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Bacteriostatic and Bactericidal Activities of Benzoxazinorifamycin KRM-1648 against *Mycobacterium tuberculosis* and *Mycobacterium avium* in Human Macrophages

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Inhibitory and bactericidal activities of KRM-1648 were determined against *Mycobacterium tuberculosis* and *M. avium* residing in human monocyte-derived macrophages and extracellular *M. tuberculosis* and *M. avium*. MICs and MBCs of KRM-1648 against intracellular and extracellular bacteria were substantially lower than those of rifampin. The MICs and MBCs of either drug against the intracellular bacteria were only twofold lower than or equal to the values found for extracellular bacteria. The prolonged effect of KRM-1648 found in this study is probably associated with high ratios of intracellular accumulation, which were 50- to 100-fold higher than that found for rifampin. Further studies on intracellular distribution of KRM-1648 and on the sites of actual interaction between the drug and bacteria residing in macrophages are necessary, as well as evaluation of combined effects of KRM-1648 with other drugs in long-term macrophage culture experiments.

Combined use of isoniazid, rifampin (RMP), and pyrazinamide provided the basis for successful short-course tuberculosis therapy. The discovery of long-lasting rifamycins created the possibility of intermittent drug regimens as has been shown in a number of observations with rifapentine (1–3, 6, 9), which accumulates to higher concentrations than does RMP in macrophages (10). A new rifamycin, has been developed by Kaneka Corporation (Osaka, Japan). The preliminary data in animal experiments have shown that it also produces long-lasting antimicrobial activities (7, 8, 14, 16). KRM-1648 accumulates to higher concentrations than does RMP in macrophages (10). The MICs and MBCs of KRM-1648 are substantially lower than those of RMP or rifabutin (4, 7, 15), but the pharmacokinetic parameters in humans and therefore the potential effectiveness of the new drug are not known yet and cannot be projected without relating these values to the concentrations attainable in blood.

In anticipation of a controlled clinical trial of KRM-1648 in the treatment of mycobacterial infections, the aim of the present study was to examine the potential activity of this rifamycin against *Mycobacterium tuberculosis* and *M. avium* residing intracellularly. Inhibitory and bactericidal activities (MIC and MBC) against intracellular bacteria were determined in human monocyte-derived macrophages and compared with the MICs and MBCs against extracellular bacteria, as well as with the actual accumulation of these drugs in human monocytes. Attention was also given to the evaluation of the long-lasting effect of KRM-1648 after pulsed exposures of the infected human macrophages, followed by a 4-week-long cultivation in drug-free media.

MATERIALS AND METHODS

Antimicrobial agents. KRM-1648 was obtained from Kaneka Corporation (Osaka, Japan). The drug was dissolved in dimethyl sulfoxide (DMSO) according to the manufacturer's instructions. Stock solutions were made at 1,280 μg/ml in 100% DMSO and then diluted in appropriate culture media (RPMI 1640 and 7H12 broth). KRM-1648 is a very lipophilic substance and presents a significant problem with solubility in water-based media. We determined that the macrophages would tolerate not more than 5% DMSO in the cell culture media. Thereafter all drug solutions were made such that the final medium contained not more than 5% DMSO. We tested the solubility by making various concentrations in the different media. The tubes of medium were left undisturbed overnight (18 h) at 37°C, after which they were examined for drug precipitation. Solubility was better in physiological saline versus RPMI 1640, Hanks' balanced salt solution, or Dulbecco's modified Eagle medium. The solubility in 7H12 broth was not a problem since the drug concentrations in MIC and MBC experiments with extracellular bacteria were within the solubility limits of KRM-1648. Solubility improved when the saline was alkalized with potassium hydroxide to pH 7.2. Even with these manipulations the maximum concentration that did not show visible precipitation was 4.0 μg/ml. In some experiments we observed a slight blue haze on the glassware and plasticware used in the 4-μg/ml and 1-μg/ml concentrations, presumably from adherence of KRM-1648 to the surfaces. The actual solubility of KRM-1648 is probably well below 1 μg/ml. We were surprised that these solubility problems had not been addressed in the literature, although in vitro concentrations of up to 100 μg/ml have reportedly been used by other authors (15).

