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Mechanism of Action of Antimycobacterial Activity of the New Benzoxazinorifamycin KRM-1648

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The mechanism of antimicrobial activity of KRM-1648 (KRM), a new rifamycin derivative with potent antimycobacterial activity, was studied. Both KRM and rifampin (RMP) inhibited RNA polymerases from *Escherichia coli* and *Mycobacterium avium* at low concentrations: the 50% inhibitory concentrations (IC_{50}s) of KRM and RMP for *E. coli* RNA polymerase were 0.13 and 0.10 μg/ml, respectively, while the IC_{50}s for *M. avium* RNA polymerase were 0.20 and 0.07 μg/ml. Both KRM and RMP exerted weak inhibitory activity against *Mycobacterium fortuitum* RNA polymerase, rabbit thymus RNA polymerases, *E. coli* DNA polymerase I, and two types of reverse transcriptases. Uptake of ^{14}C-KRM by *M. avium* reached 18,000 dpm/mg (dry weight) 1.5 h after incubation, while uptake by *E. coli* cells was slight. KRM was much more effective in inhibiting uptake of ^{14}C-uracil than was RMP (IC_{50} of KRM, 0.04 μg/ml; IC_{50} of RMP, 0.12 μg/ml). These findings suggest, first, that the potent antimycobacterial activity of KRM is due to inhibition of bacterial RNA polymerase and, second, that the activity of KRM against target organisms depends on target cell wall permeability.

3′-Hydroxy-5′-(4-isobutyl-1-piperazinyl)benzoxazinorifamycin (KRM-1648 [KRM]), a newly synthesized rifamycin derivative, exerts much more potent in vitro and in vivo activities against slowly growing mycobacteria, including those of the *Mycobacterium avium* complex and *Mycobacterium tuberculosis*, than does rifampin (RMP) (2, 9, 14, 17, 18, 22, 23). Furthermore, KRM is known to exhibit potent activity in vitro and in vivo against gram-positive bacteria (3, 23) but is not effective against gram-negative bacteria or rapidly growing mycobacteria such as *Mycobacterium fortuitum* (3, 14, 23). The spectrum of activity of KRM against gram-positive and gram-negative bacteria is nearly the same as that of RMP (3). Since it is well known that the antimicrobial activity of RMP is due to inhibition of microbial DNA-dependent RNA polymerases (4, 5, 8, 16, 19, 20), we studied the effects of KRM on mycobacterial RNA polymerases and its ability to permeate bacterial cells.

MATERIALS AND METHODS

Organisms. *M. avium*, *M. fortuitum*, and *Escherichia coli* were derived from our stock cultures. The MICs of test agents for *M. avium* were determined with the BACTEC 460 TB system as previously described (17).

Special agents. ^{14}C-KRM (0.51 MBq/mg) and KRM were obtained from KANEKA Corp. (Takasago, Japan). Other radiolabelled compounds were purchased from Daiichi Pure Chemical (Tokyo, Japan). Rifampin was the kind gift of Daiichi Pharmaceutical Co. (Tokyo, Japan). RNA polymerase (from *E. coli*) and DNA (type I from calf thymus and type VIII from *E. coli*) were purchased from Sigma (St. Louis, Mo.). DNA polymerase (type I from *E. coli*) and TTP were purchased from Takara (Kyoto, Japan). Avian myeloblastosis virus (AMV) reverse transcriptase and Moloney murine leukemia virus (MMLV) reverse transcriptase were purchased from Pharmacia Biotech (Tokyo, Japan). All other chemicals were obtained from Wako Pure Chemical Industries (Tokyo, Japan).

Buffers. The following buffers were used for purification of RNA polymerase. Buffer A contained 50 mM Tris-HCl (pH 7.9), 1 mM $MgCl_2$, 0.2 M KCl, 0.1 mM dithiothreitol (DTT), 0.1 mM EDTA, and 5% (vol/vol) glycerol. Buffer B contained 0.01 M Tris-HCl (pH 7.9), 1 mM $MgCl_2$, 0.1 mM DTT, and 5% glycerol. Buffer C contained 20 mM Tris-HCl (pH 7.9), 0.08 mM DTT, 0.04 mM EDTA, 4 mM $MgCl_2$, and 60% glycerol.

Test drugs. KRM and RMP were dissolved in dimethyl sulfoxide at 1 mg/ml and stored at −20°C, and drug solutions were diluted with distilled water at various concentrations just before use.

