Accumulation of KRM-1648 by *Mycobacterium aurum* and *Mycobacterium tuberculosis*

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After exposure to 2 mg/L 

14C-labelled KRM-1648 (a new broad-spectrum benzoxazinorifamycin antibiotic) for 5 min, a steady-state concentration of 31.3 ± 3 ng/mg cells KRM-1648 and 12.6 ± 0.3 ng/mg cells KRM-1648 was accumulated by wild-type antibiotic-susceptible *Mycobacterium aurum* (A+) and *Mycobacterium tuberculosis* (H37Rv), respectively. However, 2 mg/L KRM-1648 was bactericidal for *M. tuberculosis*. A steady-state concentration of 3.7 ± 0.1 ng/mg cells KRM-1648 was accumulated after exposure to 0.5 mg/L. At pH 4 higher concentrations were accumulated than at pH 7. A sub-inhibitory concentration of ethambutol increased the concentration of KRM-1648 accumulated, but Tween 80 and reserpine had little or no effect.

**Introduction**

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

KRM-1648 is a new broad-spectrum benzoxazinorifamycin antibiotic with good *in vitro* activity against mycobacteria, including *M. tuberculosis*. In a murine model, good activity of KRM-1648 has also been shown for *M. tuberculosis*. KRM-1648 inhibits the bacterial RNA polymerase activity of *M. tuberculosis*, presumably (as for rifampicin) by binding to the β-subunit encoded by rpoB, and forming a stable drug–enzyme complex, preventing transcription of RNA from the DNA template. Mutations giving rise to rifampicin resistance have been shown to cluster in rpoB. Clinical isolates of *M. tuberculosis* with mutations in rpoB are more susceptible to KRM-1648 than rifampicin, and some strains require <16 mg/L KRM-1648 for inhibition and may be considered clinically susceptible. KRM-1648 also accumulates rapidly within human macrophages, explaining in part, the improved therapeutic efficacy demonstrated in animal models of *Mycobacterium avium* and *M. tuberculosis* infection.

We have already established a procedure for measuring accumulation of rifampicin by bacteria, including mycobacteria. The aim of this study was to investigate the accumulation of KRM-1648 by wild-type *M. tuberculosis*.

**Materials and methods**

**Antibiotics and chemicals**

14C-labelled KRM-1648 (specific activity 15 μCi/mg) was generously provided by The Kaneka Corporation (Osaka, Japan). Radiochemical purity was determined by HPLC by Corning Hazleton and was found to be 94.1%. Biological activity of the radiolabelled KRM-1648 and non-radiolabelled drug was determined by measuring the MIC. All other agents were from Sigma (Poole, UK). KRM-1648 was dissolved in 10 mM Tris–HCl, pH 3; ethambutol, Tween 80 and reserpine were prepared according to their manufacturer’s instructions.

**Bacterial strains, growth conditions and antibiotic susceptibility testing**

*Mycobacterium aurum* A+ (Pasteur Institute, Paris, France) and *M. tuberculosis* H37Rv were maintained on...
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Lowenstein–Jensen slopes and cultured on Middlebrook 7H11 agar (Difco, West Molesey, 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. The MIC of each agent was determined as described previously. The plates were incubated for 48 h for \textit{M. aurum}, and 21 days for \textit{M. tuberculosis}. The MIC was defined as the lowest concentration of drug at which no visible growth was observed.

Measurement of KRM-1648 accumulation by mycobacteria

The concentration of 14C-labelled KRM-1648 accumulated was determined essentially as for rifampicin and as described previously. The effects of ethambutol, Tween 80 and reserpine on the accumulation of radiolabelled KRM-1648 by \textit{M. tuberculosis} were also determined as described previously for rifampicin.

Statistical analysis

The differences in the accumulation data obtained for each species were compared and the mean steady-state concentration values were analysed by Student’s \( t \) test. A \( P \) value of <0.05 was considered significant.

