Antimicrobial activities of benzoxazinorifamycin KRM-1648, clarithromycin and levofloxacin against intracellular Mycobacterium avium complex phagocytosed by murine peritoneal macrophages

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The in-vitro activities of KRM-1648, a new benzoxazinorifamycin, clarithromycin and levofloxacin against clinical isolates of Mycobacterium avium complex (MAC) were measured using various methods of assay and compared with their in-vivo therapeutic activities against MAC infection in mice. The MICs varied according to drug in the order KRM-1648 < clarithromycin < levofloxacin. However, KRM-1648 and clarithromycin but not levofloxacin had similar therapeutic outcomes in MAC-infected mice. KRM-1648 and clarithromycin given at clinical dosages caused 1 to 2 log unit reductions in bacterial loads in the lungs of host mice. The values of $C_{\text{max}}$ (lung)/MBC were more closely related to the therapeutic efficacy of these drugs in mice than were MICs and MBCs alone. Potent microbicidal activity was observed with KRM-1648 and clarithromycin but not with levofloxacin against extracellularly growing MAC (EG-MAC) in a liquid medium. These two agents caused more than 3 log unit killing of MAC during a 5 day incubation, when added at concentrations equivalent to $C_{\text{max}}$ (lung). The anti-EG-MAC bactericidal activity of these drugs was greater than their efficacy in mice in vivo. KRM-1648 and clarithromycin but not levofloxacin caused respectively 2 and 0.5 log unit killing of intracellularly growing MAC (IG-MAC) in murine peritoneal macrophages. The profiles of bacterial killing effects of these agents against IG-MAC accurately reflected their therapeutic effects in mice, although the in-vivo activity of KRM-1648 was still overestimated using even this parameter.

Introduc}on

Mycobacterium avium complex (MAC) causes disseminated infections in HIV-infected patients in the advanced stage of AIDS, and such infections are largely intractable, in part because of the severely depressed state of host defence mechanisms in AIDS patients due to suppression of T cell-mediated immunity accompanied by severe reduction in macrophage antimicrobial capacity.

A new benzoxazinorifamycin, KRM-1648, and clarithromycin have been shown to have potent anti-MAC activity in vitro. These two drugs also have significant therapeutic efficacy against MAC infection induced in mice. Although KRM-1648 had much lower MICs against MAC than other antituberculosis drugs, its therapeutic efficacy against MAC infection in mice was not as good as expected. Since macrophages are central effector cells of host defence mechanisms against mycobacteria, this discrepancy between in-vitro and in-vivo results appears to be in part due to the difference in the effect on intracellularly growing MAC (IG-MAC) in host macrophages from that on extracellularly growing MAC (EG-MAC) in culture medium. The bacteriological response in MAC patients to treatment with clarithromycin depends on the in-vitro susceptibility of individual MAC strains to the drug. The profiles of the antimicrobial effects of KRM-1648 and clarithromycin against IG-MAC in host macrophages are of particular interest, especially whether IG-MAC killing ability of these drugs is closely related to their therapeutic efficacy.

In this study, we evaluated the anti-MAC antimicrobial activities of KRM-1648, clarithromycin and levofloxacin, a new quinolone with potent activity against Mycobacterium tuberculosis and Mycobacterium kansasii in vitro and in vivo.
as judged by their MICs and MBCs, killing of EG-MAC and IG-MAC, and therapeutic efficacy against MAC infection in mice in vivo.

Materials and methods

Organisms

M. avium strain N-444 and Mycobacterium intracellulare strain N-260, both of which we had isolated from patients with MAC infection, were cultured in 7H9 broth (Difco Laboratories, Detroit, MI, USA) supplemented with 10% (v/v) ADC (albumin–dextrose-catalase) enrichment and 0.05% (v/v) Tween 80, and bacterial suspensions prepared with phosphate buffered saline containing 0.1% (w/v) bovine serum albumin were frozen at −80°C until use.

Mice

Female BALB/c mice were purchased from Japan Clea Co., Osaka, Japan.

Antimicrobial agents

KRM-1648 (KANEKA Corporation, Hyogo, Japan) and clarithromycin (Taisho Pharmaceutical Co., Tokyo, Japan) initially dissolved in dimethyl sulphoxide and levofloxacin (Daiichi Pharmaceutical Co., Tokyo, Japan) dissolved in 0.1 N NaOH, were diluted in the prescribed media before use. The $C_{\text{max}}$ (blood) and $C_{\text{max}}$ (lung) in mice orally administered each drug at the dose equivalent to the clinical dosage were as follows: KRM-1648 (2 mg/kg), 0.7 and 0.8 mg/L; clarithromycin (10 mg/kg), 0.8 and 7.0 mg/L; levofloxacin (5 mg/kg), 2.0 and 2.0 mg/L.