Test strains. Three strains of *M. tuberculosis* were used in this study: H37Rv, Erdman, and a clinical isolate (Atencio), which is one of the strains used in our laboratory for quality control. All three strains are susceptible to all conventional antituberculosis drugs. Subcultures in 7H9 broth were preserved in aliquots frozen at −70°C. For each experiment, a subculture of frozen stock was made in fresh 7H9 broth. After 10 days of cultivation at 37°C, the culture was forced through a 27-gauge needle and was then centrifuged at 250 × *g* for 5 min to remove large clumps of bacteria. Three strains of *M. avium* were also used in this study: 453, 3010, and 3354. These strains, isolated from blood samples obtained from patients with AIDS, have been used in our previous studies (5, 12). Subcultures in 7H9 broth were preserved in aliquots frozen at −70°C. For each experiment, a subculture of frozen stock was made in fresh 7H9 broth.

Infection of human macrophages. Infection of human macrophages has been described in detail in our previous publications (11, 12). The human mononuclear cells were separated by Ficoll-Hypaque gradient centrifugation and placed in 35-mm plastic petri plates. After incubation at 37°C for 1 h, the nonadherent cells were washed away and plates were cultivated for 7 days at 37°C in RPMI 1640 containing 5% unheated human serum. At this point the monocytes have matured into macrophages and formed a monolayer. The medium was removed from the plates and was replaced with a suspension containing about 10^6 bacterial cells per ml, in order to have an average of five bacteria per macrophage. After incubation for 1 h the plates were washed twice to remove the extracellular bacteria. The infected macrophages were incubated in RPMI 1640 supplemented with 1% unheated human serum at 37°C in the presence of 7% CO_2. The number of bacterial cells in the inoculum (CFU/ml) was estimated by plating samples of the bacterial suspension on the 7H11 agar plates with subsequent colony count.

MIC and MBC determination. The MICs and MBCs were determined against extra- and intracellular bacteria. The MIC was defined as the lowest drug concentration that inhibited more than 99% of the bacterial population within 7 or 8 days of observation. The MBC was defined as the lowest drug concentration in the medium that decreased the bacterial population by 2 or more $\log_{10}$ units

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TABLE 1. MICs and MBCs of KRM-1648 and RMP against *M. tuberculosis*

Strain	Concn (μg/ml) against:							
	Intracellular bacteria				Extracellular bacteria			
	KRM-1648		RMP		KRM-1648		RMP	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
H37Rv	0.004	0.016	0.25	1.0	0.008	0.031	0.12	0.5
Erdman	0.008	0.008	0.12	0.5	0.002	0.004	0.12	0.5
Atencio	0.004	0.008	0.25	0.5	0.001	0.008	0.25	0.25

within the same period of incubation. For the extracellular bacteria, MICs and MBCs were determined in 7H12 broth (12B BACTEC vials; Becton Dickinson Diagnostic Instrument Systems, Sparks, Md.). The MIC for intracellular bacteria was defined as the lowest drug concentration in the medium that completely inhibited (>99%) the intracellular bacterial growth, and the MBC was defined as the lowest drug concentration in the medium killing more than 99% of the intracellular bacteria. The vials containing 4.0 ml of broth were supplemented with various concentrations of the drug, ranging in twofold fashion from 1.0 to 0.000468 μg/ml. Drug-containing and drug-free vials were inoculated with a bacterial suspension to produce 10^4 to 10^5 CFU/ml. Duplicate vials of each drug concentration and drug-free control were inoculated and cultivated. During the subsequent 7 or 8 days of cultivation, samples from alternate vials were taken for determination of the number of CFU per ml by plating on 7H11 agar. To determine the MIC and MBC against intracellular bacteria, the infected macrophages were exposed to various drug concentrations for a period of 8 days. The viable counts on days 0, 4 and 7 or 8 were determined by colony counting on 7H11 agar plates inoculated with serial dilutions of the macrophage lysates.

