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In Vitro Activity of the Benzoxazinorifamycin KRM-1648 against Drug-Susceptible and Multidrug-Resistant Tubercle Bacilli

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We investigated the activity of benzoxazinorifamycin (KRM-1648) against several drug-susceptible and multidrug-resistant strains of tubercle bacilli. Since KRM-1648 is a rifamycin derivative, we included some strains of *Mycobacterium tuberculosis* resistant to rifampin (RIF) among the multidrug-resistant strains. For RIF-susceptible strains, the MIC of KRM-1648 was much lower than that of RIF (MICs of KRM-1648 and RIF at which 90% of strains are inhibited, ≤0.015 and ≤0.25 μg/ml, respectively). The MBC of KRM-1648 (range, 0.007 to 0.03 μg/ml) was also much lower than that of RIF (range, 0.5 to 1.0 μg/ml). Postantibiotic effect studies with KRM-1648 showed a rapid reduction in the CFU counts with an exposure of 24 h or more, and its sterilizing effect was maintained even up to 21 days thereafter. Parallel postantibiotic effect studies with RIF showed a less significant effect with a faster recovery of growth, and RIF failed to sterilize the organisms even after 72 h of exposure. KRM-1648 at 0.125 and 0.25 μg/ml caused complete inhibition of intracellular growth of *M. tuberculosis* in J774 A.1 macrophages after 48 h of exposure. After a similar exposure time RIF at a concentration of 0.25 μg/ml caused complete inhibition of growth, but a concentration of 0.125 μg/ml caused only a 50% reduction in growth compared with that of controls at day 7. With 24 h of pulsed exposure of the intracellular organisms to 0.25 μg of the drugs per ml, KRM-1648 caused complete inhibition of intracellular growth, while RIF caused only moderate inhibition of intracellular growth. These findings suggest that KRM-1648 is a potentially useful drug for the treatment of tuberculosis.

Recent outbreaks of multidrug-resistant (MDR) tuberculosis created the urgency to discover new antituberculosis drugs. Besides random screening of several new compounds, investigations of structural analogs of existing drugs will be a useful approach. Rifampin (RIF) has been one of the most effective drugs for the treatment of tuberculosis; however, in the recent past RIF-resistant strains have been on the increase. Moreover, RIF is not effective against *Mycobacterium avium* complex strains. With the objective of developing an effective drug for the treatment of these mycobacterial diseases, several analogs (e.g., KRM-1648, KRM-1657, KRM-1668, and KRM-1687) of RIF were synthesized by Kaneka Corporation, Osaka, Japan. Essentially, these analogs are modifications of the isobutyl, propyl, secondary butyl groups (7, 8) of benzoxazinorifamycin. Of these, KRM-1648 was found to be highly active against *M. avium* complex strains (6, 7); however, its activity against *Mycobacterium tuberculosis* has not been thoroughly investigated. Here we describe our detailed in vitro studies of KRM-1648 against several drug-susceptible and MDR strains of tubercle bacilli in which we used quantitative assessments of MICs, MBCs, and postantibiotic effect (PAE). The intracellular activity of the drug against these organisms was also investigated with the J774 A.1 macrophage cell line.

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MATERIALS AND METHODS

Drugs. KRM-1648 was supplied by Kaneka Corporation. RIF was purchased from Sigma Chemical Company, St. Louis, Mo. Initial stock solutions of these drugs were made in dimethyl sulfoxide, from which further dilutions were made in sterile distilled water.

Mycobacterial strains. The *M. tuberculosis* strains used in the study and their susceptibility patterns are listed in Table 1. The organisms were grown in Middlebrook 7H9 broth to a density of 10^8 CFU/ml and were stored at −80°C in 1.0-ml aliquots. For each experiment, a single vial was thawed and used, and the unused portion was discarded.

