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In Vitro Antimycobacterial Activities of Newly Synthesized Benzoxazinorifamycins

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Newly synthesized rifamycin derivatives, KRM-1648, KRM-1657, KRM-1668, KRM-1686, and KRM-1687, having the chemical structures of 3′-hydroxy-5′-(4-alkylpiperazinyl)-benzoxazinorifamycins (alkyl residues: isobutyl, propyl, *sec*-butyl, *sec*-butyl [*R* configuration], and *sec*-butyl [*S* configuration], respectively), were studied for their in vitro antimycobacterial activities. Representative (KRM-1648) MICs for 90% of the strains tested, determined by the agar dilution method on 7H11 medium, of various pathogenic mycobacteria (9 species, 174 strains) were as follows (in micrograms per milliliter): *Mycobacterium tuberculosis* (rifampin [RMP]-susceptible strains), ≤0.0125; *M. tuberculosis* (RMP-resistant strains), 12.5; *M. kansasii*, 0.05; *M. marinum*, ≤0.0125; *M. scrofulaceum*, 0.1; *M. avium*, 1.56; *M. intracellulare*, 0.1; *M. fortuitum*, >100; and *M. chelonae* subsp. *abscessus* and *M. chelonae* subsp. *chelonae*, >100. These values are more than 64 times lower than those of RMP, except for the values against RMP-resistant *M. tuberculosis* (8 times lower) and those against rapid growers, including *M. fortuitum* and *M. chelonae* (the same as those of RMP). The other derivatives had similar levels of in vitro activity against these mycobacteria. When murine peritoneal macrophages in which *M. intracellulare* was phagocytosed in vitro were cultured in the presence of the benzoxazinorifamycins (1 μg/ml), much more rapid killing of the organisms ingested in the macrophages was seen compared with when the same amount of RMP was added to the medium. The addition of benzoxazinorifamycins at the concentration of 0.05 μg/ml caused more marked suppression of intracellular growth of the organisms compared with addition of RMP. KRM-1648 and KRM-1657 inhibited intracellular growth of *M. tuberculosis*, and their efficacies were much greater than that of RMP.

Rifampin (RMP), a rifamycin derivative, is highly active against a number of mycobacteria, especially slow growers such as *Mycobacterium tuberculosis*, *M. kansasii*, and *M. marinum* (3, 22, 23), and is used for the treatment of patients with tuberculosis and some atypical mycobacterial infections (4, 8, 17, 21, 27). However, certain disadvantages have recently arisen in the clinical use of RMP. First, there are increasing numbers of RMP-resistant strains in human-derived *M. tuberculosis* (26). Second, RMP combination therapy exhibits relatively unsatisfactory efficacy against infections caused by *M. avium* complex (MAC) (4, 21, 27) because of its considerably weak in vitro activity against the MAC (3, 22, 23), possibly because of the permeability barrier of the organisms (7, 20). Although other types of rifamycin derivatives, e.g., rifabutin (RBT) (2), rifapentine (1), FCE22807 (6), CGP40/469A (6), CGP-7040 (10), and P-DEA (10), have been developed and although the drugs have higher in vitro antimycobacterial activities than RMP (2, 3, 5, 9, 23, 28), the drugs are generally not so active against MAC infection in humans, particularly immunocompromised hosts (12, 14, 29). Since MAC infections are increasing remarkably in immunocompromised hosts, particularly in AIDS patients (30), the need to develop new antimicrobial agents including rifamycin derivatives which have a strong activity against the MAC is urgent. In this study, we described the in vitro activities of newly synthesized benzoxazinorifamycins against various pathogenic mycobacteria, especially the MAC.

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MATERIALS AND METHODS

Organisms. Mycobacterial species and number of strains (nine species and 174 strains total) used are as follows: *M. tuberculosis* (RMP susceptible), 16; *M. tuberculosis* (RMP resistant), 6; *M. kansasii*, 19; *M. marinum*, 10; *M. scrofulaceum*, 19; *M. avium*, 18; *M. intracellulare*, 31; *M. fortuitum*, 20; *M. chelonae* subsp. *abscessus*, 15; and *M. chelonae* subsp. *chelonae*, 20. These strains are derived from our stock cultures on 1% Ogawa egg medium (15) or 7H9 broth (Difco Laboratories, Detroit, Mich.); most of these strains are disease-associated organisms. All the test strains of the MAC produced smooth, transparent, and irregular colonies on 7H11 agar medium (Difco) and were identified as *M. avium* or *M. intracellulare* by a DNA probe test using Gen-Probe Rapid Diagnostic System for the *M. avium* complex (Gen-Probe Inc., San Diego, Calif.) in our laboratory.