Pulsed exposure of the infected macrophages. The aim of these experiments was to examine the long-term effect of short exposure to the drug. We used two different concentrations of KRM-1648: 0.05 μg/ml and 0.3 μg/ml, which approximates the peak concentration in mouse serum after a single dose of 20 mg/kg (16). For the experiments using 0.05 μg/ml, the macrophages were exposed to the drug-containing media and incubated at 37°C. After a 2- or 6-h incubation period, the medium was removed and replaced with a drug-free medium and the monolayers were incubated for 4 weeks. The samples for determining the number of CFU were taken 0, 4, 7, 14, 21, and 28 days after infection. In experiments using 0.3 μg/ml, the macrophages were exposed to the drug media and incubated at 37°C. After a 2- or 6-h incubation period, the medium was removed and replaced with a drug-free medium and the monolayers were incubated for 4 weeks. The samples for determining the number of CFU were taken 0, 3, 7, 14, 21, and 28 days after infection.

In long-term experiments (3 or 4 weeks) the number of macrophages in the monolayers gradually decreased in drug-free controls because of the intensive intracellular multiplication of the tubercle bacilli. This did not occur in cultures treated with KRM-1648. The decrease in the number of macrophages in drug-free cultures started after 2 weeks of cultivation, and the loss was about 50% at the end of the third week and up to 100% at the end of the fourth week. Therefore, to estimate the actual number of bacteria in the drug-free cultures, the samples taken for CFU counts at any time point included the total number of bacteria per plate, both from still-intact remaining monolayers and from the supernate containing floating cells. In parallel experiments we previously confirmed that *M. tuberculosis* does not grow in cell-free RPMI 1640 with 1% human serum. This gave us confidence that the total number of bacteria recovered from macrophage cultures represented the outcome of intracellular growth only.

Method for determining intracellular concentrations. Mononuclear cells obtained through Ficoll-Hypaque gradient centrifugation were purified through adherence to the surface of the plastic petri plates. After incubation for 10 min at 37°C, the nonadherent cells were removed by washing three times with cold saline. The drug in various concentrations (0.25, 1, and 4 μg/ml) was added to the plates. After incubation at 37°C for 2 h the cells were detached by vigorous pipetting and scraping with a rubber policeman and separated from the extracellular fluid by velocity gradient centrifugation. The gradient was prepared in 1.8-ml microcentrifuge tubes (Sarstedt, Inc., Newton, N.C.) from two oil products: five parts of DC550 (specific gravity = 1.07) (Dow Corning, Midland, Mich.) plus 1.1 parts of paraffin oil (specific gravity = 0.838) (Aldrich Chemical, Milwaukee, Wis.). The gradient (1.0 ml) was layered in the bottom of each tube and overlaid with 0.5 ml of the monocyte suspension. After centrifugation at 14,000 × *g* for 3 min in a microcentrifuge (Eppendorf, Netheler, Germany), the cells had passed through the gradient to form a pellet in the bottom of the tube. After removal of the cell supernate, the oil was removed and the pellet was resuspended in 0.4 ml of saline. The number of cells was counted in a Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.), and the cells were lysed by sonication with a sonicator cell disrupter for 15 s at a power control setting of 2.5 (Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) and centrifuged to remove any cell debris. All cell samples, standards, and supernates were diluted 1:1 with DMSO to prevent further precipitation during the course of the experiment.

To determine the concentrations of the drugs, a bioassay was performed in 100-mm-diameter petri dishes filled with 10 ml of antibiotic agar no. 5 (Difco Laboratories, Detroit, Mich.) inoculated with *Micrococcus luteus* (ATCC9341). Four 8-mm-diameter wells per plate were filled with the various dilutions of the cell lysate or supernate (0.04 ml per well). For controls, the solutions of drugs were made in saline and diluted 1:1 with DMSO. Plates were incubated for 18 h at 37°C, after which the diameters of the growth inhibition zones were measured. The results from controls were used to construct a standard curve using regression analysis. The drug concentrations in experimental samples were calculated by using this standard dose-response curve. The results were expressed in micrograms per milliliter of the original (undiluted) sample. The volume of one monocyte was reported as 421 μm^3 (13); therefore, the number of cells in a sample multiplied by 421 μm^3 represented the total volume of cells before lysing. The ratio of cellular volume to sample volume was used to translate the results obtained in the bioassay into the intracellular concentration of the drug in micrograms per milliliter of cell mass.