Determination of the MIC by radiometric (BACTEC) method. Initially, all of the strains to be tested were grown in BACTEC 12B medium until the growth index (GI) reached 999 (GI is a scale in the BACTEC system which reflects the amount of growth) and were then used as the inocula. The vials were dispensed with different dilutions of KRM-1648 or RIF to reach final concentrations ranging from 0.06 to 2.0 μg/ml. All of the drug-containing vials were inoculated with 0.1 ml of the bacterial suspensions prepared as described above. Two drug-free controls were included with each test: one was inoculated with 0.1 ml of the suspension used for drug-containing vials, and the other was inoculated with 0.1 ml of a 1/100 dilution of the suspension. The vials were incubated at 37°C and were read in a BACTEC 460 reader (Johnston Laboratories, Towson, Md.) every day until the GI in the control diluted 1/100 reached ≥30, with an increase in the GI of at least 10 for 3 consecutive days. If the control diluted 1/100 reached a GI of ≥30 within 4 days or after 8 days of incubation, the test was repeated. The MIC was defined as the lowest concentration of the drug that caused an increase in the GI equal to or less than the increase in the GI of the control diluted 1/100 (3). If the MICs of the drug(s) fell out of the range used, the test was repeated with higher or lower concentrations of the drugs until the end point was reached.

Determination of MBC. Ten-milliliter volumes of Middlebrook 7H9 broth containing serial dilutions of KRM-1648 (0.25 to 0.007 μg/ml) and RIF (2.0 to 0.06 μg/ml) were prepared in screw-cap tubes. A drug-free control was included in tests with each strain. All of the drug-containing and drug-free control tubes were inoculated with 0.1 ml of an *M. tuberculosis* suspension whose turbidity was adjusted to equal that of a McFarland no. 1 standard. An aliquot (0.5 ml) from the drug-free control tube was serially diluted and was plated onto 7H11 agar plates to determine the initial CFU of the inoculum. All of the inoculated tubes were incubated at 37°C on a roller drum protected from light. After 8 to 10 days of incubation, when the control tube showed visible turbidity, those drug-containing tubes showing no turbidity were appropriately diluted (to below the MIC to prevent drug carryover), and 0.1-ml volumes were plated onto 7H11 agar. The CFUs were read after 3 weeks of incubation in a 5% CO_2 atmosphere. The

TABLE 1. *M. tuberculosis* strains used in the study and their drug susceptibility patterns[a]

Laboratory strain no.	Source	Remarks	Drug susceptibility pattern (μg/ml)[b]			
			RIF	INH	SM	EMB
9	UIC	RIF-R	>16.0	0.12	1.25	2.5
11	UIC	EMB-R	0.25		0.3	>10.0
12	UIC	PZA-R	0.12	0.12	0.3	
13	UIC	INH-R	0.06	>4.0	1.25	4.0
14	UIC	Susceptible	0.25	0.12	0.3	≤4.0
15	UIC	INH-R	0.06	>0.5	0.25	1.0
2218	CDC	MDR	0.06	4.0	>10.0	1.25
2219	CDC	MDR	>16.0	>4.0	2.5	5.0
2225	CDC	MDR	>16.0	>4.0	>10.0	5.0
2227	CDC	MDR	4.0	>4.0	>10.0	10.0
2230	CDC	MDR	>16.0	>4.0	>10.0	2.5
2235	CDC	Susceptible	0.12	≤0.12	0.3	2.5
2337	CDC	Susceptible	0.12	0.12	0.3	1.0
2242	CDC	Susceptible	0.12	0.12	0.3	
2257	CDC	Susceptible	0.25	0.12	0.3	
27	UIC	MDR	1.0	>0.5	1.0	2.0
28	UIC	SM-R	0.12	≤0.06	>2.0	1.0
29	UIC	MDR	>16.0	>0.5	>2.0	>4.0
31	UIC	MDR	>16.0	0.06	>2.0	>4.0
32	UIC	INH-R	0.25	>0.5	1.0	2.0
33	UIC	RIF-R	2.0	0.5	1.0	>4.0
37	L.A.	RIF-R	>16.0	≤0.12	≤2.0	1.0
50	UIC	RIF-R	>16.0	≤0.25	0.3	≤2.5
H37Rv	ATCC	Susceptible	0.12	0.12	0.3	5.0

