Activity of KRM 1648 Alone or in Combination with Ethambutol or Clarithromycin against *Mycobacterium avium* in Beige Mouse Model of Disseminated Infection

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Rifamycins are active against slowly growing mycobacteria, such as *Mycobacterium tuberculosis* and *Mycobacterium kansasii*, but the majority of rifamycins thus far investigated both in vitro and in vivo are inactive or have only modest activity against the *Mycobacterium avium* complex (MAC). We investigated the activity of three doses of the semisynthetic benzoxazinorifamycin KRM 1648, alone or in combination with ethambutol or clarithromycin, in beige mice challenged with the MAC strain 101. Our results show the following. (i) KRM 1648 was significantly effective against MAC infection as determined by the reduction of the number of bacteria in the blood, liver, and spleen when administered at doses of 20 and 40 mg/kg of body weight per day but not at 10 mg/kg/day, compared with untreated controls. (ii) KRM 1648 (40 mg/kg/day) administered in combination with ethambutol (100 mg/kg/day) resulted in significant reduction in bacteremia compared with values for untreated controls ($P < 0.001$), KRM 1648 alone ($P = 0.019$), and ethambutol alone ($P = 0.003$). Furthermore, the combination of KRM 1648 and ethambutol was associated with a significant decrease of the number of bacteria in the spleen and the liver compared with values for both untreated controls and each drug alone ($P < 0.001$ for all comparisons). (iii) KRM 1648 (40 mg/kg/day) administered in combination with clarithromycin (200 mg/kg/day) resulted in a significant decrease of the number of bacteria in the blood and the spleen compared with the number for untreated controls ($P < 0.001$ for all comparisons). In our experience, using MAC 101 as the challenging organism, KRM 1648 is the first rifamycin with significant activity in vivo against MAC infection in beige mice.

Organisms belonging to the *Mycobacterium avium* complex (MAC) are the most common cause of bacteremia in patients with AIDS (10, 12, 24). MAC has caused bacteremia in approximately 50% of patients monitored for 30 months after a diagnosis of AIDS (19). The survival rate of those AIDS patients with MAC bacteremia is half that of patients who have similar CD4 lymphocyte counts but who do not have disseminated MAC infection (11).

A general principle of antimycobacterial therapy is the use of combinations of agents to minimize emergence of resistance and shorten the duration of therapy. Rifampin and other rifamycins, including rifabutin and rifapentine, are active against slowly growing mycobacteria, such as *Mycobacterium tuberculosis* and *Mycobacterium kansasii* (8, 9). However, the majority of rifamycins thus far investigated in vitro and in vivo are either inactive or have borderline activity against MAC (2, 5, 6, 14–17). Although prophylactic activity has been demonstrated (18, 20), none of the rifamycins that we have previously tested exert bactericidal activity (12a).

Recent reports have indicated that a group of semisynthetic rifamycin derivatives, benzoxazinorifamycins, are active against MAC in vitro (21, 23) and in vivo (4, 22). We recently showed that one of the benzoxazinorifamycins, KRM 1648, was active against AIDS-derived strains of MAC in a BACTEC susceptibility system and in a tissue active system employing MAC-infected macrophages (13). In addition, KRM 1648 seems to be bactericidal against MAC (12b). Recognizing the

need to improve the chemotherapy of severe MAC diseases, we carried out the present studies to evaluate the activity of KRM 1648 in beige mice. We have assessed the response to increasing doses of KRM 1648 therapy alone as well as combinations of KRM 1648 plus either clarithromycin or ethambutol, two antimicrobial agents with established anti-MAC activity (3, 15).

MATERIALS AND METHODS

Mycobacteria. MAC strain 101 (serovar 1) was isolated from a patient with AIDS. MAC 101 is the most mouse-virulent strain we have tested in experimental animals, and it causes reproducible levels of infection and mortality in beige mice (1). MAC organisms were cultured for 10 days at 37°C in Middlebrook agar 7H10 medium (Difco Laboratories, Detroit, Mich.) supplemented with oleic acid, albumin, dextrose, and catalase (OADC; Difco). Transparent colonies were harvested in Hanks' buffered salt solution and washed twice, and the suspension was adjusted to 3×10^8 bacteria per ml (therapeutic model) by comparison with a McFarland turbidity standard. Samples obtained from the bacterial suspension were plated on Middlebrook 7H10 to confirm the inoculum size. One hundred microliters of the original suspension was used to infect mice. Before the mice were infected, the final suspension was vortex agitated for 2 min to avoid clumping.