Purification procedure for RNA polymerase. The purification procedure used for RNA polymerase was essentially that described by Harshey et al. (4) with slight modifications. Briefly, *M. avium* N-265 with smooth, transparent, irregularly shaped colonies was grown on 1% Ogawa egg medium at 37°C for 5 weeks. Ten grams of cultivated cells was then suspended in 15 ml of buffer A and subjected to sonication for 30 min. The suspension was centrifuged at 20,000 × *g* for 30 min, and the supernatant was centrifuged at 100,000 × *g* for 90 min. The resulting supernatant was subjected to ammonium sulfate fractionation (30 to 50%). The precipitate was dissolved in a small volume of buffer B and dialyzed overnight against the same buffer to remove excess ammonium sulfate. The dialyzed fraction was applied to a DEAE-cellulose column (10 ml of column volume per 10 g of cells). The column was washed with buffer B and eluted with a linear gradient of KCl (0 to 0.3 M) in the same buffer. The fractions containing activity were pooled and brought to 50% saturation with ammonium sulfate. The precipitate was dissolved in a small volume of buffer C and dialyzed overnight against the same buffer. The specific activity of this enzyme was 4.4 U/mg of protein.

The procedure for purification of *M. fortuitum* F-65 was almost the same as that described above for *M. avium* N-265, except F-65 cells were grown on 7H11 agar plates. The specific activity of the enzyme from *M. fortuitum* was 1.3 U/mg of protein.

Rabbit thymus RNA polymerase activity was obtained from crude extract of rabbit thymus homogenate by the method of Kedinger et al. (6).

Assay of RNA polymerase. The activity of bacterial RNA polymerase was measured by the method of Burgess (1) with slight modifications. The reaction mixture (0.2 ml) consisted of 120 mM Tris-HCl (pH 7.8); 5 mM $(CH_3COO)_2Mg$; 2 mM $MnSO_4$; 0.2 mM DTT; 0.5 mg of calf thymus DNA per ml (assay for RNA polymerase from *M. avium*) or 0.5 mg of *E. coli* DNA per ml (assay for *E. coli* RNA polymerase); 0.16 mM (each) ATP, CTP, and GTP; 0.16 mM [^{3}H]UTP (3.7 MBq/μmol); 10 to 50 μl of enzyme solution; and an appropriate amount of the test drug. The reaction was begun with the addition of [^{3}H]UTP, and incubation proceeded at 37°C for 10 min. The reaction was stopped by chilling the reaction mixture on ice, and a 40-μl aliquot of the reaction mixture was directly applied to Whatman no. 52 filter paper (2 by 2 cm). The filter paper was rinsed four times with 5% trichloroacetic acid and once with 95% ethanol and then was air dried. Radioactivity was determined with a Packard 3255 liquid scintillation counter. The method used for the assay of rabbit thymus RNA polymerase was the same as that of Kedinger et al. (6).

Assay of DNA polymerase. The activity of DNA polymerase was assayed by the method of Richardson et al. (13) with slight modifications. Briefly, the reaction mixture (0.2 ml) consisted of 57 mM potassium phosphate buffer (pH 7.4), 6.7 mM $MgCl_2$, 1 mM DTT, 45 μg of poly(dA-dT) per ml, 35 μM dATP and

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[^{3}H]TTP (26 KBq) each, 1.2 U of DNA polymerase I, and an appropriate amount of the test drug. The reaction was initiated by the addition of enzyme and incubation at 37°C for 20 min. Subsequent procedures were performed as described above for the RNA polymerase assay.

Reverse transcriptase assay. The reverse transcriptase assay was performed by the method of Komiyama et al. (7) with some modifications. For MMLV reverse transcriptase, the reaction mixture (0.1 ml) consisted of 50 mM Tris-HCl (pH 8.3), 8 mM $MgCl_2$, 10 mM DTT, 0.2 mM TTP, 0.6 U of MMLV reverse transcriptase, and an appropriate amount of the test drug. This mixture was preincubated at 37°C for 30 min. After preincubation, the reaction was begun with the addition of 0.5 μg of poly(rA), 0.5 mU of oligo(dT)$_{12-18}$, and 37 KBq of [^{3}H]TTP, and then the mixture was incubated at 37°C for 30 min. The reaction was terminated by chilling of the reaction mixture on ice, and a 50-μl aliquot of the reaction mixture was applied to Whatman DE-51 filter paper (2 by 2 cm squares). The filter paper was rinsed three times with 5% Na_2HPO_4, once with water, and once more with 95% ethanol. After drying, the radioactivity was measured with a scintillation counter. For AMV reverse transcriptase, the reaction mixture consisted of 50 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$, 30 mM NaCl, 5 mM DTT, 0.1 mg of bovine serum albumin per ml, 2 mM [^{3}H]TTP (37 KBq), 2 U of AMV reverse transcriptase, and a suitable amount of test drug. The mixture was incubated at 37°C for 30 min, and the reaction was begun with the addition of 0.5 μg of poly(rA) and 0.5 mU of oligo(dT)$_{12-18}$. After incubation at 37°C for 30 min, the reaction was stopped by chilling the reaction mixture on ice, and subsequent procedures were the same as those for the MMLV reverse transcriptase assay.