[a] UIC, University of Illinois at Chicago; CDC, Centers for Disease Control and Prevention; L.A., Los Angeles; ATCC, American Type Culture Collection; RIF, rifampin; INH, isoniazid; SM, streptomycin; EMB, ethambutol; PZA, pyrazinamide; R, resistant; MDR, multidrug resistant.
[b] Determined by BACTEC system.

lowest concentration of the drug in 7H9 broth tubes that caused no visible turbidity was considered the MIC of the drug, and the lowest concentration of the drug that killed 99% of the organisms was considered the MBC of the drug (4). The MIC and MBC data from 7H9 broth were used to calculate the MBC/MIC ratio of KRM-1648 and RIF for each strain.

Continuous exposure study. Different concentrations of KRM-1648 (0.03 and 0.125 μg/ml for susceptible strains; 0.125 and 1.0 μg/ml for the MDR strain) and RIF (0.5 and 2.0 μg/ml for H37Rv) were prepared in Middlebrook 7H9 broth. A drug-free control was included in tests with each strain. All of the tubes were inoculated with different strains, and the tubes were incubated at 37°C on a roller drum protected from light. Aliquots of samples were collected from each tube on alternate days from day 1 to day 15. The CFUs in the samples were determined by plating appropriate dilutions on 7H11 agar.

Assessment of PAE. The PAE was determined as described by Dickinson and Mitchison (1). In brief, a log-phase culture of *M. tuberculosis* H37Rv was exposed to KRM-1648 (0.075 μg/ml) or RIF (0.625 μg/ml) for 6, 12, 18, 24, 48, or 72 h. After each period of exposure, the drug was removed by filtration through a 0.22-μm-pore-size membrane filter and the bacilli were washed five times each with 10 ml of 7H9 broth. The bacilli remaining on the filter membrane after the final wash were resuspended to the original volume of medium and were incubated at 37°C. The viable numbers of organisms in the resuspended samples from drug-treated or control tubes were determined at different time points by plating on 7H11 agar medium as described above.

Determination of the activity on intracellular bacilli. Twenty-four-well tissue culture chambers were seeded with J774 A.1 macrophages (10^6 cells per ml per well) in tissue culture medium (Dulbecco's modified Eagle's medium [DMEM] with 1% fetal calf serum), and the tissue culture chambers were incubated at 37°C in a 5% CO_2 atmosphere. After 2 h, the macrophage monolayers were washed once with warm Hanks' balanced salt solution (HBSS) to remove nonadherent cells and the cells were infected with *M. tuberculosis* H37Rv or 2227 at a multiplicity of infection of 10:1 (bacteria:macrophages). After 2 h of incubation, nonphagocytosed organisms were removed by washing three times with warm HBSS. The cells were replaced with fresh tissue culture medium containing different concentrations (0.25 and 0.125 μg/ml) of KRM-1648 or RIF. Control chambers contained only tissue culture medium. The tissue culture plates were incubated at 37°C in an atmosphere of 5% CO_2. After 48 h of exposure to the drugs the macrophages were replaced with fresh medium without the drugs. At 0, 4, and 7 days postinfection, macrophages in duplicate wells from each group were lysed with 0.25% sodium dodecyl sulfate. The number of viable organisms in each group was determined by injecting the aliquots of macrophage lysates into BACTEC 12B vials and determining the GI after 24 h of incubation (5).

In another set of experiments, the same procedure was followed except that the drug was removed from the wells after a single pulsed exposure for 24 h by washing with HBSS and replacing with fresh DMEM. The macrophage monolayers were lysed, and the GI readings were obtained as described above.