Drugs. Benzoxazinorifamycins, e.g., KRM-1648, KRM-1657, KRM-1668, KRM-1686, and KRM-1687, having the chemical structures of 3′-hydroxy-5′-(4-alkylpiperazinyl)-benzoxazinorifamycins (alkyl residues: isobutyl, propyl, *sec*-butyl, *sec*-butyl [*R* configuration], and *sec*-butyl [*S* configuration], respectively), were newly synthesized by Kanegafuchi Chemical Industry, Co., Hyogo, Japan (Fig. 1). RMP and RBT were obtained from Daiichi Pharmaceutical Co., Tokyo, Japan, and Farmitalia Carlo Erba Research Laboratories, Milan, Italy, respectively. These agents were dissolved in dimethyl sulfoxide at the concentration of 5 mg/ml and then diluted with appropriate media.

Drug susceptibility testing. The bacterial suspension was prepared by diluting organisms grown in 7H9 broth (7H9 broth containing 0.05% Tween 80 was used to prepare a finely dispersed inoculum) at 37°C (33°C for *M. marinum* and

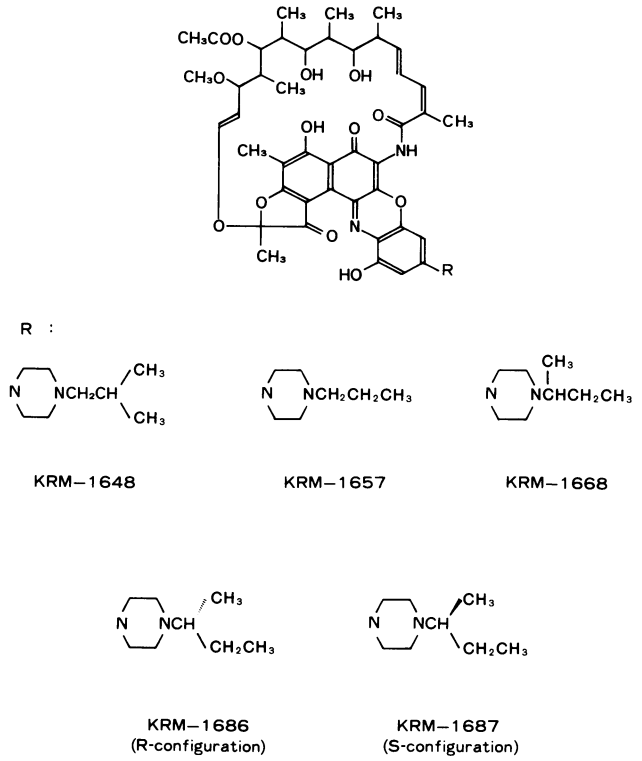

FIG. 1. Chemical structures of KRM-1648, KRM-1657, KRM-1668, KRM-1686, and KRM-1687.

M. chelonae subsp. *chelonae*) for 3 to 7 days with 0.1% Tween 80 in saline to give an optical density at 540 nm of 0.1 (approximately 10^7 CFU/ml) as measured by the Hitachi model 100-10 spectrophotometer (Hitachi Industry Co., Hitachi, Japan); the organisms were then diluted 10 times in the saline. Bacterial suspension (5 μl) was spotted on 7H11 agar plates containing 100 ~0.0125-μg/ml doses (twofold dilution) of drugs. The MICs of the drugs were determined 7 days (rapid growers) or 14 days (slow growers) after cultivation at 37°C (33°C for *M. marinum* and *M. chelonae* subsp. *chelonae*) in a CO_2 incubator (5% CO_2–95% humidified air). The MICs were read as minimum concentrations of drugs completely inhibiting the growth of organisms or allowing no more than five colonies to grow.

