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Activities of the Benzoxazinorifamycin KRM 1648 and Ethambutol against *Mycobacterium avium* Complex In Vitro and in Macrophages

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KRM 1648 is a 4-aminobenzoxazine derivative of rifamycin S with potent in vitro activity against the *Mycobacterium avium* complex (MAC); the MIC for 90% of 24 MAC isolates from AIDS patients was 0.25 μg/ml as determined by a radiometric broth macrodilution assay. KRM 1648 was bactericidal for MAC isolates in Middlebrook 7H9 broth, with a reduction in viability of 1 to 4 orders of magnitude over 72 h. In human macrophages, KRM 1648 also was bactericidal, with a reduction of 3 to 4 orders of magnitude in CFU per ml of macrophage lysate at a concentration of 1 μg/ml; however, the bactericidal activity varied approximately 10-fold among the three MAC serovars tested. In growth medium, ethambutol potentiated the effect of KRM 1648, but this potentiation was modest when tested against MAC in macrophages and also varied between MAC strains. KRM 1648 has potential as an antimycobacterial agent for MAC disease, perhaps in combination with other agents so that the use of lower dosages of KRM 1648 than are needed with other rifamycins may be possible.

Although the therapeutic options available for the treatment of serious *Mycobacterium avium* complex (MAC) disease have improved in the past few years (7), notably by the addition of new macrolides such as clarithromycin and azithromycin (3, 4, 15), no single compound or combination that is as potent and bactericidal against the MAC as are rifampin and isoniazid against the *Mycobacterium tuberculosis* complex has been identified. Ethambutol potentiates the activity of other antimicrobial agents against MAC isolates in general and may have therapeutic activity alone against certain isolates (1, 10). Thus, the combination of ethambutol and a rifamycin might achieve multiple objectives, including improved efficacy, prevention of resistance, and lower dosages. The last objective is of increasing interest because of the adverse effects associated with multiple-drug regimens including rifamycins. Finally, there is a clear need for additional antimicrobial agents with activity against the MAC to provide options in the face of established resistance and therapeutic failure and to achieve a more rapid clinical response. KRM 1648 is a 4-alkyl-1-piperazinyl derivative of 3′-hydroxy-5′-aminobenzoxazinorifamycin in which the alkyl in the 4-position is an isobutyl moiety (14). KRM 1648 was shown to have little in vitro activity against enteric gram-negative bacteria but potent in vitro activity against gram-positive microorganisms and mycobacteria, including *M. tuberculosis* and MAC organisms (11, 14). In an effort to both confirm and expand on these observations, we tested KRM 1648 alone and in combination with ethambutol in a variety of in vitro and macrophage studies.

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MATERIALS AND METHODS

Organisms. In vitro and macrophage studies were done with a collection of 24 MAC strains isolated from human immunodeficiency virus-infected patients with culture-documented disseminated MAC disease. The strains included in this collection were chosen to be representative of the serovars commonly associated with this disease as well as a spectrum of antimicrobial susceptibility patterns on the basis of the activities of a variety of other drugs previously tested against these isolates. Some studies were restricted to a few strains, and these were chosen on the basis of certain characteristics such as virulence in the beige mouse model of infection or colony type (e.g., exclusively transparent colony type).

Drugs. KRM 1648 was kindly provided by Kaneka Corporation, Osaka, Japan; rifabutin was provided by Adria Laboratories, Columbus, Ohio; and clarithromycin was provided by Abbott Laboratories, Abbott Park, Ill. All other drugs were obtained from the manufacturer or other commercial sources.

MIC measurements. The in vitro activities of drugs were determined by measuring the MICs by a radiometric broth macrodilution method and the T100 method of datum analysis (8). Drugs were dissolved according to the directions of the manufacturers and diluted in sterile distilled water or another suitable diluent. At least five concentrations of drugs were tested in order to establish an MIC based on a dose-response curve with inocula diluted 1:100 and 1:1,000 used as controls as previously described (8).

Combination drug measurements. The combined effect of KRM 1648 with ethambutol was determined by combining the drugs at subinhibitory (sub-MIC) concentrations and measuring the MICs by the previously described radiometric broth macrodilution method. A synergistic, additive, or antagonistic effect was determined by calculating the fractional inhibitory concentration as described elsewhere (6).