TABLE 2. MICs of KRM-1648 and RIF for *M. tuberculosis* strains by BACTEC method

Strain	Susceptibility to antituberculosis drugs[a]	MIC (μg/ml)	
		KRM-1648	RIF
Susceptible strains			
14	Susceptible	0.0039	0.250
2235	Susceptible	0.0009	0.125
2237	Susceptible	0.0078	0.125
2242	Susceptible	0.0078	0.125
2257	Susceptible	0.0078	0.250
H37Rv	Susceptible	0.0156	0.125
Strains resistant to drugs other than RIF			
11	EMB-R	0.0019	0.250
12	PZA-R	0.0019	0.125
13	INH-R	0.0019	0.062
15	INH-R	0.0039	0.062
28	SM-R	0.0078	0.125
32	INH-R	0.0039	0.250
2218	MDR	0.125	0.062
RIF-resistant strains			
9	RIF-R	4.0	>16.0
27	MDR	1.0	>16.0
29	MDR	4.0	>16.0
31	MDR	4.0	>16.0
33	RIF-R	0.125	2.0
37	RIF-R	0.50	>16.0
50	RIF-R	0.125	>16.0
2219	MDR	2.0	>16.0
2225	MDR	4.0	>16.0
2227	MDR	0.062	4.0
2230	MDR	0.125	>16.0

[a] See footnote *a* of Table 1 for definitions of abbreviations.

RESULTS

MICs of KRM-1648 and RIF for drug-susceptible and MDR tubercle bacilli. KRM-1648 had MICs (0.0009 to 0.125 μg/ml) much lower than those of RIF (0.06 to 0.25 μg/ml) for the six drug-susceptible strains (Table 2) and seven MDR strains resistant to drugs other than RIF; the MIC of KRM-1648 at which 90% of the MDR strains are inhibited was ≤0.015 μg/ml and that of RIF was ≤0.25 μg/ml (Table 2). The susceptibility determined by the conventional method also showed that KRM-1648 had much lower MICs than RIF, and there was clear agreement between the two methods (data not shown).

Interestingly, for 5 of 11 RIF-resistant strains for which MICs were ≥2.0 μg/ml, MICs of KRM-1648 were ≤0.5 μg/ml. Some of these strains were resistant to rifabutin as well. For strains resistant to both the drugs, KRM-1648 still showed lower MICs (1.0 to 4.0 μg/ml) compared with those of RIF (≥16.0 μg/ml). A similar trend was seen by the conventional method of susceptibility testing.

MBCs of KRM-1648 and RIF. For all of the RIF-susceptible strains, the MBCs of KRM-1648 ranged from 0.007 to 0.03 μg/ml and those of RIF ranged from 0.5 to 1.0 μg/ml (Table 3).

TABLE 3. MBCs and MICs of KRM-1648 and RIF

Strain	KRM-1648			RIF		
	MBC (μg/ml)	MIC (μg/ml)	MBC/MIC	MBC (μg/ml)	MIC (μg/ml)	MBC/MIC
H37Rv	0.007	0.007	1	0.5	0.25	2
2257	0.015	0.007	2	1.0	0.5	2
2237	0.007	0.007	1	1.0	0.5	2
2242	0.030	0.007	4	1.0	0.5	2
2230	0.250	0.125	2	ND[a]	ND	ND

[a] ND, not determined.

The MBC/MIC ratios for KRM-1648 ranged from 1 to 4, and for RIF it was 2. The MBC of KRM-1648 for the RIF-resistant strain 2230 was highest (0.25 μg/ml), with a MBC/MIC ratio of 2.

Effect of continuous exposure of drug-susceptible and RIF-resistant strains to KRM-1648. Continuous exposure of H37Rv to 0.125 μg of KRM-1648 per ml resulted in about a 6-log-unit reduction in the CFU in 1 day (Fig. 1A). Even with a lower concentration of KRM-1648 (i.e., 0.03 μg/ml), the elimination of the bacilli was evident, although at a slower rate (3 or 5 days) (Fig. 1C). For RIF, on the other hand, much higher concentrations (0.5 and 2.0 μg/ml) were required to show similar results (Fig. 1B).