Antimicrobial agents. KRM 1648 was provided by Kaneka Corp., Osaka, Japan; clarithromycin was provided by Abbott Laboratories, Abbott Park, Ill.; and ethambutol was provided by Lederle Laboratories, Pearl River, N.Y.

All drugs were dissolved according to the recommendations

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TABLE 1. Treatment regimens, dosages, and number of
mice per experimental group

Expts and treatment	Dose (mg/kg/day)	No. of mice evaluated
Dose-response		
KRM 1648	10	26
	20	26
	40	26
None (control)		20
Combination		
KRM 1648	40	28
Ethambutol	100	30
Clarithromycin	200	30
KRM 1648 + clarithromycin	40/200	20
KRM 1648 + ethambutol	40/100	29
None (control)		29

of the manufacturers. The drugs were freshly prepared every morning before administration.

Mice. Experiments were carried out with 6- to 7-week-old female C57BL/6 bg^+/bg^+ mice (Jackson Laboratories, Bar Harbor, Maine).

Experimental design. The therapeutic efficacy of KRM 1648 alone and in combination with clarithromycin and ethambutol was examined by using the beige mouse model of infection as previously described (14). Briefly, mice were infected via the caudal vein with 3×10^7 bacteria, and after 7 days treatment with KRM 1648 (40 mg/kg of body weight per day) or KRM 1648 plus clarithromycin (200 mg/kg/day) or ethambutol (100 mg/kg/day) was initiated. Drugs were administered by gavage for 28 days. A control group of mice was infected but received only saline instead of antibiotics. An additional group of mice was harvested at day 7 after infection to establish the initial level of infection before the initiation of therapy. At the termination of treatment, the livers and spleens of control and treated mice were aseptically removed and the organs were weighed and then homogenized in 5 ml of Middlebrook 7H9 broth (Difco) with a tissue homogenizer. The tissue suspensions were serially diluted in sterile water and plated onto 7H10 agar (Difco) plates supplemented with OADC (Difco) for quantitation of viable bacteria. The level of mycobacteremia was determined by collecting 0.05 ml of blood at days 7 and 28. The number of CFU per milliliter of blood was determined by inoculating the blood into 4 ml of BACTEC 12B radiometric medium (Johnson Laboratories, Sparks, Md.) by the T100 method of data analysis as previously described (14).

Statistical analysis. The statistical significance of the differences between the numbers of viable organisms recovered from the spleen, liver, and blood were evaluated by one or two variable analyses of variance. Differences between experimental groups and between experimental groups and control groups were considered statistically significant if P values were <0.05.

RESULTS

Table 1 shows the treatment regimens and the number of mice used in each experimental group.

KRM 1648 activities at different doses. KRM 1648 was administered at doses of 10, 20, and 40 mg/kg/day for 4 weeks to treat disseminated MAC infection in beige mice. KRM 1648 at 10 mg/kg/day was associated with 23% mortality, compared with 15% mortality in the group receiving 20 mg/kg/day, 12%
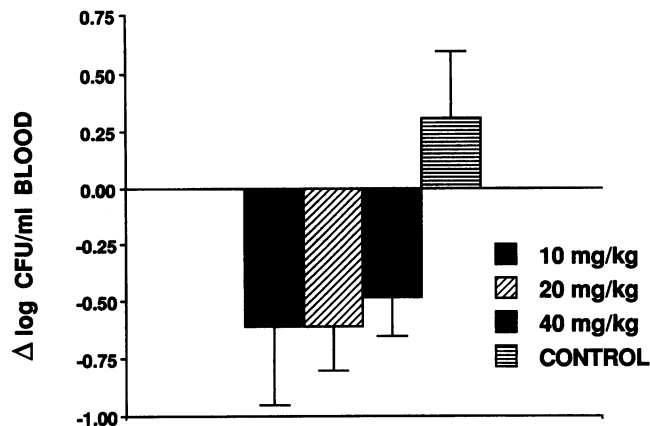