Time-kill curve measurements. The bactericidal activities of KRM 1648 alone and in combination with ethambutol were

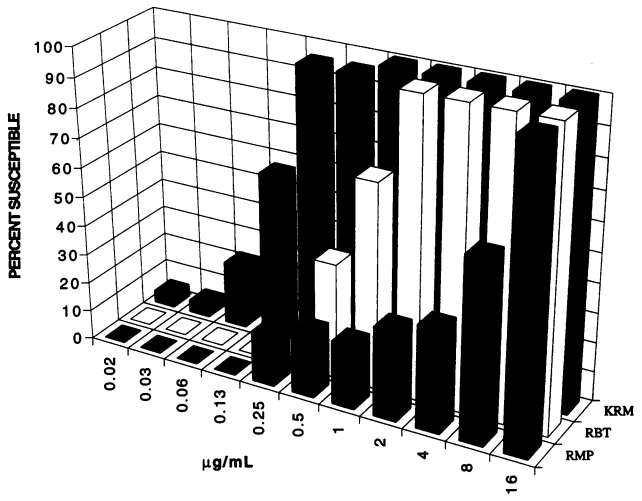

FIG. 1. Percentages of 24 MAC isolates susceptible to increasing concentrations of rifampin (RMP), rifabutin (RBT), and KRM 1648 (KRM) as determined by a radiometric broth macrodilution assay.

determined by a time-kill curve assay. The concentrations of KRM 1648 tested were 1, 5, and 10 times the MIC for the tested strain; ethambutol was tested at the MIC. The turbidity of a suspension of mycobacteria was adjusted to be equivalent to a McFarland 1.0 standard and diluted into Middlebrook 7H9 broth so that the starting inoculum was approximately 10^6 CFU/ml. The actual starting inoculum was confirmed by serial dilution and quantitative plate counts. Samples were taken at regular intervals, diluted, and plated for quantitation. Dilution of the sample was sufficient to prevent carryover of drug that could lead to a false detection of bactericidal activity.

Preparation of macrophages. The source of macrophages was the human monocyte cell line U937 cultured in RPMI 1640 medium at pH 7.2 (Gibco, Detroit, Mich.) supplemented with 5% fetal bovine serum (Sigma Chemical Co., St. Louis, Mo.) and 2 mM L-glutamine. Cells were grown to a density of 5×10^6 cells per ml and then centrifuged, washed, and resuspended in RPMI 1640 supplemented medium. The concentration of cells was adjusted to 10^6 cells per ml, and 1 ml of the cell suspension was added to each well of a 24-well tissue culture plate (Costar, Cambridge, Mass.). Monolayers were treated with 0.5 μg of phorbol myristic acetate per ml for 24 h for maturation of the monocytes. Adherent cells (approximately 5×10^5 per well) were then infected with 5×10^6 mycobacteria for 4 h and washed twice with Hanks' buffered salt solution (HBSS) to remove extracellular bacteria. Following the infection period, macrophage monolayers were treated with KRM 1648 alone and in combination with clarithromycin or ethambutol for 4 days. Medium and antibiotics were replenished daily. Control monolayers were run in parallel and treated in the same manner as the experimental monolayers; no aspect of the protocol caused untoward detachment of cells from the wells of the culture plate.

Macrophage infection. MAC strains 100, 101, and 109 were cultured for 10 days on Middlebrook 7H10 agar (Difco Laboratories, Detroit, Mich.). On the day of the experiment, mycobacteria were harvested, washed twice in HBSS, suspended in HBSS, and sonicated for 5 s to disperse clumps of