KRM-1648 was less effective against RIF-resistant strain 2230 (Fig. 1D). At a concentration of 1.0 μg/ml, which is eight times the MIC for this strain, elimination of the bacilli was seen by 7 days. With the lower concentration (0.125 μg/ml), which caused a dramatic reduction in the CFU of the susceptible strains (Fig. 1A and B), only a gradual reduction was seen over a period of 17 days (Fig. 1D).

In order to ascertain that the drugs were stable during the 2 weeks of incubation, both KRM-1648 and RIF were prepared in 7H9 broth and were incubated at 37°C on a roller drum. Aliquots of the medium collected at days 0, 7, and 14 were tested in the BACTEC system for their activities against strain H37Rv. The activities of these samples were similar to those of the freshly prepared drugs, indicating the stabilities of the drugs for up to 2 weeks.

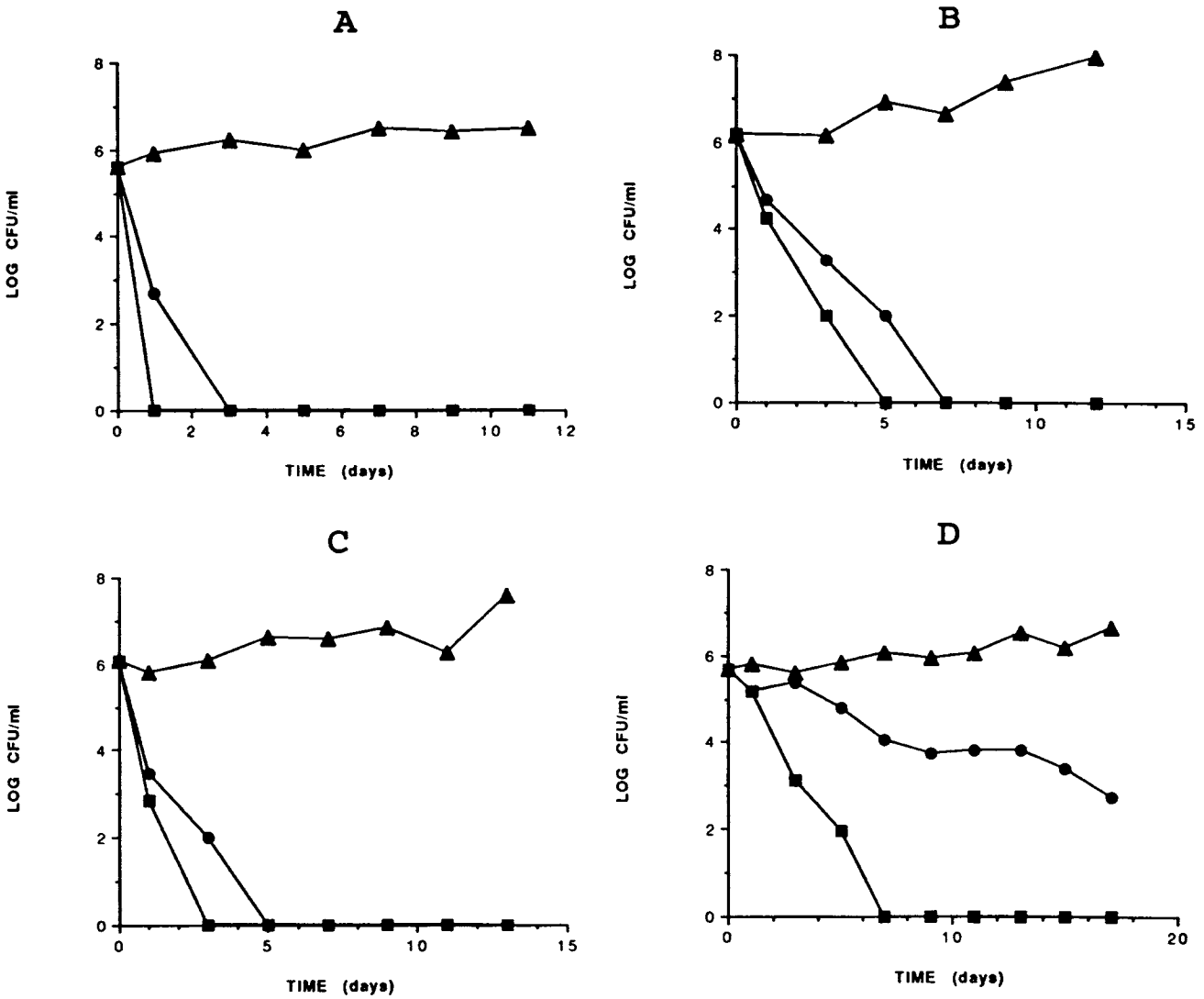