In vitro activity against intracellular organisms. Peritoneal exudate cells from female BALB/c mice (8 to 10 weeks old) (Japan SLC Inc., Shizuoka, Japan) injected intraperitoneally with 1 mg of zymosan A (Sigma Chemical Co., St. Louis, Mo.) 4 days before cell harvest were harvested with Hanks balanced salt solution (HBSS; Nissui Pharmaceutical Co., Tokyo, Japan), washed with HBSS, and then suspended in RPMI 1640 medium (Nissui) supplemented with 10% fetal bovine serum (FBS) (M. A. Bioproducts, Walkersville, Md.). The cell suspension was seeded onto plastic 16-mm culture wells (Corning Glass Works, Corning, N.Y.) at the cell density of 7.5×10^5 per well. After 2 h of incubation in a CO_2 incubator, the wells were vigorously rinsed with 2% FBS-HBSS. The resultant macrophage monolayer culture was overlaid with 1 ml of 10% FBS-RPMI 1640 medium containing 3×10^6 to 5×10^6 CFU of *M. intracellulare* N-260 or *M. tuberculosis* $H_{37}Rv$ per ml, incubated for 1 h, and washed thoroughly with 2% FBS-HBSS, and the resultant macrophages were further incubated in 1 ml of 10% FBS-RPMI 1640 medium with or without 0.005, 0.05, 0.5, or 1 μg of the drug per ml at 37°C for up to 5 days. At intervals, the macrophage monolayer culture was thoroughly rinsed with phosphate-buffered saline (PBS) to remove antimicrobial agents. Thereafter, two to three wells for each experimental group were stained with Giemsa solution to count the number of macrophages per well. The remaining macrophage monolayer cultures (three wells for each experimental group) were treated with distilled water to lyse macrophages, and the CFU of organisms in the macrophage lysate were counted on 7H11 agar plates (23). Results were expressed as CFU/100 macrophages.

RESULTS

In vitro antimicrobial activity. Table 1 shows the MICs of benzoxazinorifamycins for 50% (MIC_{50}) and 90% (MIC_{90}) of the various mycobacterial strains. All the drugs (KRM-1648, KRM-1657, KRM-1668, KRM-1686, and KRM-1687) had much smaller MICs than RMP, in particular those drugs active against slowly growing mycobacteria, such as *M. tuberculosis* (especially RMP-susceptible strains), *M. kansasii*, *M. marinum*, *M. scrofulaceum*, *M. avium*, and *M. intracellulare* (8 to 512 times lower than the MIC_{50} of RMP and 8 to 128 times lower than the MIC_{90} of RMP). In contrast, the MICs of the drugs against rapidly growing mycobacteria, such as *M. fortuitum*, *M. chelonae* subsp. *abscessus*, and *M. chelonae* subsp. *chelonae*, were not reduced in such a marked manner by these benzoxazinorifamycins compared with that of RMP, and the drugs were virtually inactive against these organisms. Among the drugs tested, KRM-1648 exhibited the strongest antimicrobial activity, followed by KRM-1657, although this was true only for slowly growing mycobacteria. However, it was noted that the activity of KRM-1648 against RMP-resistant strains of *M. tuberculosis* was considerably lower than those of other KRMs, although KRM-1648 exhibited the most potent activity against RMP-susceptible strains of *M. tuberculosis*.

Figure 2 compares cumulative susceptibilities of various species of slowly growing mycobacteria to KRM-1648, KRM-1657, and RMP. This analysis also shows remarkable superiority of the two benzoxazinorifamycins in terms of in vitro antimycobacterial activities when compared with that of RMP. It is noteworthy that the benzoxazinorifamycins had a much more narrow range of MIC distribution (in terms of log concentration) in *M. tuberculosis*, *M. kansasii*, *M. marinum*, and *M. scrofulaceum*. On the other hand, the benzoxazinorifamycins had nearly the same range of MIC distribution for *M. intracellulare* as did RMP and had a much wider range of MIC distribution than did RMP against *M. avium*. Further, it may be noted that in the test strains of *M. avium*, there was a small number of organisms highly resistant to the two KRMs (MIC, ≥100 μg/ml), whereas such a phenomenon was not observed in other mycobacterial species. The other three benzoxazinorifamycins (KRM-1668, KRM-1686, and KRM-1687) had MIC distribution patterns similar to those of KRM-1648 and KRM-1657 (data not shown).

