive to accepted dogma for the mechanism of Tween 80 synergy. There are two possible explanations: (i) Tween 80 exerts a bactericidal or bacteriostatic activity such that there are fewer viable cells present in an accumulation experiment, thereby giving rise to lower accumulation values, or (ii) the cell-associated rifampicin may represent a significant concentration of rifampicin dissolved into the lipid domains of the mycobacterial cell wall. Tween 80 has been proposed to disrupt cell wall lipids and disruption of these domains may increase the concentration of rifampicin released from the cell wall during the washing step of the accumulation protocol leading to an overall reduced SSC. All three species of mycobacteria yielded lower viable counts after growth in Tween 80 compared with glycerol, supporting the first hypothesis. Despite using an equivalent OD cell suspension in the accumulation assay, when the viable count was determined it was demonstrated that this gave rise to fewer numbers of cells after growth in Tween 80. It should be noted that the water sonication step that was used to eradicate clumping of mycobacteria after growth in glycerol was also used in the Tween 80 experiments.

The small but reproducible effect of the efflux inhibitor reserpine suggests that rifampicin can be ejected by mycobacteria. The small effect of efflux inhibitors is consistent with an efflux pump expressed at low level in wild-type bacteria, and has been observed with other wild-type bacteria and antibiotics. Reversal of the effect by glucose further supports these data, as work with Streptococcus pneumoniae has shown that glucose provides energy for the fluoroquinolone efflux pump. The presence of an efflux pump in wild-type mycobacteria suggests that mutants might arise with enhanced efflux, mediating rifampicin resistance. It is possible that rpoB-mutation-negative rifampicin-resistant mycobacteria are just such mutants. Consistent with this hypothesis is the identification of an efflux pump(s) in M. smegmatis and M. tuberculosis and a plasmid-mediated pump that removes rifampicin in Pseudomonas fluorescens.

Knowledge of drug transport in mycobacteria may aid
in the development of new agents such as those with enhanced entry into cells, or efflux pump inhibitors, and/or agents not recognized as a substrate for the efflux pump(s). Further investigations on the permeability of mycobacteria to existing and potential novel agents, and the role permeability plays in drug resistance are imperative.

Acknowledgement
This work was supported in part by the Glaxo Wellcome A ction TB Initiative studentship to Kerstin Williams.

References

Received 13 May 1999; returned 27 July 1999; revised 21 September 1999; accepted 4 October 1999