FIG. 1. Effect of KRM 1648 at three different doses on bacteremia in beige mice infected with approximately 10^7 CFU of MAC 101 injected into the caudal tail vein. Therapy was begun 1 week after injection and continued for 4 weeks. Controls were injected with saline. Error bars indicate standard errors of the means.

mortality in the group receiving 40 mg/kg/day, and 55% mortality in the untreated control group ($P = 0.055$ for the comparison between the 10-mg/kg/day group and the control group, $P < 0.05$ for the comparison between the 20- or 40-mg/kg/day group and the untreated control group, and $P = 0.07$ for the comparison between the 10-mg/kg/day group and the 20- or 40-mg/kg/day group).

Treatment with KRM 1648 at 20 and 40 mg/kg/day resulted in significant reduction in the number of bacteria in the blood compared with the numbers for untreated controls ($P = 0.02$ and 0.013 for comparisons between the 40- and 20-mg/kg/day groups, respectively, and the untreated control group) (Fig. 1).

Each of the three doses of KRM 1648 (10, 20, and 40 mg/kg/day) was associated with a significant reduction of the number of viable organisms per gram of liver tissue ($P = 0.014$, <0.001, and <0.001 for the comparisons between 10-, 20-, and 40-mg/kg/day doses, respectively, and the untreated control group) (Fig. 2) and spleen tissue ($P = 0.035, 0.003$, and <0.001 compared with the level of infection in the untreated control
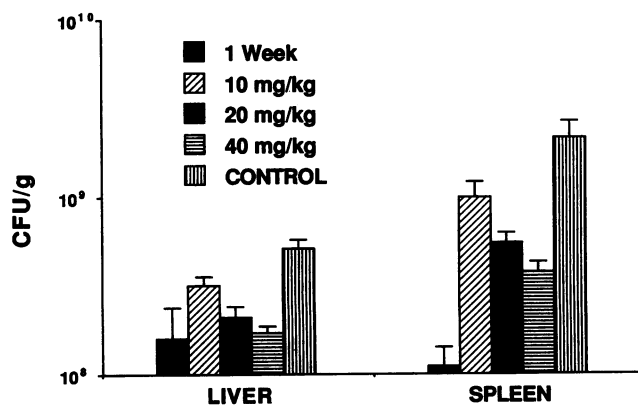

FIG. 2. Activity of KRM 1648 at three different doses on the level of infection in liver and spleen tissues from beige mice infected with MAC 101. Animals were infected with approximately 10^8 CFU of MAC 101 injected into the caudal tail vein; therapy was begun 1 week later and continued for 4 weeks. Controls were injected with saline. Error bars indicate standard errors of the means.
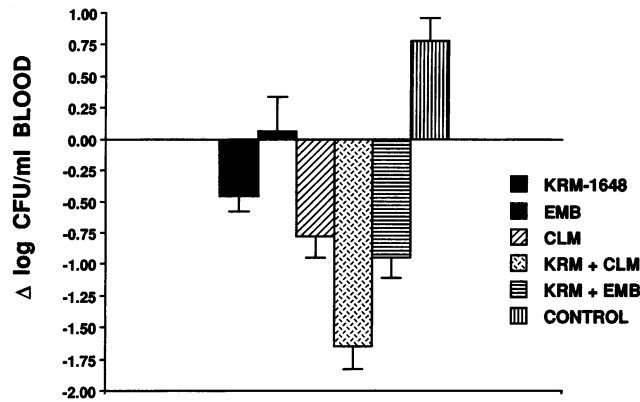