Activity against intracellular organisms. Table 2 shows the activities of benzoxazinorifamycins against *M. intracellulare* N-260 phagocytosed in murine peritoneal macrophages. MICs of the drugs against *M. intracellulare* N-260 were as follows (in micrograms per milliliter): KRM-1648, 0.05; KRM-1657, 0.1; KRM-1668, 0.2; KRM-1686, 0.1; KRM-1687, 0.1; RMP, 12.5; and RBT, 0.4. When these drugs were

TABLE 1. MICs of benzoxazinorifamycins against various mycobacteria

Species	No. of strains	MIC_{50} (μg/ml)						MIC_{90} (μg/ml)					
		KRM-1648	KRM-1657	KRM-1668	KRM-1686	KRM-1687	RMP	KRM-1648	KRM-1657	KRM-1668	KRM-1686	KRM-1687	RMP
M. tuberculosis	22	≤0.0125	≤0.0125	0.025	≤0.0125	0.025	0.2	12.5	1.56	3.13	1.56	1.56	100
RMP susceptible	16	≤0.0125	≤0.0125	≤0.0125	≤0.0125	≤0.0125	0.2	≤0.0125	0.025	0.025	0.025	0.025	0.8
RMP resistant	6	12.5	1.56	3.13	1.56	1.56	>100	12.5	3.13	6.25	3.13	3.13	>100
M. kansasii	19	≤0.0125	≤0.0125	0.025	≤0.0125	0.025	0.4	0.05	0.05	0.1	0.1	0.2	6.25
M. marinum	10	≤0.0125	0.025	0.05	0.025	0.05	1.56	≤0.0125	0.025	0.05	0.025	0.05	1.56
M. scrofulaceum	19	0.05	0.1	0.1	0.1	0.1	1.56	0.1	0.1	0.4	0.2	0.2	12.5
M. avium	18	0.05	0.1	0.1	0.1	0.1	25	1.56	6.25	6.25	3.13	3.13	100
M. intracellulare	31	0.05	0.05	0.1	0.05	0.1	6.25	0.1	0.1	0.2	0.1	0.1	12.5
M. fortuitum	20	>100	25	25	6.25	25	>100	>100	>100	>100	>100	>100	>100
M. chelonae subsp. *abscessus*	15	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
M. chelonae subsp. *chelonae*	20	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100

added into the culture medium for chase incubation of *M. intracellulare*-infected macrophages at the concentration of 1 μg/ml (this is about 1/5 to 1/14 of the concentrations in the lung of test drugs given at 20 mg/kg) (Table 2, experiment 1), a steady killing of the intracellular organisms was seen during the first 3-day incubation, although gradual regrowth of the organisms was seen during subsequent incubation. The most marked killing and subsequent inhibition of intracellular growth of the organisms was achieved by KRM-1657, followed by KRM-1668. KRM-1648 exhibited a somewhat lower efficacy. In any case, the antimicrobial activities of these benzoxazinorifamycins were much higher than that of RMP, which exhibited only growth inhibitory effects at the same concentration. The concentration of KRMs added to the medium (1 μg/ml) is about 5 to 16 times lower than those that appeared in the lungs and liver of host mice 5 h after oral administration of the drugs at the dose of 20 mg/kg (i.e., KRM-1648, 5.3 and 6.8 μg/g; KRM-1657, 13.5 and 16.3 μg/g; and RMP, 8.5 and 68.4 μg/g in the lungs and liver, respectively [unpublished data]). In the same experimental protocol, the concentrations of KRM-1648, KRM-1657, and RMP in plasma seen 5 h after oral administration at the dose of 20 mg/kg were 0.25, 0.37, and 12.7 μg/ml, respectively.

When the benzoxazinorifamycins were added at the concentration of 0.05 μg/ml (Table 2, experiment 2), only KRM-1657 and KRM-1668 showed proper activity against the organisms ingested in macrophages during the first 3 days. Other drugs (KRM-1648, KRM-1686, and KRM-1687) caused only growth inhibition. From day 3 to 5, considerable bacterial growth was seen, even in the macrophages cultivated in the medium containing KRM-1657 or KRM-1668. However, bacterial growth caused by the KRMs was slower than that in the drug-free medium compared with overall growth throughout the 5-day incubation, and KRM efficacies were also significantly higher than that of RMP. KRM-1648 and KRM-1657 seemed to exhibit higher efficacies of bacterial growth inhibition than RBT (although there was no statistical difference at $P < 0.05$), which has potent killing activity against *M. intracellulare* in resident or immunologically activated peritoneal and alveolar macrophages (16). The activities of the other KRMs were equal to that of RMP.