MIC determination

MICs of test agents were determined as previously reported with slight modifications. Briefly, the bacterial culture in 7H9 medium was diluted in 0.1% Tween 80–saline to an optical density at 540 nm of 0.01, and bacterial suspension containing about $5 \times 10^4$ cfu was spotted on to 7H11 agar plates containing test drugs using a microplanter. After cultivation at 37°C for 14 days, MICs were read as minimum concentrations of drugs allowing no more than five colonies to grow. Alternatively, MICs were determined by the broth dilution method using 7HSF medium, a broth medium with the same composition as 7H11 agar without malachite green as described by Yajko et al. The bacterial suspension in 7HSF medium (0.1 mL) containing $1 \times 10^5$ cfu was inoculated into 0.1 mL of 7HSF medium containing test drugs in microculture wells (Japan Becton Dickinson & Co., Osaka, Japan). After cultivation at 37°C for 14 days, MICs were read as minimum concentrations of drugs completely inhibiting visible growth of organisms.

MBC determination

After MIC determination using 7HSF medium, MBC was determined by inoculating 10 $\mu$L samples from the wells, in which test agents allowed no visible growth of the organisms, on to a 7H11 agar plate, followed by 14 day cultivation at 37°C in a CO$_2$ incubator. MBCs were read as minimum concentrations of drugs causing >99.9% killing of the inoculated organisms.

Antimicrobial activity against EG-MAC in a liquid medium

Test organisms ($2 \times 10^4$ cfu) were inoculated into 16 mm culture wells (Becton Dickinson) containing 1.0 mL of antibiotic-free RPMI 1640 medium supplemented with 5% (v/v) fetal bovine serum (FBS) (BioWhittaker Co., Walkersville, M D, USA) with or without the addition of the drug at either $C_{\text{max}}$ or 0.1 $\times C_{\text{max}}$ in the lungs of mice. The wells were then incubated at 37°C for up to 5 days. At intervals, the organisms were harvested and washed twice.

Table I. MICs and MBCs of KRM-1648, clarithromycin and levofloxacin against M. avium N-444 and M. intracellulare N-260 strains measured in 7H 11 or 7HSF medium

<table>
<thead>
<tr>
<th>Drug</th>
<th>Organism</th>
<th>MIC (mg/L) in 7H11</th>
<th>MIC (mg/L) in 7HSF</th>
<th>MBC (mg/L) a</th>
<th>MBC/MIC a</th>
<th>$C_{\text{max}}$/MIC a</th>
<th>$C_{\text{max}}$/MBC a</th>
</tr>
</thead>
<tbody>
<tr>
<td>KRM-1648</td>
<td>M. avium</td>
<td>0.025</td>
<td>0.06</td>
<td>1</td>
<td>16</td>
<td>11.7</td>
<td>13.3</td>
</tr>
<tr>
<td></td>
<td>M. intracellulare</td>
<td>0.025</td>
<td>0.06</td>
<td>1</td>
<td>16</td>
<td>11.7</td>
<td>13.3</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>M. avium</td>
<td>6.25</td>
<td>8</td>
<td>32</td>
<td>4</td>
<td>0.10</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>M. intracellulare</td>
<td>6.25</td>
<td>4</td>
<td>16</td>
<td>4</td>
<td>0.21</td>
<td>1.8</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>M. avium</td>
<td>25</td>
<td>16</td>
<td>32</td>
<td>2</td>
<td>0.13</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>M. intracellulare</td>
<td>25</td>
<td>16</td>
<td>32</td>
<td>2</td>
<td>0.13</td>
<td>0.13</td>
</tr>
</tbody>
</table>

a Calculated from measurements in 7HSF medium. $C_{\text{max}}$ was determined in the blood and lungs of mice.
with distilled water by centrifugation (1700g, 30 min) to reduce carryover of antimicrobial agents. The organisms were then resuspended in distilled water (1.0 mL) and 10 μL aliquots of serial ten-fold dilutions were spotted on 7H11 agar plates. The number of residual bacterial cfu was counted after 5–7 day cultivation at 37°C in a CO₂ incubator by microscopy at ×15 magnification.

Antimicrobial activity against IG-MAC

Macrophage monolayer cultures were prepared by seeding 1×10⁶ of Zymosan A-induced peritoneal exudate cells of 8–12-week-old BALB/c mice on 16 mm culture wells. The cultures were incubated in 5% FBS-RPMI 1640 medium (antibiotic-free) (1 mL), containing 2×10⁶ cfu of the MAC at 37°C in a CO₂ incubator (5% CO₂–95% air) for 2 h. After washing with 2% FBS–Hanks’ balanced salt solution (HBSS) to remove extracellular organisms, the MAC-infected macrophages were cultured for up to 5 days in 5% FBS–RPMI 1640 medium (1 mL) in the presence or absence of test drugs at the Cₘₐₓ or 0.1× Cₘₐₓ found in mouse lung. At intervals, the macrophages were lysed with 0.07% (w/v) sodium dodecysulphate, washed with distilled water by centrifugation, and the numbers of residual bacterial cfu in the resultant samples were counted as above.