FIG. 3. Effect of KRM 1648 alone (40 mg/kg/day) or in combination with clarithromycin (CLM) (200 mg/kg/day) or ethambutol (EMB) (100 mg/kg/day) on bacteremia in beige mice infected with approximately 10^8 CFU of MAC 101 injected into the caudal vein. Therapy was begun 1 week after infection and continued for 4 weeks. Controls were injected with saline. Error bars indicate standard errors of the means.
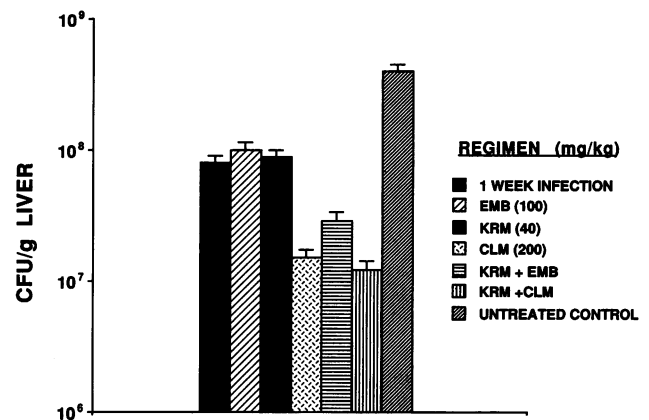

FIG. 4. Effect of KRM 1648 alone or in combination with clarithromycin (CLM) (200 mg/kg/day) or ethambutol (EMB) (100 mg/kg/day) on the level of infection in liver tissue from beige mice infected with MAC 101. Animals were infected with approximately 10^8 CFU of MAC 101 injected into the caudal vein; therapy was begun 1 week later and continued for 4 weeks. Error bars indicate the standard errors of the means.

group) (Fig. 2). In the cases of both the liver and the spleen, there were significant differences in the levels of tissue infection between the 10-mg/kg/day dose and the 20- and 40-mg/kg/day doses. The differences between the 20- and 40-mg/kg/day doses were not significant for liver tissue ($P > 0.05$) but were significant for spleen tissue ($P = 0.04$).

Efficacy of the combination of KRM 1648 with ethambutol. KRM 1648 (40 mg/kg/day) administered in combination with ethambutol (100 mg/kg/day) resulted in a percent mortality comparable to that for ethambutol alone. It was not significantly different from the control value, suggesting some toxicity associated with treatment with ethambutol. Specifically, treatment with KRM 1648 resulted in 18% mortality, ethambutol resulted in 43% mortality, and KRM 1648 plus ethambutol was associated with 39% mortality, while the untreated control group experienced 32% mortality.

Figure 3 shows the effects of KRM 1648 alone and in combination with ethambutol on bacteremia. Treatment with KRM 1648 in combination with ethambutol resulted in a significant reduction in bacteremia compared with that of the untreated control ($P < 0.001$) and compared with treatment with KRM 1648 ($P = 0.019$) or ethambutol ($P = 0.003$) alone. In addition, treatment with the combination of KRM 1648 and ethambutol led to a significant reduction in CFU per gram in both the spleen and the liver compared with treatment with KRM 1648 ($P < 0.001$) or ethambutol ($P < 0.001$) alone or no treatment (control) ($P < 0.001$) 28 days after infection (Fig. 4 and 5).

KRM 1648 activity in combination with clarithromycin. Therapy of MAC infection in the beige mouse with KRM 1648 (40 mg/kg/day) in combination with clarithromycin (200 mg/kg/day) was associated with 24% mortality, compared with 18% mortality for KRM 1648 alone, 10% mortality for clarithromycin alone, and 32% mortality for the untreated control.

The combination of KRM 1648 and clarithromycin resulted in a significant decrease in bacteremia compared with KRM 1648 alone ($P < 0.001$) or clarithromycin alone ($P = 0.001$) or no treatment (control) ($P < 0.001$) (Fig. 3). Furthermore, treatment with KRM 1648 and clarithromycin was associated with a significant reduction of the number of bacteria in the spleen compared with KRM 1648 alone ($P < 0.001$), clarithro-

mycin alone ($P < 0.001$), or no treatment (control) ($P < 0.001$) (Fig. 4).