FIG. 1. Change in log CFU counts of *M. tuberculosis* strains in the presence of KRM-1648 or RIF and in drug-free controls. (A and B) Strain H37Rv; (C) strain 2242; (D) strain 2230. The sensitivity of detection was about 10 CFU/ml. ▲, control; ●, KRM-1648 at 0.03 μg/ml; ■, KRM-1648 at 0.125 μg/ml.

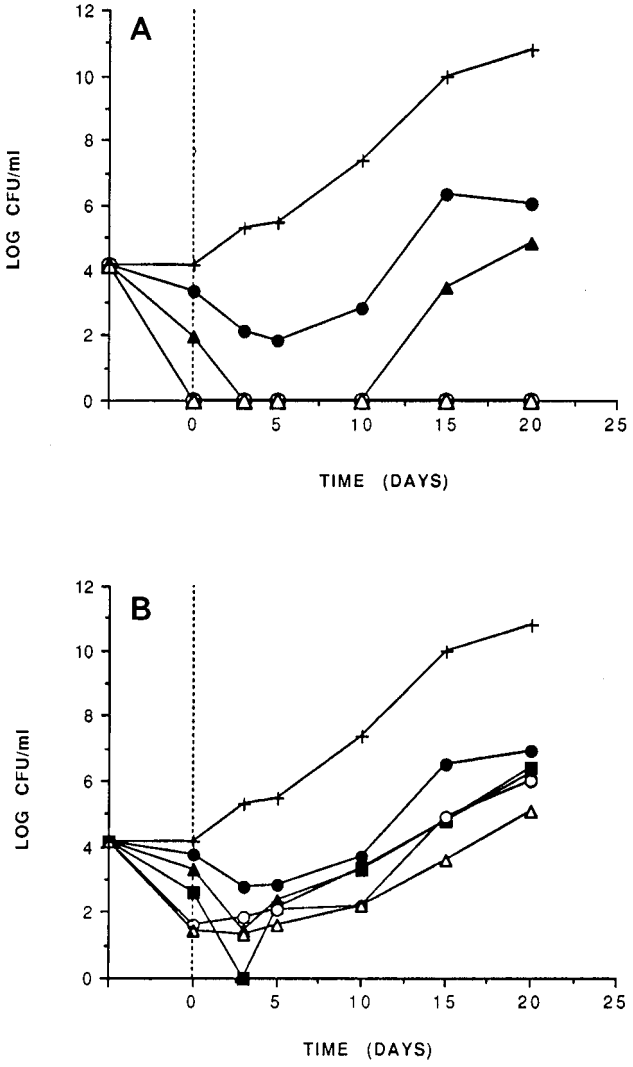

FIG. 2. Change in log CFU of *M. tuberculosis* H37Rv after exposure to drugs (KRM-1648 or RIF) for different periods of time (6, 12, 24, 48, and 72 h). (A) KRM-1648 at 0.075 μg/ml; (B) RIF at 0.625 μg/ml. After exposure to drugs for different periods of time, the bacillary suspensions were washed five times on a 0.22-μm-pore-size membrane filter and were finally resuspended in the original volume of 7H9 broth and incubated. The CFUs were determined up to 21 days postexposure. The sensitivity of detection was about 10 CFU/ml. ●, 6 h; ▲, 12 h; ■, 24 h; ○, 48 h; △, 72 h; +, control.