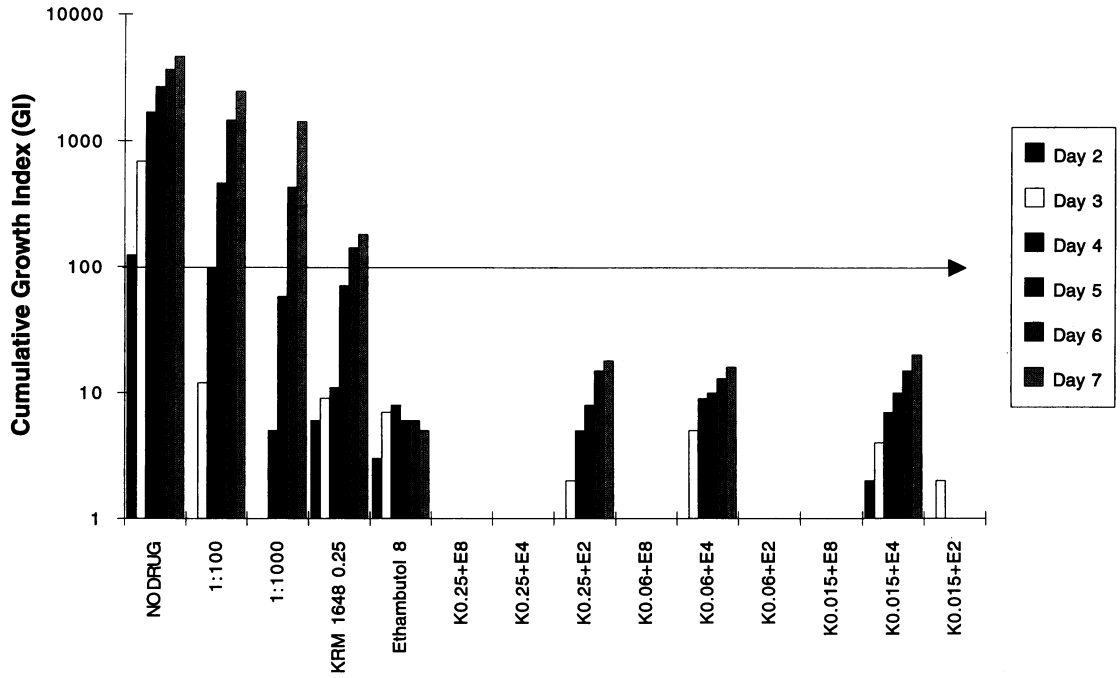

FIG. 2. Effect of KRM 1648 in combination with ethambutol against *M. avium* isolate MAC 101 (10^4 to 10^5 CFU/ml) as determined by a radiometric broth macrodilution assay and the T100 method of datum analysis. Bars indicate the cumulative growth index (GI) over 6 days, corrected for background by using a killed inoculum; the absence of bars indicates a cumulative GI less than that of the control. Additional controls included no drug, inoculum diluted 1:100, and inoculum diluted 1:1,000. The MICs of KRM 1648 and ethambutol for MAC 101 were 0.25 and ≤8 μg/ml, respectively (K0.25 and E8, respectively). Inhibition (arrow) is defined as a cumulative GI of less than 100 over the period of observation. MAC 101 was inhibited by all concentrations of KRM 1648 and ethambutol, and the fractional inhibitory concentrations were calculated on the basis of these results.