In contrast, in the liver, KRM 1648 administered in combination with clarithromycin was not significantly more effective than clarithromycin alone ($P = 0.34$), although the combination was significantly more effective than KRM 1648 alone ($P < 0.001$) or no treatment (control) ($P < 0.001$) (Fig. 5).

DISCUSSION

KRM 1648 is a newly synthesized benzoxazinorifamycin that recently has been shown to have antimycobacterial activity (21). Previous studies have used beige mice and a small number of rabbits to assess anti-MAC activity in vivo and suggested that KRM 1648 is effective against MAC (4, 22). Our study extends the previous observations with KRM 1648 by challenging beige mice with a strain of MAC that kills beige
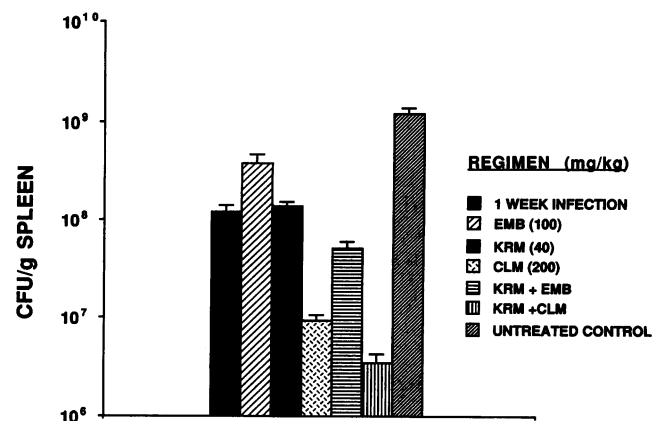

FIG. 5. Effect of KRM 1648 alone (40 mg/kg/day) or in combination with clarithromycin (CLM) (200 mg/kg/day) or ethambutol (EMB) (100 mg/kg/day) on the level of infection in splenic tissue from beige mice infected with MAC 101. Error bars indicate the standard errors of the means.

mice in a predictable manner. We have also used a larger number of mice per experimental group than previous studies, therefore increasing the statistical power of the observation. In addition, we demonstrated that combinations with ethambutol and clarithromycin are significantly more effective than each drug alone for the treatment of MAC infection in beige mice.

Recent studies in vitro have demonstrated that KRM 1648 was bactericidal for MAC at a concentration achievable in blood (13) and that combination with ethambutol was significantly more effective than each drug by itself. This observation was confirmed in vivo. However, the combination of KRM 1648 with clarithromycin was not more effective in reducing the number of bacteria in the liver than each drug alone. This finding could be due to an interaction between the two drugs in the liver. One possibility is that KRM 1648, like other rifamycins, can induce the synthesis of cytochrome P450 in the liver, an enzymatic system responsible for the catabolism of a number of compounds, including macrolides (7).

Pharmacokinetic studies of KRM 1648 in mice have shown that while concentrations in plasma are low (0.6 to 1 μg/ml) compared with those of rifampin, KRM 1648 was concentrated in the lung and in the spleen. Eight hours after the administration of 20 mg/kg, KRM 1648 had a sixfold greater concentration than rifampin (22) in splenic tissue, achieving a concentration of 7 to 9 μg/ml. Therefore, on the basis of recent data from this laboratory and other laboratories (13, 21) which showed that the MICs of KRM 1648 for the majority of MAC strains range from 0.25 to 1 μg/ml, the concentration of KRM 1648 in tissue is likely to be at least seven- to eightfold higher than the MICs of the drug for the organisms. Somewhat paradoxically, KRM 1648 appears to be more active in vivo than suggested by in vitro studies with activated macrophages (13); however, in other studies, we showed that KRM 1648 was bactericidal for MAC at a concentration that is likely to be achieved in human serum (13).

The magnitude and urgency of the problem of MAC infection in patients with AIDS require a continued search for active antimicrobial agents with potential for clinical use. Overall, the results of this study suggest that KRM 1648 is active in vivo both as a single agent and in combination with ethambutol or clarithromycin. Thus, KRM 1648 deserves further evaluation for the therapy of MAC infection.

ACKNOWLEDGMENTS

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