Table 3 compares activities of KRM-1648 and KRM-1657 against *M. intracellulare* ingested in macrophages, when added at concentrations of 0.05 and 0.5 μg/ml. It was again confirmed that KRM-1657 had somewhat higher antimicrobial activity than KRM-1648.

Table 4 shows activities of KRM-1648 and KRM-1657 against *M. tuberculosis* $H_{37}Rv$ phagocytosed in murine peritoneal macrophages. MICs of test drugs against *M. tuberculosis* $H_{37}Rv$ were as follows (in micrograms per milliliter): KRM-1648, ≤0.0125; KRM-1657, ≤0.0125; RMP, 0.2; and RBT, ≤0.0125. When these drugs were added into the culture medium for chase incubation of infected macrophages at the concentration of 0.005 μg/ml (Table 4), marked growth inhibition of the intracellular organisms was seen during the whole incubation time. KRM-1657 achieved bacterial killing from day 3 to 5. On the basis of weight, the activities of these benzoxazinorifamycins against intracellular *M. tuberculosis* were much more potent than that of RMP but nearly the same as that of RBT. However, it should be noted that this ordering for anti-*M. tuberculosis* activity cannot be directly applied to their in vivo activities, since concentrations of the KRMs in blood were markedly lower than that of RMP, although such an obvious difference was not observed for the concentrations in the lung.