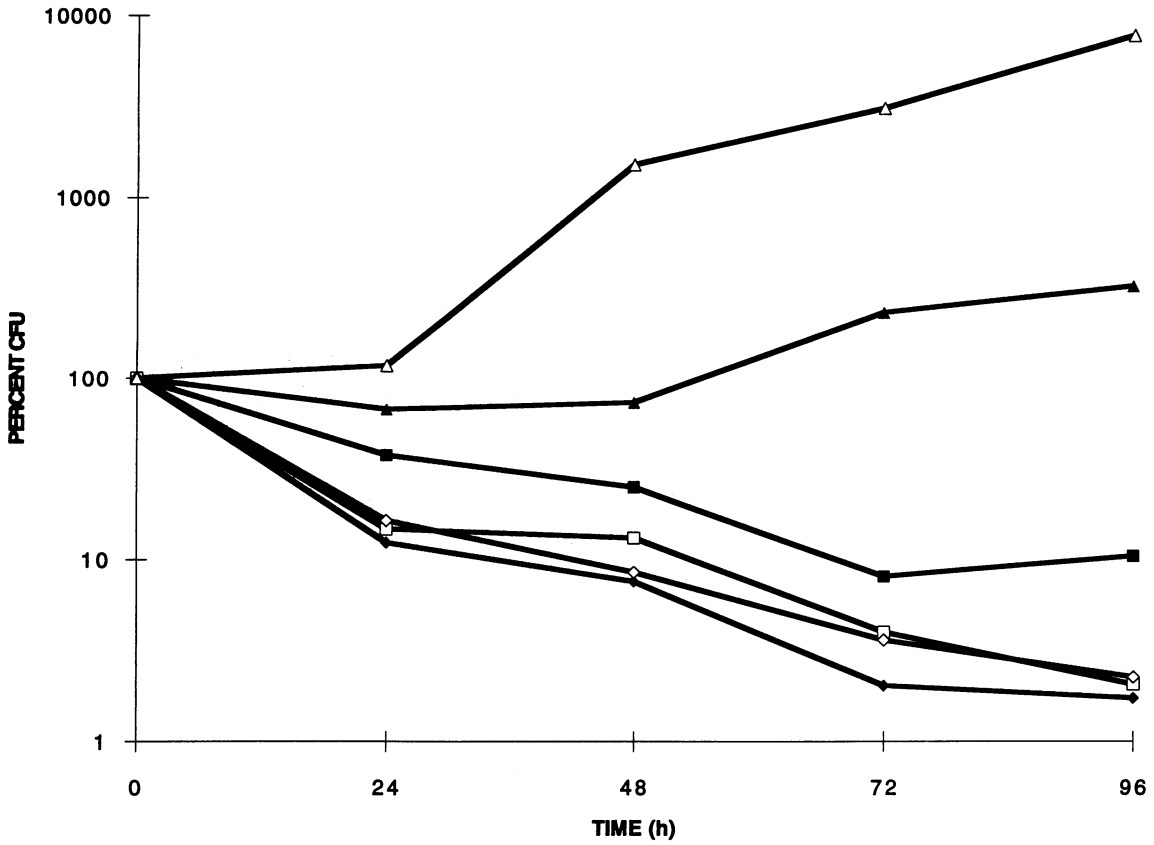

FIG. 3. KRM 1648 time-kill curve for *M. avium* isolate MAC 101, with a starting inoculum of 10^6 CFU/ml in Middlebrook 7H9 broth plotted as the percent CFU present at time zero. KRM 1648 was added at concentrations that were 1 (■), 5 (□), and 10 (◆) times the MIC (0.25 μg/ml). Ethambutol was tested at 4 μg/ml, alone (▲) and with KRM 1648 (◇). Samples were taken at 24-h intervals and plated on Middlebrook 7H11 agar for quantitation; sample volumes were sufficiently small to assure no carryover of drug, and a control was tested with no drug (△).

bacilli. The turbidity of the suspension was adjusted to be equivalent to a McFarland no. 1 standard and the suspension was diluted to a final concentration of approximately 5×10^7 CFU/ml, and each monolayer was infected with 100 μl of the final suspension. The actual CFU per milliliter of the final suspension was determined by serial dilution and quantitative plate counts.

Four hours after infection, the CFU of mycobacteria per macrophage monolayer (well) was determined by lysing the macrophages and quantitative plate counts. Ice-cold sterile water (0.5 ml) was added to each monolayer well, and the mixture was allowed to rest for 10 min at room temperature. Then, 0.5 ml of a second lysing solution (consisting of 1.1 ml of Middlebrook 7H9 broth plus 0.4 ml of 0.25% sodium dodecyl sulfate [SDS] in phosphate buffer) was added to each well, and the mixture was allowed to stand for an additional 10 min. The wells were vigorously scraped with a rubber policeman, and the macrophage lysates were resuspended in 0.5 ml of 20% bovine serum in sterile water to neutralize the SDS. Then the suspension was vortex agitated for 2 min to assure complete lysis of the macrophages. Finally, the lysate was sonicated for 5 s (using a power output of 2.5 W/s) to disrupt clumps of bacilli. To control for the osmotic stability of the mycobacteria, a suspension of mycobacteria alone was plated for quantitation before and after being subjected to the lysis procedure as described above, and in each instance there was no change in the CFU per milliliter before or after the lysis treatment procedure.