Experimental infection

Five-week-old BALB/c mice were infected intravenously with 1×10⁷ cfu of M. avium N-444 strain. They were given no drug or KRM-1648, clarithromycin or levofloxacin at doses of 2, 10 or 5 mg/kg, respectively, by gavage, five times per week, for 8 weeks from week 5 to the end of the experiment. At intervals (day 1 and weeks 5 and 13), mice were killed and the bacterial load in the lungs estimated by counting the number of cfu in the homogenates of individual organs following culture on Middlebrook 7H11 agar plates.

Results

MICs and MBCs of test drugs

Table I shows values obtained for the MIC, MBC and MBC/MIC, and the antimicrobial/pharmacokinetic para-

![Figure 1](image-url)

**Figure 1.** Antimicrobial activity of KRM-1648 against extracellularly growing (a, c) and phagocytosed intracellularly growing organisms (b, d) of M. avium N-444 (a, b) and M. intracellulare N-260 (c, d). KRM-1648 was added in 5% FBS-RPMI medium at concentrations equivalent to Cₘₐₓ (0.8 mg/L) and 0.1× Cₘₐₓ (0.08 mg/L) in the lungs of mice after oral administration at the clinical dosage (2 mg/kg). ○, None added; ●, KRM-1648 at 0.1× Cₘₐₓ; ▲, KRM-1648 at 0.1× Cₘₐₓ. Each plot indicates the mean ± S.E.M. (n = 3; error bars were omitted when the S.E.M. was < 0.1). Representative results of repeated experiments are indicated. Asterisked values were significantly different from the control value (none added) (*, P < 0.05; **, P < 0.01; Student’s t-test).
meters $C_{\text{max}}$/MIC and $C_{\text{max}}$/MBC for KRM-1648, clarithromycin and levofloxacin for M. avium N-444 and M. intracellulare N-260. The agar and broth dilution methods yielded comparable MICs and MBCs for each drug, varying according to drug in the order KRM-1648 < clarithromycin < levofloxacin. $C_{\text{max}}$ (lung)/MIC and $C_{\text{max}}$ (lung)/MBC also varied from drug to drug in the order, KRM-1648 > clarithromycin > levofloxacin. The variance was much larger for the former than for the latter ratio. $C_{\text{max}}$ (lung)/MBC values for KRM-1648 and clarithromycin were below 1.0, suggesting that effective inhibition of the in-vivo anti-MAC microbicidal activity of these drugs may not occur in the lungs.

Figure 2. Antimicrobial activity of clarithromycin against extracellularly growing (a, c) and phagocytosed intracellularly growing organisms (b, d) of M. avium N-444 (a, b) and M. intracellulare N-260 (c, d). Clarithromycin was added in the medium at concentrations equivalent to $C_{\text{max}}$ (7.0 mg/L) and $0.1 \times C_{\text{max}}$ (0.7 mg/L) in the lungs of mice after oral administration at the clinical dosage (10 mg/kg). ○, None added; ●, + clarithromycin at $0.1 \times C_{\text{max}}$; ▲, + clarithromycin at $C_{\text{max}}$. Details are the same as for Figure 1.

Antimicrobial activity of test agents against EG-MAC and IG-MAC

Next, we studied the antimicrobial activities of KRM-1648, clarithromycin and levofloxacin against EG-MAC and IG-MAC. In these experiments, test drugs were added to the culture medium at $C_{\text{max}}$ or $0.1 \times C_{\text{max}}$, which are achievable in the lungs of mice at clinical dosages. KRM-1648 caused significant killing of EG-MAC even at $0.1 \times C_{\text{max}}$ (lung) and more rapid killing at $C_{\text{max}}$ (lung) (Figure 1). KRM-1648 had significant bactericidal effects against IG-MAC at the same concentrations. However, its efficacy in killing IG-MAC was less than that in killing EG-MAC. Clarithromycin potently killed EG-MAC at $C_{\text{max}}$ (lung) and had a weak bactericidal effect even at $0.1 \times C_{\text{max}}$ (lung) (Figure 2). Clarithromycin at $C_{\text{max}}$ (lung) also exhibited significant bactericidal activity against IG-MAC but its efficacy in killing IG-MAC was much less than its activity against EG-MAC. Third, levofloxacin at $C_{\text{max}}$ (lung) exerted a weak bactericidal or only a bacteriostatic effect against EG-MAC (Figure 3). Although levofloxacin did not kill IG-MACs, it slightly inhibited the growth of IG-MAC at $C_{\text{max}}$ (lung). Similar results were obtained when the antimicrobial effects of KRM-1648, clarithromycin and levofloxacin against EG-MAC were examined using 7FSF medium (data not shown).
Therapeutic efficacies of the three drugs against MAC infection in mice