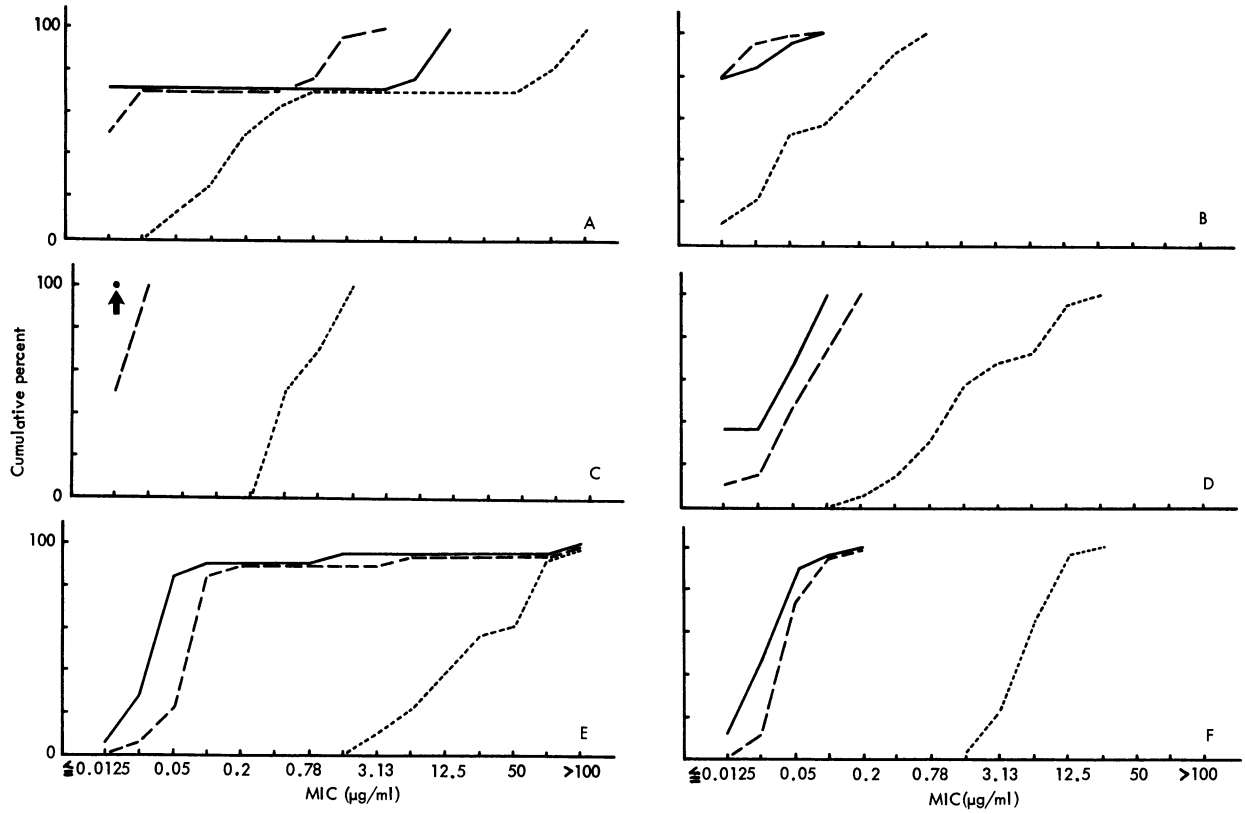

FIG. 2. Susceptibility distribution of various mycobacterial species to KRM-1648 (——), KRM-1657 (– – –), and RMP (·····). MICs against *M. tuberculosis* (A), *M. kansasii* (B), *M. marinum* (C), *M. scrofulaceum* (D), *M. avium* (E), and *M. intracellulare* (F) are shown. The numbers of test strains are indicated in Table 1.

DISCUSSION

This study elucidated that benzoxazinorifamycins, which are newly synthesized rifamycin derivatives, have remarkably excellent in vitro antimycobacterial activities, especially against pathogenic mycobacteria. Their MICs against slow growers were much lower than those of RMP. We previously found that RBT is 4 to 32 times more active than RMP, on the basis of MIC_{90}s determined by the same method as performed in the present study (23) as follows (in micrograms per milliliter): *M. tuberculosis* (RMP-resistant strains), 12.5; *M. tuberculosis* (RMP-susceptible strains), 0.05; *M. kansasii*, 0.1; *M. marinum*, 0.1; *M. scrofulaceum*, 1.56; *M. avium* complex, 1.56; *M. fortuitum*, 6.25; *M. chelonae* subsp. *abscessus*, 25; and *M. chelonae* subsp. *chelonae*, 50. These values are considerably larger than those of the benzoxazinorifamycins (Table 1), except for MICs against *M. fortuitum* and *M. chelonae*, to which RBT exhibits considerably higher activity than did the KRMs. As mentioned above, concentrations in plasma of KRMs were markedly lower than that of RMP, although the concentrations in the lung were similar. Therefore, in vivo efficacies of the KRMs, especially against organisms in the bloodstream, seem not to be so remarkably improved compared with that of RMP. However, the KRMs may exhibit a superior in vivo activity against mycobacteria growing in the lungs, which is the target organ of MAC infection.

It is noteworthy that the benzoxazinorifamycins showed a high potency of activity selective against slowly growing mycobacteria. The MICs of benzoxazinorifamycins against rapidly growing mycobacteria were in the same level as that

TABLE 2. Activities of benzoxazinorifamycins against *M. intracellulare* phagocytosed in murine peritoneal macrophages

Expt no. and drug[a]	CFU/100 macrophages[b] after incubation for:	
	3 days	5 days
1		
None	19.5 ± 1.15	33.4 ± 2.98
RMP	4.10 ± 0.21	14.6 ± 6.69
KRM-1648	0.25 ± 0.10	0.36 ± 0.30
KRM-1657	0.03 ± 0.01	0.06 ± 0.03
KRM-1668	0.04 ± 0.01	0.20 ± 0.05
KRM-1686	0.05 ± 0.01	0.27 ± 0.04
KRM-1687	0.05 ± 0.02	0.41 ± 0.39
2		
None	32.5 ± 4.97	109 ± 4.06
RMP	22.6 ± 1.48	80.0 ± 12.4
RBT	18.7 ± 3.51	47.5 ± 6.78
KRM-1648	10.1 ± 1.40	36.5 ± 8.55
KRM-1657	6.06 ± 0.79	29.9 ± 7.41
KRM-1668	7.33 ± 0.96	45.5 ± 7.31
KRM-1686	11.6 ± 1.37	47.9 ± 6.99
KRM-1687	13.5 ± 1.87	50.5 ± 5.25

[a] Concentrations of drugs were 1 and 0.05 μg/ml for experiments 1 and 2, respectively.

[b] The zero time values were 3.00 ± 0.02 and 10.6 ± 0.12, for experiments 1 and 2, respectively. Values are means ± standard errors of the mean (n = 3).