Protein was determined by the method of Lowry et al. (10).

Incorporation of ^{14}C-KRM into *M. avium* or *E. coli*. *M. avium* N-265 was grown in 7H9 broth containing 0.5% glycerol. The bacterial suspension was centrifuged at 20,000 × *g* for 30 min. The bacterial pellet was resuspended in the same broth and sonicated to achieve dispersion. The bacterial suspension was diluted with culture medium to an optical density at 540 nm of 0.1. ^{14}C-KRM was added at a concentration of 1 μg/ml to the bacterial suspension, and this mixture was incubated at 37°C for various periods of time. One milliliter of each of the incubated suspensions was withdrawn and filtered with a cellulose nitrate filter (0.45-μm pore size [Toyo]) and washed with 30 ml of distilled water–30 ml of 0.5% trichloroacetic acid–2 ml of 95% ethanol. After air drying, the radioactivity was determined with a scintillation counter. *E. coli* IFO 12734 was cultured in Mueller-Hinton broth (Difco). A bacterial suspension at an optical density of 0.1 was used for the experiments. Other assay procedures were performed as described above for *M. avium.*

Uptake of [^{14}C]uracil into RNA by *M. avium.* Measurement of incorporation of uracil into RNA was performed essentially in accordance with the method of White et al. (21). First, 5-ml bacterial suspensions (optical density at 540 nm of 0.08) were incubated with drug solutions and [^{14}C]uracil (2.04 KBq/nmol) in a 30-ml conical flask in a water bath with a shaker at 37°C. Bacterial suspension without drug was used as a control. Four hours later, 1 ml of sample was withdrawn and precipitated with 1 ml of cold 10% trichloroacetic acid. The precipitate was cooled in an ice bath for 10 min and then heated in a 90°C water bath for 1 min. The sample was filtered through glass fiber filters (GF/C; Whatman). The filters were washed with 5 ml of 2 N HCl–2 ml of 95% ethanol and air dried. The radioactivity was determined with a scintillation counter.

RESULTS AND DISCUSSION

Effects of KRM on RNA polymerases and other nucleic acid synthetases. We first examined the effect of KRM on RNA polymerases from *E. coli*, *M. avium*, and *M. fortuitum* (Fig. 1). Both KRM and RMP inhibited *E. coli* and *M. avium* RNA polymerases at low concentrations. The 50% inhibitory concentration (IC_{50}) of KRM was higher than that of RMP for both of these RNA polymerases (*E. coli*, KRM, 0.13 μg/ml; RMP, 0.10 μg/ml; *M. avium*, KRM, 0.20 μg/ml; RMP, 0.07 μg/ml). These rifamycin compounds exhibited the same inhibitory effect on RNA polymerase from *M. avium* N-333 (data not shown). Thus, the antimicrobial activity of KRM appears to be due to inhibition of RNA polymerase. Moreover, KRM and RMP each only partially inhibited *M. fortuitum* RNA polymerase (29 and 42% inhibition, respectively) even at much higher concentrations of 1 to 10 μg/ml. This is consistent with the finding that these rifamycins lack activity in vitro against *M. fortuitum* (14). It is possible that the molecular structure of *M. fortuitum* RNA polymerase differs from that of *M. avium* RNA polymerase.

Table 1 summarizes the IC_{50}s of KRM and RMP for various nucleic acid synthetases. Neither had any appreciable inhibitory effect on rabbit thymus RNA polymerase, *E. coli* DNA polymerase I, or two types of reverse transcriptase (no inhibition observed at 10 μg/ml for either drug). The spectrum of inhibitory effects of KRM on various nucleic acid synthetases is the same as that of RMP.

