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In Vitro Bactericidal and In Vivo Therapeutic Activities of a New Rifamycin Derivative, KRM-1648, against *Mycobacterium tuberculosis*

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The in vitro and in vivo activities of a new rifamycin derivative, KRM-1648, against *Mycobacterium tuberculosis* H37Rv were compared with those of rifampin. Bactericidal activity was evaluated by using a silicone-coated slide culture method. The MBC of KRM-1648 was 0.15 to 0.3 μg/ml for 24 h of exposure, while that of rifampin was >160 μg/ml under the same conditions. Against experimental murine tuberculosis, KRM-1648 exhibited significant therapeutic effects, in terms of prolonged survival times for mice compared with those with rifampin treatment, even at lower doses, such as 1 and 3 mg/kg. At a dose of 3 mg/kg, KRM-1648 was at least as effective as rifampin at 10 mg/kg. The combination of KRM-1648 (3 mg/kg) plus isoniazid (3 mg/kg) plus ethambutol (10 mg/kg) exhibited much more activity than did rifampin (10 mg/kg) plus isoniazid (3 mg/kg) plus ethambutol (10 mg/kg). These findings suggest that KRM-1648 is a promising candidate for the treatment of tuberculosis.

We originally reported the potent in vitro and in vivo activities of 5-benzoxazinorifamycins synthesized by the Biochemical Research Laboratory of Kaneka Corporation, Takasago, Japan, against both *Mycobacterium tuberculosis* and *Mycobacterium avium* complex (14, 21). One such derivative, 3′-hydroxy-5′-(4-isobutyl-1-piperazinyl)benzoxazinorifamycin (KRM-1648), has consistently been reported to exhibit significant activity both in vitro (15, 17, 19, 21) and in vivo (14) against mycobacteria, including principally *M. avium* complex and *M. tuberculosis*. We found that the MIC at which 90% of the isolates are inhibited of KRM-1648 for 20 rifampin (RFP)-susceptible clinical isolates of *M. tuberculosis* was 1/16 to 1/32 of that of RFP (21). In an experimental murine model of tuberculosis, KRM-1648 was markedly more potent than RFP in prolonging the survival of infected mice at a dose of 10 mg/kg/day (14).

Here we report the bactericidal activity, which is important for the evaluation of the potential activities of antimicrobial agents in the treatment of human infectious disease, of KRM-1648. The purposes of this study were to compare the MBC of KRM-1648 with that of RFP against *M. tuberculosis* H37Rv and, further, to confirm the chemotherapeutic activity of KRM-1648 when used in place of RFP, with emphasis on combination chemotherapy in our experimental murine tuberculosis.

MATERIALS AND METHODS

Drugs. KRM-1648 was obtained from Kaneka Corporation, RFP was obtained from Daiichi Pharmaceutical Co. (Tokyo, Japan), isoniazid (INH) was obtained from Meiji Seika Co. (Tokyo, Japan), and ethambutol (EB) was obtained from Lederle Japan Co. (Tokyo, Japan).

Organism. *M. tuberculosis* H37Rv had been stocked frozen in our laboratory prior to experiments. This strain was grown in 1% Ogawa egg medium before use (16). For studies of infection, the strain was passed twice through ddY mice to maintain virulence and then was finally grown in modified Dubos Tween-albumin medium (13).

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MBC determination. The MBC was determined by the silicone-coated slide culture method as previously described (9, 20) with minor modifications (12). Standard glass microscope slides which were cleaned and coated with silicone were used in this experiment. *M. tuberculosis* H37Rv suspended in petroleum benzine (12) was adjusted to a density of 1 mg/ml by comparison with a no. 1 McFarland turbidity standard. The silicone-coated slides were immersed in a bacillary suspension so that tubercle bacilli would adhere to the surface. The slides were transferred to test tubes containing drug solution diluted with Kirchner's medium supplemented with 10% bovine serum and incubated at 37°C for 2, 5, or 24 h.

At various intervals, the slides were removed from the tubes and then incubated in the same medium without drugs at 37°C for 21 days. After 21 days of incubation, the slides were observed macroscopically; the MBC was defined as the lowest concentration of the drug at which no visible growth of bacilli was observed on the slides.

Drugs were dissolved in dimethyl sulfoxide and then diluted with Kirchner's medium to obtain concentrations of between 10 and 0.017 μg/ml for KRM-1648 and between 160 and 0.3 μg/ml for RFP.

In vivo activity in an experimental murine model of tuberculosis. Five-week-old male ddY mice, 20 to 26 g, purchased from Japan SLC Inc., Shizuoka, Japan, were used. In vivo studies were carried out in two separate experiments. In the dose-response study, *M. tuberculosis* H37Rv was grown in Dubos Tween-albumin medium (Difco Laboratories, Detroit, Mich.) at 37°C for 4 weeks, and the bacterial suspension was prepared by discarding the supernatant of the medium in order to acquire a lethal dose of organisms. One hundred mice were inoculated intravenously with approximately 10^8 viable organisms and divided into 10 groups of 10 mice each. KRM-1648 or RFP, finely suspended in 2.5% gum arabic–0.2% Tween 80, was administered directly into the stomach through a small metal tube attached to a 1-ml syringe once daily six times per week from day 1 to day 40 after infection. Control infected mice received vehicle alone delivered in a similar fashion. The survival times for each group were recorded for 160 days after infection, and efficacy was assessed by using the rate of survival of infected mice.