TABLE 2. MICs and MBCs of KRM-1648 against *M. avium*

Strain	Concn (μg/ml) against:			
	Extracellular bacteria		Intracellular bacteria	
	MIC	MBC	MIC	MBC
453	0.016	0.125	0.031	>0.125
3010	0.031	0.250	0.062	>0.125
3354	0.016	0.250	0.062	>0.125

RESULTS

MICs and MBCs. The MICs and MBCs of KRM-1648 against intracellular and extracellular *M. tuberculosis* were similar (Table 1). The MICs ranged from 0.001 to 0.008 μg/ml, and the MBCs ranged from 0.008 to 0.03 μg/ml, resulting in MBC/MIC ratios from 8 to 1. The MICs and MBCs of KRM-1648 against intracellular and extracellular *M. avium* were notably higher than those for *M. tuberculosis* (Table 2). The MICs for extracellular *M. avium* ranged from 0.016 to 0.03 μg/ml, and the MBCs ranged from 0.03 to 0.06 μg/ml, resulting in an MBC/MIC ratio of 8 or 16. The MICs for intracellular *M. avium* ranged from 0.03 to 0.06 μg/ml. The MBCs for intracellular *M. avium* were determined to be greater than the highest concentration tested (>0.125 μg/ml).

Intracellular concentrations. The concentrations of KRM-1648 in macrophages were significantly greater than those in the extracellular medium. The intracellular concentration of KRM-1648 was a function of the drug concentration to which the macrophages were exposed. The intracellular/extracellular (I/E) ratios were within the same range in the presence of 0.25, 1.0, or 4.0 μg/ml (Table 3). The mean I/E ratio was 236 ± 28 (Table 3). We were not able to use lower concentrations in these experiments because of the limitations of the bioassay to determine the drug concentration of the samples. However, this was not important, since the experiments showed about

TABLE 3. Accumulation of KRM-1648 and RMP in human monocytes

Concn in medium (μg/ml)	I/E ratio (mean ± SE)[a]	
	KRM-1648	RMP
0.25	288 ± 36	4.2 ± 1.9
1.0	191 ± 46	4.5 ± 2.5
4.0	230 ± 56	4.5 ± 1.7
Overall mean	236 ± 28	4.4 ± 1.9

[a] Each value represents the mean of three to five different experiments.

TABLE 4. Effect of one 2- or 6-h pulsed exposure of infected macrophages to 0.05 μg of KRM-1648 per ml

Test strain and exposure	Avg no. of CFU per 5×10^5 macrophages at day					
	0	4	7	14	21	28
Erdman						
Control	2.1×10^3	9.4×10^3	4.1×10^4	2.4×10^6	5.8×10^7	7.5×10^7
2 h	2.1×10^3	1.3×10^0	1.6×10^1	9.6×10^1	1.5×10^3	2.8×10^2
6 h	2.1×10^3	0	5.2×10^1	3.7×10^1	6.7×10^2	3.3×10^3
H37Rv						
Control	2.6×10^4	3.3×10^3	3.2×10^5	4.8×10^6	1.3×10^7	4.6×10^6
2 h	2.6×10^4	1.3×10^1	3.5×10^2	1.0×10^3	5.0×10^1	6.6×10^1
6 h	2.6×10^4	2.5×10^1	6.3×10^1	9.6×10^2	5.0×10^2	8.2×10^1
Atencio						
Control	7.5×10^2	5.0×10^3	8.0×10^4	1.5×10^6	1.4×10^7	2.7×10^7
2 h	7.5×10^2	6.3×10^0	0	1.3×10^1	0	Not done
6 h	7.5×10^2	0	5.0×10^1	3.8×10^1	0	5.7×10^2

the same I/E ratio after exposure to various drug concentrations. Despite this intracellular accumulation, the concentrations of this agent in the medium required for complete inhibition of growth of the intracellular bacteria (MIC) or for a 100-fold or greater decrease in the number by killing (MBC) were similar to the MICs and MBCs against extracellular bacteria (Tables 1 and 2).

Effect of pulsed exposure to KRM-1648. In the experiments using 0.05 μg/ml, the number of viable bacteria in the drug-free controls increased during the 28-day period of incubation from an average of 7.5×10^2 to 2.6×10^4 to an average of 4.6×10^6 to 2.7×10^7 CFU/5×10^5 macrophages (Table 4). The number of viable bacteria in macrophages exposed to KRM-1648 had declined during the first 7 days of incubation by 1 to 3 $\log_{10}$ units (Table 4). During the following period of observation the effect of the drug was only inhibitory with some fluctuation of the number of bacteria and rebound to higher numbers toward the end of observation. The fluctuating number of bacteria found during this stationary phase of incubation may be due, in part, to the technical errors inevitable when quantitating samples containing a small number of bacteria.