Table II shows the therapeutic efficacy of KRM-1648, clarithromycin and levofloxacin against M. avium N-444 infection in mice. KRM-1648 and clarithromycin each caused an approximately 1.0 log unit reduction ($P < 0.001$) in bacterial loads in the lungs compared with those in control mice, when they were given to mice at doses of 2 and 10 mg/kg, respectively, once daily, five times per week, for 8 weeks from week 5 to the end of the experiment, starting from week 5.
8 weeks from week 5 to the end of the experiment. On the other hand, levofloxacin exhibited no significant therapeutic effect.

**Discussion**

In the present study, we evaluated the anti-MAC activities of KRM-1648, clarithromycin and levofloxacin in vitro using various methods of assay. The parameters of in vitro activity, MIC, MBC, C_max/MIC and C_max/MBC, varied according to drug in the order KRM-1648 > clarithromycin > levofloxacin. This variation was most marked in the case of MIC. When the anti-MAC activity of test agents was expressed using a parameter based on the killing of IG-MAC, the degree of variation by drug was much decreased.

In this study, KRM-1648 and clarithromycin exhibited similar levels of therapeutic effects against M. avium infection in mice, when doses equivalent to clinical regimens (2 and 10 mg/kg, respectively) were begun 5 weeks after infection (Table II). We previously observed similar therapeutic effects in M. avium- and M. intracellulare-infected mice which were given these drugs from the day after infection. On the other hand, levofloxacin at the clinical dose (5 mg/kg) failed to exhibit therapeutic effects (Table II). Related to this, we found previously that ofloxacin (half as potent as levofloxacin) had no significant therapeutic effects in MAC-infected mice, and levofloxacin has been reported to be ineffective in controlling murine MAC infection even at a dose of 50 mg/kg.

Generally, the MIC and MBC of drugs are poorly predictive of their therapeutic efficacy in patients with MAC infection, although the therapeutic effect of clarithromycin can be predicted fairly well from its MIC. In the present study, the in-vitro anti-MAC activity of KRM-1648 in terms of MIC was also stronger than its therapeutic effects in mice (Tables I and II). However, the use of a pharmacokinetic/antimicrobial parameter, in particular C_{max} /MIC, enabled prediction of the bacteriologic response in drug-treated mice more precisely than did use of MIC or MBC alone.

Other parameters of the efficacy of KRM-1648, clarithromycin and levofloxacin, i.e., bactericidal activity against EG-MAC and IG-MAC (Figures 1-3), were examined to determine their usefulness in predicting therapeutic response in drug-treated mice (Table II). The anti-MAC activities of these agents were again in the order KRM-1648 > clarithromycin > levofloxacin. Both KRM-1648 and clarithromycin at C_{max} (lung) caused potent killing of EG-MAC. The observed activities of these drugs against EG-MAC was much stronger than their therapeutic efficacy in mice. Bactericidal effects of these drugs against the IG-MAC were much less than against EG-MAC. The anti-IG-MAC activity of clarithromycin at C_{max} (lung) was reduced to levels comparable to its therapeutic effects against murine MAC infection. On the other hand, KRM-1648 still had fairly potent microbicidal effects against IG-MAC, almost completely killing the organisms during cultivation for 5 days. The anti-IG-MAC activity of KRM-1648 was stronger than its therapeutic effects in mice, since KRM-1648 had only a weak to moderate therapeutic effect in mice and failed to overcome bacterial growth in the lungs and spleens of host mice in the late period of MAC infection (Table II). Therefore, it is difficult to predict the therapeutic effect of KRM-1648 in mice, even based on the profiles of its efficacy of killing IG-MAC.

The reasons for failure to predict outcome using the parameters of anti-MAC killing determined with the macrophage cocultivation system are unclear. However, Mehta et al. have reported that intracellular multiplication of M. tuberculosis is much more vigorous in the human alveolar pneumocyte epithelial cell line A-549 than in human monocytes. In addition, they found that the organisms that invaded lung epithelial cells resisted the antimicrobial effects of amikacin, while the parasites phagocytosed by monocytes were easily killed by this antibiotic. Our preliminary study also indicated that MAC grew much more rapidly in A-549 cells than in murine peritoneal macrophages. Moreover, MAC organisms living in A-549 cells were more resistant to the microbicidal effects of KRM-1648 and clarithromycin than were the MAC in macrophages. These findings suggest that some non-professional phagocytes play important roles as sites of infection and multiplication of MAC. Further studies are currently under way to examine the antimicrobial effects of these agents against IG-MAC in A-549 cells.

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**References**


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