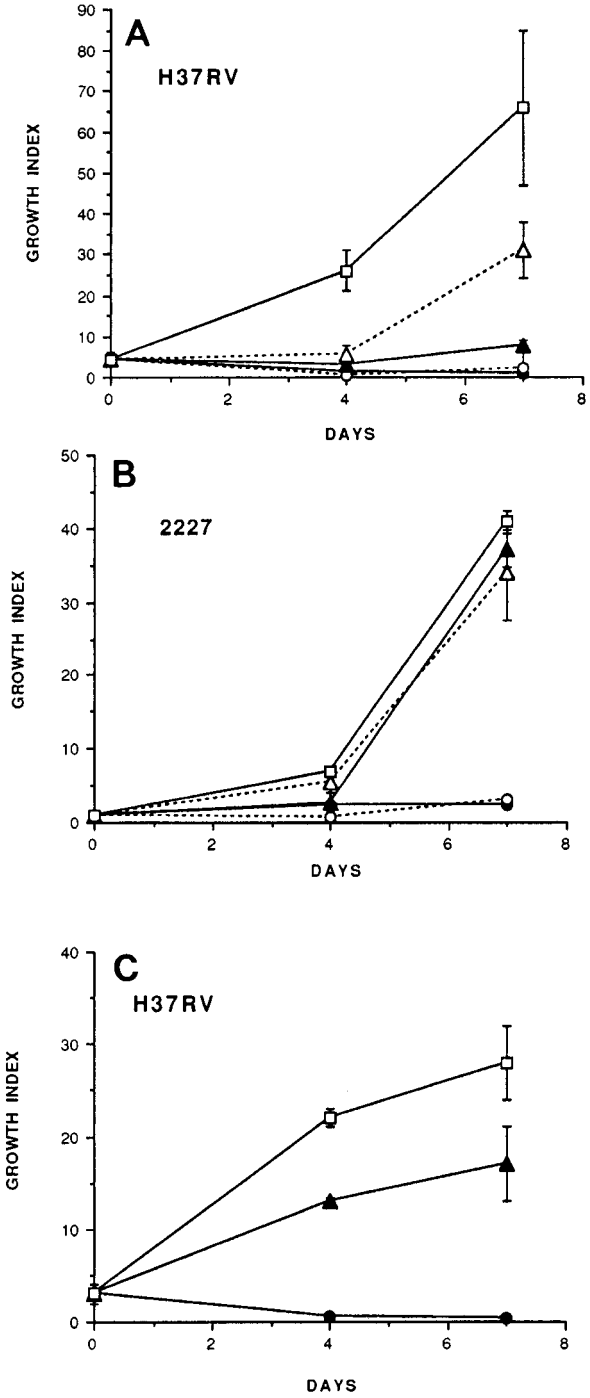

FIG. 3. Intracellular activities of KRM-1648 and RIF against *M. tuberculosis* strains. Both of the drugs were tested at 0.25 and 0.125 μg/ml. (A and B) Strains H37Rv and 2227, respectively; macrophages were exposed to the drugs for 48 h postinfection. (C) Strain H37Rv; macrophages were exposed to the drugs for 24 h postinfection. ●, KRM-1648 at 0.250 μg/ml; ○, KRM-1648 at 0.125 μg/ml; ▲, RIF at 0.250 μg/ml; △, RIF at 0.125 μg/ml; □, control.