In the experiments using 0.3 μg/ml, the number of viable bacteria in the drug-free controls increased during the 28-day period of incubation from an average of 5.1×10^3 to 5.7×10^3 to an average of 8.6×10^6 to 5.5×10^7 CFU per 5×10^5 macrophages (Table 5). During the same period, the number of viable bacteria in macrophages exposed for 2 h to 0.3 μg of KRM-1648 per ml had declined by 3 $\log_{10}$ units after 7 days of incubation. The bacterial populations began to rebound somewhat after 14 or 21 days in two of the strains tested (Table 5). In comparison, pulsed exposure to 3 μg of RMP per ml caused a decline of only 1 or 2 log units after 7 days of incubation (Table 5).

DISCUSSION

KRM-1648 has shown higher bacteriostatic and bactericidal activities than RMP, especially against intracellular bacteria growing in human monocyte-derived macrophages. The intracellular accumulation of KRM-1648 in human monocytes was much higher than that of RMP, with an average extracellular/intracellular ratio of 236 for KRM-1648. The method used in this study cannot determine the intracellular distribution of the drug in macrophages. Therefore, our assessment of the intracellular concentration should be interpreted rather as the concentration of the cell-associated drug. Evaluation of the drug intracellular traffic will require other types of studies, which are now in progress in our laboratory. The need for such studies is justified by the controversial findings in the current study. We have found that despite the high intracellular accumulation of KRM-1648, the lowest extracellular concentrations required to induce complete inhibition of growth of the intracellular *M. tuberculosis* populations was no different from the MIC ranges found in cell-free liquid medium. The MBCs against intracel-

TABLE 5. Effect of one 2-h pulsed exposure of infected macrophages to 0.3 μg of KRM-1648 per ml or 3 μg of RMP per ml

Test strain and drug	Avg no. of CFU per 5×10^5 macrophages at day					
	0	3	7	14	21	28
H37Rv						
KRM	5.6×10^3	6.3×10^0	1.1×10^1	2.5×10^0	2.2×10^2	6.5×10^2
Control	5.6×10^3	4.4×10^3	1.8×10^4	7.3×10^4	6.5×10^6	8.6×10^6
RMP	6.6×10^3	3.0×10^2	1.1×10^2	2.3×10^2		
Control	6.6×10^3	5.3×10^4	9.8×10^5	1.8×10^7		
Erdman						
KRM	5.7×10^3	2.5×10^0	7.5×10^0	2.5×10^0	2.1×10^2	0
Control	5.7×10^3	5.2×10^4	2.0×10^5	9.2×10^6	1.7×10^7	1.7×10^7
RMP	1.0×10^4	7.4×10^2	3.7×10^2	1.2×10^3		
Control	1.0×10^4	3.4×10^4	8.2×10^5	8.3×10^6		
Atencio						
KRM	5.1×10^3	2.5×10^0	7.5×10^0	1.8×10^2	7.5×10^0	2.4×10^3
Control	5.1×10^3	4.4×10^4	7.5×10^5	1.3×10^7	3.7×10^7	5.5×10^7
RMP	9.7×10^3	1.9×10^2	1.2×10^2	6.4×10^2		
Control	9.7×10^3	9.5×10^3	6.0×10^4	3.9×10^6		

lular bacteria were also in the same range as the MBCs against extracellular bacteria. The controversy between these data and the substantial intracellular accumulation may be explained by the possibility that only a portion of the cell-associated drug interacted with the intracellular bacteria. Short 2- or 6-h pulsed exposures of the infected macrophages to 0.05 μg of KRM-1648 per ml resulted in a prolonged inhibitory effect without complete sterilization. The prolonged effect during 28 days of incubation in drug-free media after pulsed exposure to KRM-1648 may be due to the high intracellular accumulation with a subsequent release of the drug over time. The solution to these problems requires further studies, particularly on the intracellular traffic and distribution of KRM-1648 in different cellular compartments. We conclude from the observations presented in this report that KRM-1648 may have potential for effective use in intermittent drug regimens; however, the poor solubility of the drug may present some problems.

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