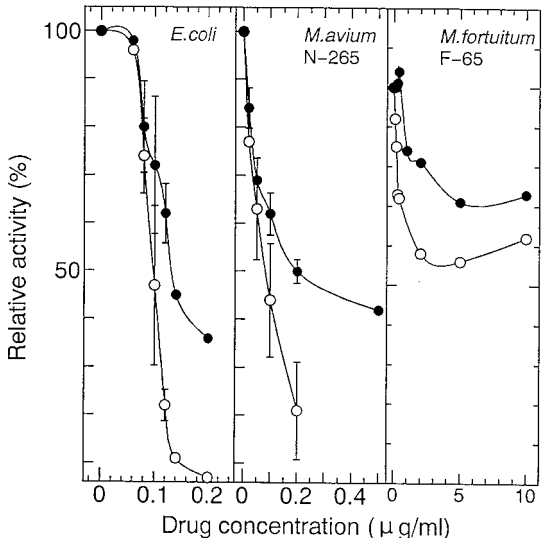

FIG. 1. Effects of KRM and RMP on the activities of three bacterial RNA polymerases. Changes in enzyme activity in the presence of the indicated rifamycins are illustrated. Each point represents the mean ± standard deviation of two to six determinations. ●, KRM. ○, RMP.

We note here two issues concerning the effect of KRM on bacterial RNA polymerases. First, although KRM potently inhibited the RNA polymerases of both *M. avium* and *E. coli* to the same extent, the activity of KRM against *E. coli* (3, 23) was much weaker than that against *M. avium* (9, 14, 17, 18). Second, despite these findings that the inhibitory effect of KRM was weaker than that of RMP, KRM had much greater antimycobacterial activity than did RMP (the MIC of KRM for *M. avium* N-265 was about 10 times lower than that of RMP).

It has been shown that the outer membrane of *E. coli* acts as a permeability barrier for hydrophobic agents (11). The hydrophobicity of KRM is greater than that of RMP (i.e., the log *P* values of the partition coefficients of KRM and RMP at pH 7 are 5.96 [unpublished data] and 1.19 [15], respectively). Moreover, the permeability barrier of mycobacteria determines the extent of the activity of RMP against them (5, 12). Thus, we hypothesized that the ability of KRM to permeate bacterial membrane would have an effect on those issues. Therefore, we compared the incorporation of KRM into *M. avium* with that into *E. coli.*

TABLE 1. IC_{50}s of KRM and RMP for various types of nucleic acid synthetases

Enzyme(s)	IC_{50} (μg/ml) of:	
	KRM	RMP
M. avium N-265 RNA polymerase	0.20	0.07
E. coli RNA polymerase	0.13	0.10
M. fortuitum F-65 RNA polymerase	>10	>10
Rabbit thymus RNA polymerases	>10	>10
E. coli DNA polymerase I	>10	>10
AMV reverse transcriptase	>10	>10
MMLV reverse transcriptase	>10	>10

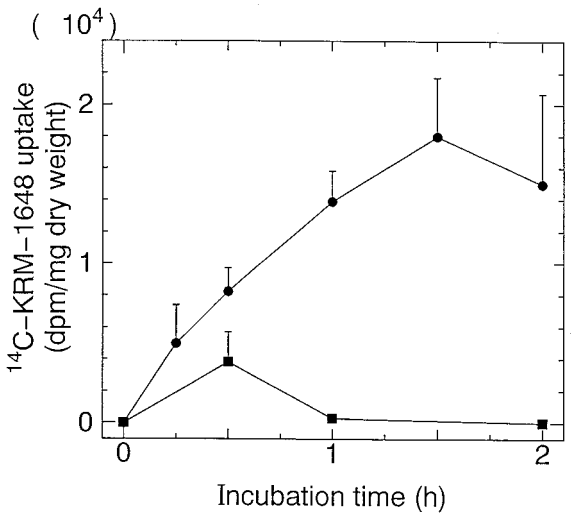

FIG. 2. Uptake of ^{14}C-KRM by *M. avium* and *E. coli*. Each point indicates the mean ± standard deviation of four determinations. ●, *M. avium*. ■, *E. coli.*

Incorporation of ^{14}C-KRM into *M. avium* and *E. coli.* Figure 2 shows the incorporation of KRM into *M. avium* and *E. coli*. The incorporation of ^{14}C-KRM into *M. avium* increased, depending on time of incubation (about 18,000 dpm/mg [dry weight] during the first 1.5-h incubation). The radioactivity of KRM in *E. coli* cells rapidly increased during the first 0.5-h incubation, reaching ca. 4,000 dpm/mg (dry weight) but thereafter decreased to ca. 300 dpm/mg (dry weight) at 1 h. By 2 h later, the radioactivity of *E. coli* cells had returned to the background level.