The final macrophage lysate suspension was serially diluted, and 0.1 ml was plated onto 7H10 agar. The plates were allowed to dry at room temperature for 15 min and incubated at 37°C in 5% CO_2 for 2 weeks. Duplicate plates were prepared for each well, and the results are reported as the mean CFU per milliliter of macrophage lysate. Each experiment was repeated three times. The statistical significance of differences between the control (untreated) and experimental (treated) macrophage cultures at identical time points was determined by Student's *t* test.

RESULTS

MICs of KRM 1648 compared with those of other rifamycins. KRM 1648 was tested against 24 MAC strains, with a concentration range of 0.015 to 0.25 μg/ml. The range of MICs was 0.015 to >0.25 μg/ml, and the MIC for 50% of strains tested (MIC_{50}) and MIC_{90} were 0.12 and 0.25 μg/ml, respectively. By comparison, when these same 24 strains were tested against rifampin, the range of MICs was <0.5 to >8 μg/ml and the MIC_{50} and MIC_{90} were 8 and >8 μg/ml, respectively. When tested against rifabutin, the range of MICs was 0.25 to 2 μg/ml and the MIC_{50} and MIC_{90} were 1 and 2 μg/ml, respectively. A graphic comparison of KRM 1648, rifabutin, and rifampin, shown as the percentages of strains susceptible at $\log_2$ increments of increasing concentrations of drug, is shown in Fig. 1. This analysis demonstrates that there is a 3- to 4-doubling difference between the MICs of KRM 1648 and