PAE. Exposure of *M. tuberculosis* H37Rv to 0.075 μg of KRM-1648 per ml for 24 h or more caused the rapid elimination of the bacilli, and no resurgence of growth was seen even after 3 weeks of incubation (Fig. 2A). With an exposure time of 12 h, such a reduction was seen at 3 days and the growth commenced at 10 days. With 6 h of contact, only a gradual and slight reduction in CFU was seen by up to 5 days, at which time the bacterial growth started to increase. Thus, a contact period of 24 h or more would ensure complete elimination of the tubercle bacilli, while 12 h of contact would cause only a transient activity for about a week. In contrast, only a slight reduction in growth was seen after 24 to 72 h of contact with 0.625 μg of RIF per ml (Fig. 2B).

Action of KRM-1648 on intracellular tubercle bacilli. Initial studies assessed the viability of the J774 A.1 macrophages by the trypan blue exclusion test following exposure to KRM-1648 or RIF. No change in viability was seen with up to 5.0 μg of the drugs per ml. Exposure to 0.125 and 0.25 μg of KRM-1648 per ml for 48 h caused complete inhibition of intracellular multiplication of strain H37Rv (Fig. 3A). In contrast, RIF showed inhibition only at the higher concentration (0.25 μg/ml). KRM-

1648 also demonstrated similar levels of inhibition of the RIF-resistant strain 2227 (Fig. 3B); as was expected, RIF did not show any activity. KRM-1648 demonstrated inhibitory activity even after 24 h of exposure of the macrophage monolayer (Fig. 3C). Under similar circumstances, the effect of RIF was much less pronounced.

DISCUSSION

The high incidence of MDR tuberculosis brought to the forefront the necessity of discovering effective antituberculosis drugs. In this endeavor, in addition to searching for new compounds, investigating structural modifications of the existing lead drugs will be a useful alternate approach. The discovery of KRM-1648 as a promising drug is one good example of such attempts. Several studies with this drug have shown its value against members of the *M. avium* complex (6, 7), and the current studies with *M. tuberculosis* add equally important dimensions to its potential role as an antituberculosis drug.

The results presented here have shown the many facets of the activity of KRM-1648 in vitro and on intracellular tubercle bacilli. First, KRM-1648 demonstrated significantly greater in vitro activity with much lower MICs than those of RIF for several strains of tubercle bacilli. The MICs for some susceptible strains were as low as 1 ng/ml. Of particular value is its activity against MDR strains of tubercle bacilli, including those resistant to RIF. About 45% of the RIF-resistant strains were found to be susceptible to KRM-1648; on the other hand, none of the KRM-1648-resistant strains was susceptible to RIF. Second, the drug showed encouraging MBCs. Finally, the sustained killing effect after continuous exposure and, more importantly, after pulsed exposure add more weight to its antimycobacterial activity. Until recently, among the available antituberculosis drugs, RIF was acclaimed to be a very powerful one, in that it demonstrated a great PAE. The current studies demonstrate that KRM-1648 is superior to RIF in this respect. This property is seen not only in conventional in vitro PAE studies but also in macrophage models. These types of PAE studies with macrophage models will offer useful information along with information on the other parameters obtained by conventional macrophage studies.

In addition to its excellent in vitro and intracellular activities, KRM-1648 was found to possess better pharmacokinetics than RIF. Even though the levels of KRM-1648 in the plasma of mice were much lower than those of RIF, its concentrations in tissues, especially lungs and spleens, were significantly higher than those of RIF and the levels of the drug in these tissues persisted longer than those of RIF (8). The in vitro activity and the pharmacokinetic data have been substantiated by its effective in vivo activity against *M. avium* complex strains in mouse (7) and rabbit (2) models. Our preliminary in vivo data in C57BL/6 mice also indicate the superiority of KRM-1648 over RIF in rapidly eliminating tubercle bacilli from the organs. Overall, the data discussed in this and other reports (6, 7) show that KRM-1648 is a powerful antimycobacterial drug, suggesting that it has great potential for clinical application for the treatment of tuberculosis and, more importantly, disease caused by MDR strains.

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