In the drug combination study, 5-week-old male ddY mice were infected intravenously with approximately 3×10^8 viable *M. tuberculosis* H37Rv organisms. Sixty-four mice were divided into eight groups of eight mice each. Treatment was begun 7 days after infection, and the various regimens were orally administered once daily six times per week for 14 days. The tested drugs and dosages were as follows: KRM-1648, 3 mg/kg; RFP, 10 mg/kg; INH, 3 mg/kg; and EB, 10 mg/kg. The drug combinations INH-EB, RFP-INH-EB, and KRM-1648-INH-EB were studied, along with single-drug regimens of INH, EB, RFP, and KRM-1648 for comparison. In order to evaluate the effects of chemotherapeutic regimens, the rate of survival of infected mice was assessed for 160 days after infection.

Statistical analysis. The survival rates were analyzed by the generalized Wilcoxon test by using a computerized statistical analysis (SAS). Differences between each group were considered statistically significant if the P value was <0.05.

TABLE 1. MBCs with various contact times of KRM-1648 and RFP against *M. tuberculosis* H37Rv[a]

Expt	Drug	MBC (μg/ml) with the following contact time (h):		
		2	5	24
1	RFP	>160	>160	>160
	KRM-1648	0.6	0.6	0.3
2	RFP	>160	>160	>160
	KRM-1648	0.6	0.6	0.3
3	RFP	>160, >160	>160, >160	>160, >160
	KRM-1648	0.3, 0.3	0.15, 0.3	0.15, 0.15

[a] Three experiments were conducted under the same conditions by using the silicone-coated slide culture method. Detailed procedures are described in Materials and Methods.

RESULTS

MICs and MBCs of KRM-1648 and RFP. Table 1 shows the MBCs of KRM-1648 against *M. tuberculosis* H37Rv compared with those of RFP determined by the silicone-coated slide culture method. Three experiments were conducted under the same conditions. The MBCs of KRM-1648 ranged from 0.15 to 0.6 μg/ml for the contact periods tested, and there were rather slight changes of MBC between 2 and 24 h of drug contact, demonstrating very potent bactericidal activity. The MBCs of RFP were >160 μg/ml even when incubation was for 24 h. KRM-1648 was at least 500 times more potent than RFP over a 24-h contact period.

In experiment 3, the MICs (defined as the lowest concentrations at which no visible growth of bacilli is observed on slides incubated with drugs for 3 weeks) of the drugs against *M. tuberculosis* were 0.035 μg/ml for KRM-1648 and 2.5 μg/ml for RFP.

Comparison of in vivo efficacies of two single-drug regimens (KRM-1648 and RFP). The rates of survival of infected mice treated with KRM-1648 and RFP are shown in Fig. 1. Treatment dosages were 1, 3, and 10 mg/kg/day for KRM-1648 and 3, 10, and 30 mg/kg/day for RFP. In the infected control group, 8 of 10 mice died by 30 days after infection and the remaining 2 animals died by 90 days after infection. The survival curve for 3 mg of RFP per kg was similar to that for the control, and no significant difference was observed between the survival rate of mice given 3 mg of RFP per kg and that of the infected control group ($P > 0.05$). A dose-response effect was detected for both drugs. All of the mice treated with 10 mg of RFP per kg died by day 130, while 2 of 10 mice treated with 1 mg of KRM-1648 per kg survived at 160 days of infection. The effect of 1 mg of KRM-1648 per kg was comparable to that of 10 mg of RFP per kg, and the superiority of 3 mg of KRM-1648 per kg to 10 mg of RFP per kg was clearly demonstrated ($P < 0.05$).

No significant difference between survival rates with KRM-1648 and RFP at their highest doses was found ($P > 0.05$).

Effect of combinations of KRM-1648 with INH and EB. The survival rates for each combination regimen are shown in Fig. 2. There was no difference between the rate of survival of the mice treated with EB (10 mg/kg) alone and that of the control group ($P > 0.05$). The rate of survival of the mice treated with the INH-EB combination regimen was somewhat higher than that of mice treated with INH (3 mg/kg) alone, and the efficacy of combination chemotherapy with INH-EB was similar to that of RFP (10 mg/kg) alone up to 80 days after infection (only one mouse treated with RFP alone survived at 160 days after infection).