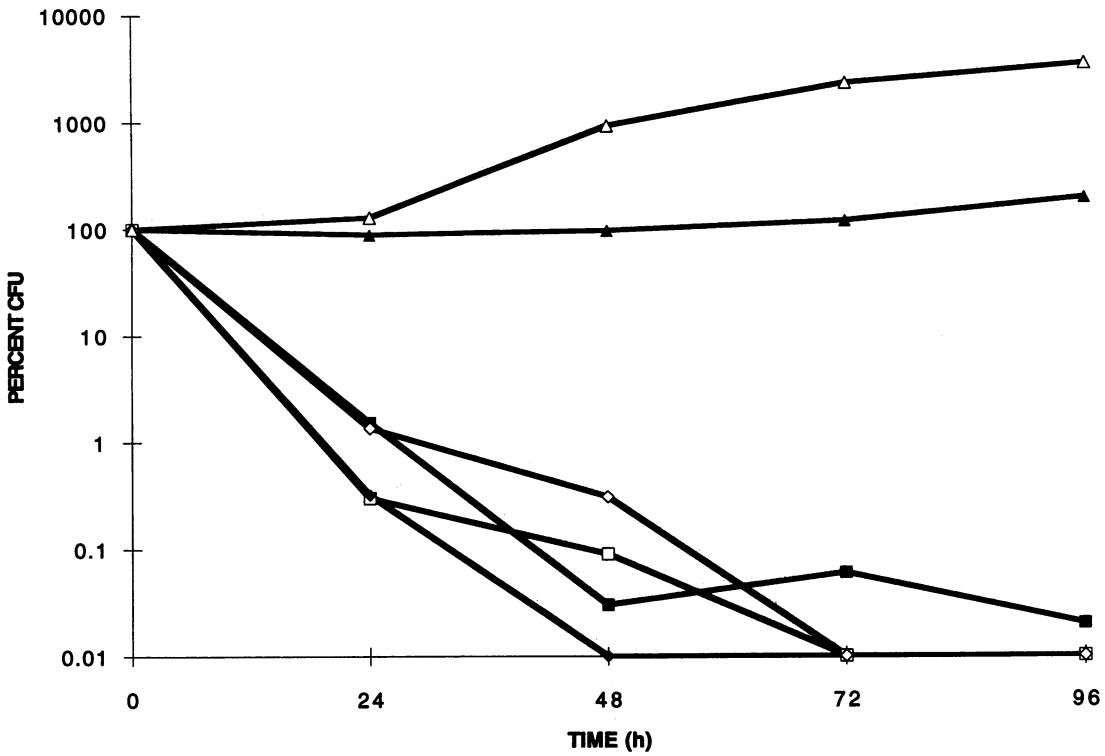

FIG. 4. KRM 1648 time-kill curve for *M. avium* isolate MAC 108, with a starting inoculum of 10^6 CFU/ml in Middlebrook 7H9 broth plotted as the percent CFU present at time zero. KRM 1648 was added at concentrations that were 1 (■), 5 (□), and 10 (◆) times the MIC (0.25 μg/ml); ethambutol was tested at 4 μg/ml, alone (▲) and with KRM 1648 (◇). Samples were taken at 24-h intervals and plated on Middlebrook 7H11 agar for quantitation; sample volumes were sufficiently small to assure no carryover of drug, and a control was tested with no drug (△).

rifabutin and the MICs of KRM 1648 and rifampin for 80% or more of the 24 MAC strains tested.

Activity of KRM 1648 in combination with ethambutol. KRM 1648 in combination with ethambutol was tested against two strains, MAC 101 and 108. The MICs of KRM 1648 for the two strains are approximately the same, but the MICs of ethambutol differ by 1 dilution (MAC 101, 4 μg/ml; MAC 108, 8 μg/ml). KRM 1648 was tested at three concentrations at and below the MIC for each strain, i.e., 0.25, 0.06, and 0.015 μg/ml, while ethambutol was tested at 2, 4, and 8 μg/ml. The interaction between the drugs was determined by the broth radiometric macrodilution method and by the T100 method of datum analysis, which allows a precise assessment of drug-drug interactions as shown, for example, for MAC 101 in Fig. 2. KRM 1648 (alone) is at or slightly below the MIC for MAC 101 at 0.25 μg/ml, depending on the threshold. With a threshold of 99% inhibition (1:100 control), the MIC is ≤0.25 μg/ml; however, with a threshold of 99.9% inhibition (1:1,000 control), the MIC is equal to or slightly higher than 0.25 μg/ml. Upon the addition of ethambutol at 2, 4, or 8 μg/ml, MAC 101 is inhibited by a KRM 1648 concentration as low as 0.015 μg/ml. The KRM 1648 MIC for MAC 108 was 0.25 μg/ml at both 1:100 and 1:1,000 thresholds. Upon the addition of ethambutol at 2, 4, or 8 μg/ml, MAC 108 is inhibited by a KRM 1648 concentration of 0.015 μg/ml. The fractional inhibitory concentrations for the combination of KRM 1648 and ethambutol against MAC 101 and MAC 108 were 0.56 and 0.25, respectively. On the basis of the results with these two MAC strains, it appears that the combination of KRM 1648 and ethambutol is additive with marginal synergy.

Bactericidal activity of KRM 1648 with or without ethambutol. The bactericidal activity of KRM 1648 was determined by a time-kill curve analysis, using MAC 101 and MAC 108 at starting inocula of 10^6 CFU/ml. Killing activity was measured over a period of 96 h, and drug was added to the growth medium at concentrations that were 1, 5, and 10 times the MIC; ethambutol was added at only one concentration, which was equivalent to the MIC. With KRM 1648 at 5 to 10 times the MIC, there was a reduction in the viability of MAC 101 bacilli of 1 to 2 orders of magnitude by 72 h (Fig. 3). Ethambutol by itself was bacteriostatic, but in combination with KRM 1648 at the MIC, there was increased bactericidal activity to a level equivalent to the activity at 10 times the MIC for KRM 1648 alone. KRM 1648 caused a reduction of 3 to 4 orders of magnitude in the viability of MAC 108 bacilli at all concentrations (Fig. 4); ethambutol had little effect on either the degree or the rate of killing of MAC 108 by KRM 1648.