TABLE 3. Activities of KRM-1648 and KRM-1657 against *M. intracellulare* in murine peritoneal macrophages

Drug and concn (μg/ml)	CFU/100 macrophages[a] after incubation for:	
	3 days	5 days
None	40.8 ± 4.16	121 ± 3.61
KRM-1648		
0.05	9.02 ± 1.04	18.3 ± 3.52
0.5	3.46 ± 1.17	2.75 ± 0.34
KRM-1657		
0.05	8.76 ± 1.06	12.2 ± 1.88
0.5	2.73 ± 0.97	2.36 ± 0.25

[a] The zero time values were 5.48 ± 0.09; all values are means ± standard errors of the mean (n = 3).

of RMP (Table 1). This may imply a possible diversity either in cellular mechanisms of initiation step of transcription (25) or in cell wall permeability for drugs (7) between slowly growing and rapidly growing mycobacteria. It is interesting to know the combined effect of the KRMs with other drugs, such as ethambutol, which gives synergistic in vitro activity with RBT (9). Studies examining this point are under way.

Studies on the effects of the benzoxazinorifamycins against *M. intracellulare* and *M. tuberculosis* inside host macrophages, which are the most important effector cells in host defense mechanisms against mycobacterial infections (11, 13, 18, 19) (Table 2, 3, and 4), indicate the superiority of these drugs compared with RMP on the basis of bacterial killing or growth inhibitory activity against organisms located inside cells. In vitro activities of the test rifamycin derivatives against *M. intracellulare* growing in culture media were determined on the basis of their MICs, as follows: KRM-1648 > KRM-1657, KRM-1686, and KRM-1687 > KRM-1668 > RBT >> RMP. On the other hand, activities against the organisms growing in host phagocytic cells were ranked from the results in Table 2, as follows: KRM-1657 > KRM-1668 > KRM-1686 and KRM-1648 > KRM-1687 > RBT >> RMP. These orderings substantially coincided with each other, but some discrepancies are also noted. Thus, the efficiency of a given rifamycin derivative against organisms located in phagocytes is fixed not only by MIC (a parameter of direct action against the organisms) but also by other factors, such as the efficiency of drug transfer to the inside of phagocytic cells by penetration through cell membrane or by pinocytosis, stability under the intracellular microenvironment, and so on. These findings suggest the possibility that the benzoxazinorifamycins may exhibit much improved in vivo activity. In fact, in our preliminary experiments, we found that KRM-1468 and KRM-1657 exhibited much higher therapeutic efficacies against *M. intracellulare* infection induced in mice than did RMP, which virtually lacked such an in vivo activity (unpublished data). Similar results have been obtained (10a).

TABLE 4. Activities of benzoxazinorifamycins against *M. tuberculosis* in murine peritoneal macrophages

Drug[a]	CFU/100 macrophages[b] after incubation for:	
	3 days	5 days
None	42.3 ± 2.59	226 ± 12.6
RMP	40.1 ± 1.66	98.4 ± 21.4
RBT	20.6 ± 2.84	17.4 ± 1.27
KRM-1648	18.4 ± 1.73	23.5 ± 3.81
KRM-1657	14.2 ± 0.76	7.88 ± 1.24

[a] All drugs were given at 0.005 μg/ml.

[b] The zero time values were 14.4 ± 0.68; all values are means ± standard errors of the mean (n = 3).

In any case, the benzoxazinorifamycins seem promising for clinical use in the treatment of MAC infections. Our preliminary experiments showed that these drugs had lower toxicities in mice and rats than RMP. Successive oral administration of KRM-1648 or KRM-1657 at doses of 100 up to 600 mg/day/kg of body weight to mice for 5 days and to rats for 2 weeks did not cause any serious toxic symptoms (unpublished data), but a certain level of toxicity was noted for RPM at 600 mg/kg. We previously found that the therapeutic efficacy of RMP could be markedly increased by a drug delivery system in which liposome vesicles were used (24). Therefore, therapeutic efficacies of the benzoxazinorifamycins may be much improved by using an appropriate delivery system. Further detailed studies concerning in vivo therapeutic activities of the drugs against various mycobacterial infections, in particular MAC infections, induced in mice are now under way.

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