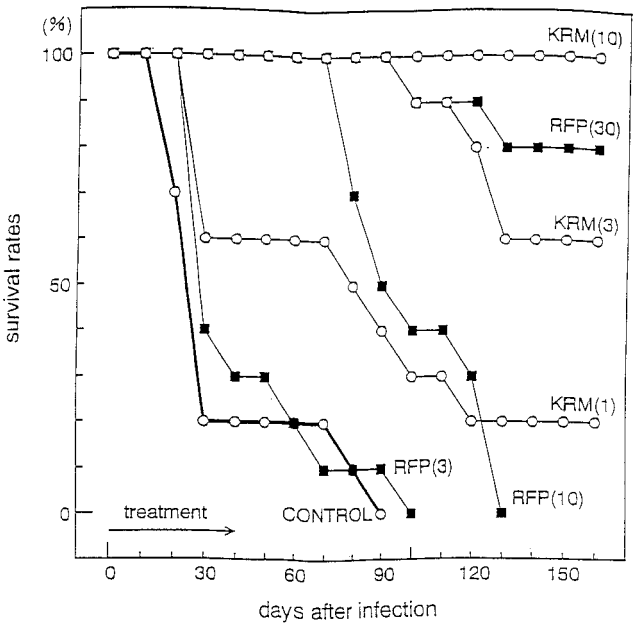

FIG. 1. Dose-response effects of KRM-1648 (KRM) on the rates of survival of mice infected with a lethal dose of *M. tuberculosis* H37Rv. Treatment was started on day 1 after infection and continued for 40 days. The values in parentheses are doses in milligrams per kilogram per day.

The prolongation of survival time with the 3-mg/kg dose of KRM-1648 was significantly greater than that with INH or RFP alone ($P < 0.05$). The prolongation of survival time with three-drug combination chemotherapy with KRM-1648 plus INH plus EB was significantly greater than that with RFP-INH-EB ($P < 0.05$), the efficacy of which was similar to that of KRM-1648 alone.

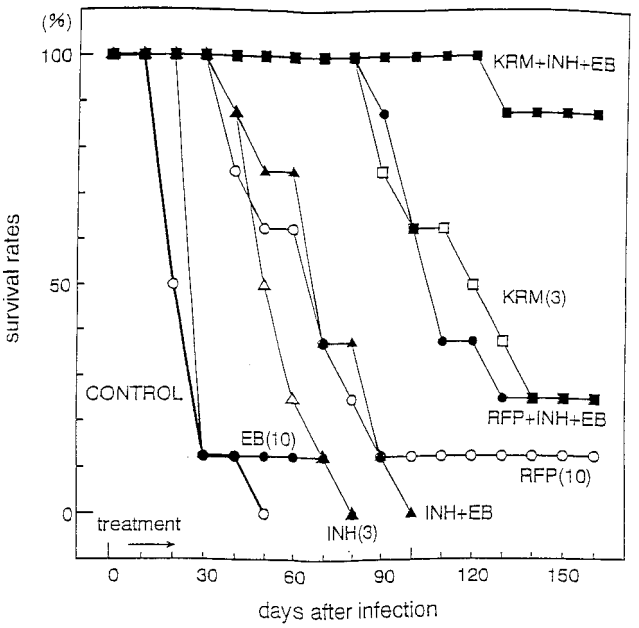

FIG. 2. Effects of combinations of KRM-1648 (KRM) with INH and EB on the rates of survival of mice infected with a lethal dose of *M. tuberculosis* H37Rv. Treatment was started on day 7 after infection and continued for 21 days. The values in parentheses are doses in milligrams per kilogram per day.

DISCUSSION

A number of studies have already demonstrated that the new rifamycin derivative KRM-1648 exhibits remarkable activity both in vitro (15, 17, 21) and in vivo (10, 14) against *M. tuberculosis*. In the present study, using a silicone-coated slide culture method, we found that KRM-1648 had an MBC much lower than that of RFP against *M. tuberculosis* H37Rv and that KRM-1648 exhibited superior therapeutic efficacy when used alone or in combination with other antituberculous drugs against experimental murine tuberculosis.

Within the past few years, a number of new rifamycins have been reported to have significant in vitro and/or in vivo activity against *M. tuberculosis* (1, 6–8). One of them, rifabutin, was found to have greater activity in vitro and to be six to seven times more active in vivo against *M. tuberculosis* (3, 18, 21) than RFP, which is a major antituberculous drug with strong activity against *M. tuberculosis* (2, 4, 5). Klemens et al. reported promising activity of KRM-1648 against *M. tuberculosis* ATCC35801 in CD-1 mice as indicated by the number of bacteria in tissues. KRM-1648 was more active than either RFP or rifabutin against organisms in spleens and lungs (10).

Luna-Herrera et al. reported in vitro activity of KRM-1648 against drug-susceptible and multidrug-resistant tubercle bacilli. For RFP-susceptible strains, the MIC and the MBC of KRM-1648 were much lower than those of RFP (15).

In the present study, the MBC of KRM-1648 for *M. tuberculosis* showed KRM-1648 to be at least 500 times more potent than RFP. This potent in vitro bactericidal activity was confirmed by the finding of prolonged survival times for mice with experimentally induced tuberculosis.

The prolongation of the survival time of the infected animals directly reflects an in vivo defense against bacterial infection. Kradolfer has reported that increases in the prolongation of survival time are strongly correlated with decreases in the viable counts of *M. tuberculosis* in lungs (11).

These findings suggest that KRM-1648 may play an important role in shorter-course regimens for tuberculosis.

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