Results

Accumulation of 14C-labelled KRM-1648

A method to determine accumulation of 14C-labelled rifampicin was established for \textit{Staphylococcus aureus} and mycobacteria including \textit{M. tuberculosis} in two previous studies. Although the MIC of rifampicin for \textit{S. aureus} and \textit{M. tuberculosis} is 0.002 mg/L and 0.25 mg/L, respectively, the optimum concentration of radiolabelled rifampicin for accumulation studies was 2 mg/L with both species; in the time frame (≈20 min) of the accumulation experiment this concentration had no deleterious effect upon cell viability or growth. Therefore, although the MIC of KRM-1648 was 0.25 mg/L for \textit{M. aurum} and \textit{M. tuberculosis}, all initial experiments were performed with 2 mg/L radiolabelled KRM-1648. The MIC values of the radiolabelled and unradioled KRM-1648 were identical (data not shown). With 2 mg/L KRM-1648 and \textit{M. aurum} a steady-state concentration of 21.3 ± 2.9 ng/mg cells 14C-labelled KRM-1648 was obtained within 5 min of exposure to 14C-labelled KRM-1648 (Figure 1).

With 2 mg/L KRM-1648 and \textit{M. tuberculosis}, a steady-state concentration of 12.64 ± 0.3 ng/mg cells 14C-labelled KRM-1648 was obtained after 5 min exposure (Figure 1), but decreased slightly over the next 15 min. At 0°C, 3.99 ± 0.4 ng/mg cells radiolabelled KRM-1648 was adsorbed, and the calculated ‘intracellular’ concentration was 8.5 ± 0.6 ng/mg cells radiolabelled KRM-1648. After 15 min exposure to 2 mg/L radiolabelled KRM-1648, a decrease in the viable count from \( 2.7 \times 10^7 \) cfu/mL to \( 2.0 \times 10^7 \) cfu/mL was observed. Therefore, subsequent experiments were performed with two concentrations of radiolabelled KRM-1648: 0.5 and 2 mg/L. At 0.5 mg/L there was no significant decrease in viable count over the time course of the accumulation experiment, and because of low specific activity of 14C-labelled KRM-1648 and low concentrations accumulated, this concentration was the lowest limit of detection. At 0.5 mg/L and 37°C, 3.7 ± 0.1 ng/mg cells radiolabelled KRM-1648 was accumulated; at 0°C 0.8 ± 0.04 ng/mg cells radiolabelled KRM-1648 was adsorbed, and the calculated ‘intracellular’ concentration was 2.95 ± 0.1 ng/mg cells radiolabelled KRM-1648.

Effect of pH on accumulation of radiolabelled KRM-1648

KRM-1648 dissolves more readily at acid pH (Hidaka, personal communication), so the effect of measuring accumulation at acid versus neutral pH was determined. After exposure to 0.5 or 2 mg/L KRM-1648, slightly greater concentrations were accumulated at pH 4 than at pH 7 (Figure 2). The increase from 12.99 ± 0.4 ng/mg cells at pH 7 to 14.6 ± 0.3 ng/mg cells at pH 4 was statistically significant (\( P < 0.005 \)).

Effect of ethambutol on accumulation of radiolabelled KRM-1648

The MIC of KRM-1648 was reduced from 0.25 mg/L to 0.12 mg/L in the presence of 0.25 mg/L ethambutol (four-fold less than the MIC of ethambutol alone). The concentration of radiolabelled KRM-1648 accumulated by
Mycobacterial accumulation of KRM-1648

Figure 2. Summary of the concentration of KRM-1648 (0.5 and 2 mg/L) accumulated after 5 min exposure at pH 7 in the presence and absence of ethambutol, Tween 80 and reserpine. (a) 0.5 mg/L KRM-1648; (b) 0.5 mg/L KRM-1648 + 0.25 mg/L ethambutol; (c) 0.5 mg/L KRM-1648 + 20 mg/L reserpine; (d) 0.5 mg/L KRM-1648 + 0.05% Tween 80; (e) 2 mg/L KRM-1648; (f) 2 mg/L KRM-1648 + 0.25 mg/L ethambutol; (g) 2 mg/L KRM-1648 + 20 mg/L reserpine; (h) 2 mg/L KRM-1648 + 0.05% Tween 80.

M. tuberculosis after exposure to 2 mg/L was increased in the presence of 0.25 mg/L ethambutol from 13.09 ± 0.6 ng/mg dry cells to 18.45 ± 0.9 ng/mg dry cells (Figure 2). This increase was statistically significant (P < 0.005).