We found that the incorporation of KRM into *M. avium* was much greater than that into *E. coli*. This suggests that the weak activity of KRM against *E. coli* is due to the poor ability of KRM to permeate the *E. coli* membrane. Next, in order to examine the rate of accumulation of these rifamycins by *M. avium*, we determined the effects of KRM and RMP on bacterial RNA synthesis.

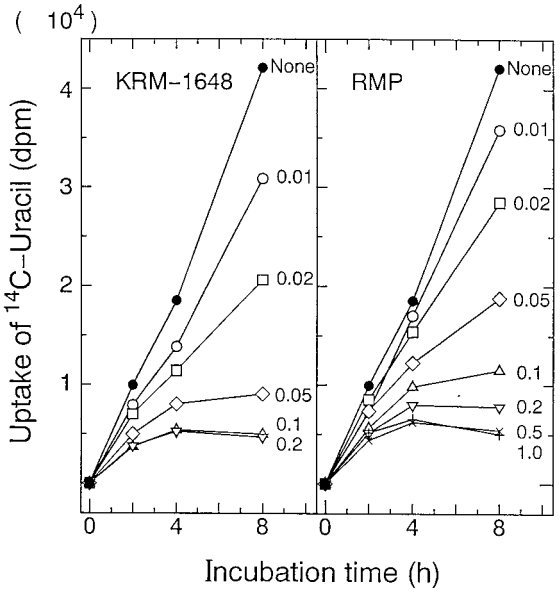

FIG. 3. Effects of KRM and RMP on RNA synthesis by *M. avium* N-265. Drug concentrations (symbols) are given in micrograms per milliliter. Each point indicates the mean of two determinations.

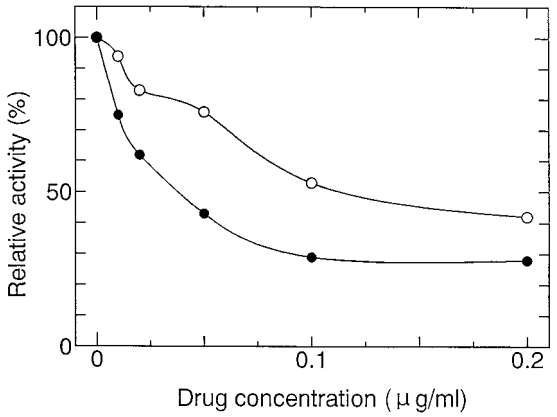

FIG. 4. Effects of KRM and RMP on RNA synthesis by *M. avium* N-265. Values are given as relative activities of the enzyme in the presence of rifamycins. Each point indicates the mean of two determinations. ●, KRM. ○, RMP. The IC_{50}s of KRM and RMP for this experiment were 0.04 and 0.13 μg/ml, respectively.

Effect of KRM on [^{14}C]uracil incorporation into RNA by *M. avium.* Figure 3 shows the effects of KRM and RMP on the uptake of [^{14}C]uracil by *M. avium*. KRM inhibited RNA synthesis in a dose-dependent manner. The inhibitory effect of KRM was maximal at 0.1 μg/ml and almost the same as that of RMP at 0.5 μg/ml. Two different phases of inhibition were observed for both KRM and RMP. During the first 4-h incubation, RNA synthesis increased almost linearly with time even in the presence of the highest concentrations of the test agents. Thereafter, RNA synthesis of *M. avium* almost completely ceased in the presence of high concentrations of each of the drugs (KRM, >0.05 μg/ml; RMP, >0.1 μg/ml). The time courses of inhibition with these rifamycins were almost the same, but they were obtained at different concentrations.

Figure 4 shows the effects of these drugs on bacterial RNA synthesis when added at various concentrations 4 h after incubation. The IC_{50}s of KRM and RMP were estimated to be 0.04 and 0.13 μg/ml, respectively. Similar findings were obtained for *M. avium* N-333 (data not shown). The effect of KRM on RNA synthesis by *M. avium* was greater than that of RMP.

The degree of inhibition of RNA polymerase obtained with KRM in our cell-free system was somewhat lower than that with RMP, but RNA synthesis of the organisms was more strongly inhibited by KRM than by RMP. This suggests that the rate of accumulation of KRM by *M. avium* may be higher than that of RMP and that the higher hydrophobicity of KRM may help increase this rate.

In summary, we studied the mechanism of antimicrobial activity of KRM and found that KRM inhibited *M. avium* and *E. coli* RNA polymerase at low concentrations. *M. avium* cells were found to be more permeable to KRM than *E. coli* cells. Thus, the bactericidal activity of KRM is largely determined by cell permeability and its efficacy in decreasing the RNA polymerase activity of target organisms.

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