Effect of KRM 1648, ethambutol, or clarithromycin alone on *M. avium* in macrophages. All three strains of the MAC used in these studies infected the U937 human monocytes to an equivalent degree; i.e., between 4×10^5 and 8×10^5 CFU per monolayer. Macrophages infected with MAC 100, MAC 101, or MAC 109 were treated with KRM 1648 at concentrations that ranged from 0.0156 to 1 μg/ml for 4 days; growth medium and KRM 1648 were replenished daily. The results show that KRM 1648 was bactericidal for all three MAC strains tested, even at the lowest concentration tested, 0.0156 μg/ml (Fig. 5). In each instance, there was a statistically significant ($P < 0.05$) decrease in the number of viable bacilli within the macrophages of nearly 3 orders of magnitude compared with the number in the untreated control at day 4. In the absence of drug, there was an increase in the number of viable bacilli of

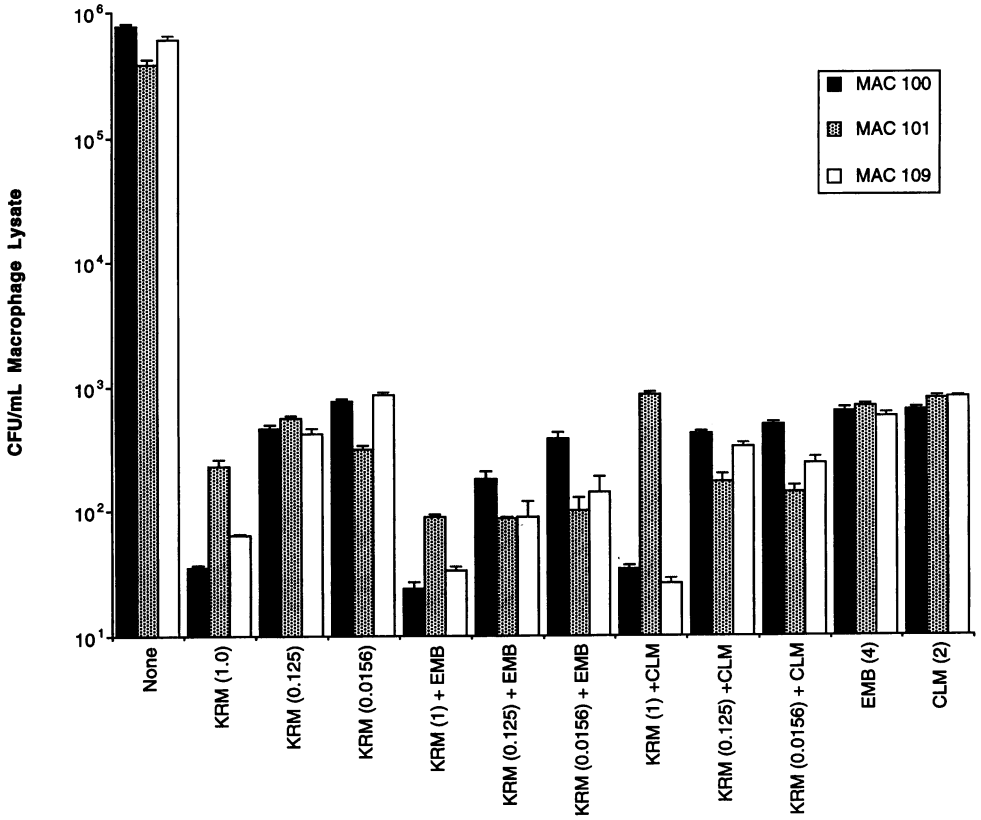

FIG. 5. Effect of KRM 1648 alone or in combination with ethambutol or clarithromycin on three *M. avium* isolates in infected human macrophages. Cultured human macrophages (U937 cell line) were infected with 5×10^6 mycobacteria for 4 h and then washed free of extracellular organisms. The infected macrophage monolayers were treated for 4 days with KRM 1648 (KRM) at three concentrations (0.0156, 0.125, and 1 μg/ml) alone or in combination with ethambutol (EMB) (4 μg/ml) or clarithromycin (CLM) (2 μg/ml). Growth medium and antibiotics were replenished each day for the 4-day incubation period. Macrophage monolayers were harvested and lysed and plated on Middlebrook agar for quantitation.

greater than 1 order of magnitude from the time of inoculation (day zero). Either ethambutol or clarithromycin alone, at the maximum concentrations in serum for these agents, also resulted in a statistically significant ($P < 0.05$) decrease of 3 orders of magnitude in the viability of MAC bacilli within macrophages.