Effect of Tween 80 on the accumulation of radiolabelled KRM-1648

The MIC of KRM-1648 was reduced from 0.25 mg/L to 0.12 mg/L in the presence of 0.05% Tween 80 (four-fold less than the MIC of Tween 80 alone). Although Tween 80 had a slight synergic effect on the antimicrobial activity of KRM-1648, the concentration of radiolabelled KRM-1648 accumulated by M. tuberculosis was unchanged in the presence of 0.05% Tween 80 (Figure 2). One explanation for these data is that growth in 0.05% Tween 80 gives rise to lower numbers of M. tuberculosis cells than growth in glycerol, and that it is this lower number that gives rise to the lower steady-state concentrations. To examine this, the viable count of M. tuberculosis was determined after growth in 0.05% Tween 80 with or without KRM-1648 compared with parallel cultures grown with glycerol. The viable count was slightly lower for the cultures grown in 0.05% Tween 80 (2.6 × 10^7 cfu/mL) than those grown with glycerol (2.7 × 10^7 cfu/mL) and in the presence of KRM-1648 plus Tween 80 it was further reduced (1.9 × 10^7 cfu/mL).

Effect of the efflux inhibitor reserpine on the concentration of radiolabelled KRM-1648 accumulated

Reserpine (20 mg/L) had no effect upon the MIC of KRM-1648 and a minimal effect upon accumulation, increasing the concentration of radiolabelled KRM-1648 accumulated by 0.5 ± 0.2 mg/mg dry cells. This was not statistically significant.

Discussion

In the present study, accumulation of a new rifamycin, KRM-1648, was investigated. It took 5 min for a steady-state concentration of KRM-1648 to be accumulated, whereas for rifampicin an apparent steady state was reached within 1–2 min. In addition, lower concentrations of KRM-1648 were accumulated than of rifampicin. However, KRM-1648 and rifampicin had the same MIC for this strain of M. tuberculosis. It was previously postulated that the rate of influx of rifampicin was associated with activity and not with the steady-state concentration, and (as for rifampicin) the concentrations of KRM-1648 accumulated do not correlate with the MIC value. For rifampicin accumulation in Escherichia coli and S. aureus we postulated that as the concentration accumulated mirrored the IC_{50} value for rifampicin inhibition of the target enzyme, then the steady-state concentration reflected binding of the drug to RNA polymerase. The same may also be true for KRM-1648, and the rate of influx is sufficiently rapid to ensure that KRM-1648 reaches the target to give rise to a similar MIC value to that for rifampicin. For strains of M. tuberculosis with greater susceptibility to KRM-1648 than rifampicin, it is likely that the target enzyme is more susceptible to the drug.

A well-established effect of ethambutol is to lower the MIC values of several antimycobacterial agents. We have previously shown that the concentration of rifampicin accumulated in the presence of ethambutol increased, supporting the hypothesis that ethambutol interacts with components of the mycobacterial cell wall increasing cell wall permeability. The data obtained for KRM-1648 further support the proposal that the synergy between antibiotics and ethambutol is a direct consequence of increased cell wall permeability to the drugs.

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 the study with rifampicin, and also in the present study with KRM-1648, it was found that Tween 80 had no effect upon the concentration of these antibiotics accumulated by M. tuberculosis. Although this is counter-intuitive to accepted dogma for the mechanism of action of Tween 80, we have also found that Tween 80 has slight bactericidal activity such that there are less viable cells present in an accumulation experiment and in our opinion thus giving rise to lower accumulation values. The antimicrobial synergy probably results from the additive effect of the bactericidal activity and not with the steady-state concentration.
of Tween 80 plus that of KRM-1648. The mechanism of bactericidal action of the Tween 80 is unknown.

KRM-1648 dissolves more readily at acid pH, and was also found to accumulate to higher concentrations when the experiment was performed at pH 4. These data suggest that an acidic environment may enhance the activity of KRM-1648 against \textit{M. tuberculosis} in vivo. As for rifampicin, there was a small, statistically insignificant, effect of the efflux inhibitor reserpine upon the concentration of KRM-1648 accumulated. The role of efflux in attenuating the activity of KRM-1648, if any, will need evaluating with a characterized efflux mutant of \textit{M. tuberculosis}.

In conclusion, the good antimycobacterial activity of KRM-1648 probably results from the rapid influx of this agent combined with high affinity for the target enzyme.

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References


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