Effect of KRM-1648 in combination with ethambutol or clarithromycin on *M. avium* in macrophages. For two of the three strains tested (MAC 101 and 109), the combination of KRM 1648 with ethambutol resulted in significantly ($P < 0.05$) greater killing than either KRM 1648 or ethambutol alone (Fig. 5). Treatment of MAC 100-infected macrophages with KRM 1648 and ethambutol was not associated with significantly greater killing than treatment with KRM 1648 alone. Similarly, treatment of MAC-infected macrophages with the combination of KRM 1648 and clarithromycin led to statistically significant ($P < 0.05$) greater killing (but only modest biological improvement) than treatment with either KRM 1648 or clarithromycin alone for MAC 101 and 109 but not MAC 100 (Fig. 5).

DISCUSSION

Although clarithromycin and azithromycin have proven efficacy in the treatment of disseminated MAC disease (2, 15), resistance to these agents appears to rapidly develop during therapy (5), and the current recommendation is that these agents be combined with a second or third agent such as ethambutol and clofazimine (9). The premise for this suggestion is based on the extensive experience with *M. tuberculosis*, for which resistance is readily controlled with multiple-drug therapy (e.g., isoniazid, rifampin, and pyrazinamide). However, this premise has not been tested with the MAC, which is only variably susceptible to agents such as ethambutol. In addition, macrolides and ethambutol are primarily bacteriostatic agents. Thus, there is a clear need for potent antimycobacterial agents that will prevent resistance and improve the bactericidal activity of MAC disease treatment regimens.

Saito and colleagues (11) tested KRM 1648 against 49 MAC strains, using a Middlebrook 7H11 agar modified-proportion method, and Tomioka et al. (12) tested 30 MAC strains, using a broth radiometric macrodilution assay. In each of these studies, the MICs of KRM 1648 were significantly lower than those of both rifampin and rifabutin, and the MICs for KRM 1648 were somewhat lower as determined by the radiometric method than by the agar method (12). Saito et al. (11) observed a significant difference between the MIC_{90}s for 18 strains of *M. avium* (1.56 μg/ml) versus 31 strains of *Mycobacterium intracellulare* (0.1 μg/ml), but there was no difference between *M. avium* strains isolated from AIDS and non-AIDS patients (13).

Saito et al. (11) also showed that KRM 1648 was more bactericidal than rifampin at the same concentration in a

murine peritoneal macrophage assay using *M. intracellulare*-infected macrophages tested over a 5-day period. In animal test systems (BALB/c or C57BL/6 beige mice), KRM 1648 was significantly more active than rifampin or rifabutin against both a virulent and a less virulent strain of *M. intracellulare* (11). Although KRM 1648-treated animals survived longer than untreated animals, these results appeared to be at least partially dependent on the strain of *M. intracellulare* used to infect the animals. Furthermore, the levels of infection of the lung and the spleen were not significantly different in the treated and untreated animals which survived for the longest periods. Murine pharmacokinetic studies showed that while the levels of KRM 1648 in plasma were lower than those of rifampin and rifabutin, the levels (measured as either micrograms per gram or milliliter or as the area under the concentration-time curve) in the spleen and the lung were substantially higher without significant toxicity (13, 14).

We have found that in vitro, on a weight basis, KRM 1648 is the most active rifamycin derivative against the MAC yet identified, and in combination with ethambutol there appears to be a synergistic or additive interaction. The bactericidal activity of KRM 1648 against the MAC is variable and appears to be strain dependent, but ethambutol may improve the killing activity against strains for which the MICs of KRM 1648 are higher. The bactericidal activity of KRM 1648 against the MAC also was demonstrated in experiments with human macrophages infected with three different MAC strains; however, in these experiments, there was little improvement in the bactericidal activity of KRM 1648 when combined with ethambutol or clarithromycin. Animal studies of KRM 1648 alone and in combination with other agents indicate that this benzoxazinorifamycin derivative is perhaps the most potent rifamycin identified to date with activity against the MAC. It will be important to assess the potential role of KRM 1648 in the prevention of drug resistance to macrolides and other agents used in the treatment of MAC disease. In vivo and toxicology studies are needed before human studies can be initiated, but the present results are encouraging.

ACKNOWLEDGMENT

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