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# Therapeutic Efficacy of Benzoxazinorifamycin, KRM-1648, in Combination with Other Antimicrobials Against *Mycobacterium leprae* Infection Induced in Nude Mice[1]

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Multidrug therapy (MDT) consisting of rifampin (RMP), clofazimine (CFZ), and diaminodiphenylsulfone (DDS, dapsone) is considered to be the most effective treatment for patients with leprosy ([10]). However, it takes from at least 6 months to more than 4 years of treatment to achieve appreciable results in the control of multibacillary leprosy, even with this multidrug regimen ([3]). The development of new protocols which could provide more rapid therapy for leprosy patients and that contain other types of antileprosy drugs is, therefore, considered urgent.

The new benzoxazinorifamycin derivative KRM-1648 (Kaneka Corporation, Hyogo, Japan) has excellent *in vitro* antimycobacterial activities, and is much more potent than RMP ([1,8]). KRM-1648 exhibited a potent therapeutic efficacy against *Mycobacterium avium* complex infections induced in mice and rabbits ([1,9]). Moreover, we previously found that KRM-1648 exhibited much more potent therapeutic efficacy against *M. leprae* infection induced in mice when compared to RMP ([7]). In this study, *in vivo* anti-*M. leprae* activity of KRM-1648 was evaluated in combination with DDS and CFZ.

## MATERIALS AND METHODS

**Special agents.** KRM-1648, CFZ, and DDS were obtained from Kaneka Corporation, Hyogo, Japan; Ciba Geigy Co., Tokyo, Japan, and Wako Pure Chemical Ind., Osaka, Japan, respectively.

***In vitro* anti-*M. leprae* activity.** Drug susceptibility testing using the BACTEC 460 TB System (Becton Dickinson, Towson, Maryland, U.S.A.) was performed according to Franzblau ([2]), with some modifications. The inoculum was prepared from the foot pads of *M. leprae*-infected BALB/c nude mice by homogenizing the infected tissue with a glass homogenizer in RPMI 1640 medium containing fetal bovine serum. Cell debris were removed by subsequent centrifugation at 150 × *g* for 5 min. The resultant bacterial suspension was mixed with an equal volume of 4% NaOH. After dilution with 2 volumes of distilled water, the organisms were collected by centrifugation at 1500 × *g* for 20 min, washed twice with RPMI 1640 medium, and finally suspended in Tween 80-free 7H9 medium. KRM-1648, DDS, and CFZ were dissolved in ethanol at a concentration of 2 mg/ml and diluted to 80 μg/ml in Tween 80-free 7H9 medium. BACTEC 12B medium (4 ml; without PANTA, containing $^{14}$C-palmitic acid) was inoculated with 0.1 ml of *M. leprae* suspension ($10^8$/ml) and 0.1 ml of diluted drug solution. Then, the air space of the BACTEC 12B vial was flushed out with 5% $CO_2$-air and the vial incubated at 33°C without agitation for up to 4 weeks. The growth index (GI) was read on days 4, 11, 18, and 27.

**Experimental infection.** *M. leprae* Thai-53 was harvested from the infected foot pads of BALB/c nude mice, and the bacterial suspension was prepared as follows. The infected foot pads were homogenized in Hanks' balanced salt solution containing 5% fetal bovine serum and centrifuged at 150

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TABLE 1. In vitro *anti*-M. leprae *activity of KRM-1648 (KRM), CFZ, and DDS measured by the BACTEC 460 TB System.*

| Drug | Concentration (μg/ml) | GI value[a] (% reduction) | | | |
|---|---|---|---|---|---|
| | | Day 4 | Day 11 | Day 18 | Day 27 |
| Controls | — | 132 ± 9 | 267 ± 18 | 200 ± 15 | 106 ± 5 |
| KRM | 0.01 | 63 ± 6 (52) | 110 ± 2 (59) | 44 ± 1 (78) | 22 ± 2 (79) |
| CFZ | 0.5 | 120 ± 9 (9) | 232 ± 7 (13) | 141 ± 3 (30) | 74 ± 2 (30) |
| DDS | 2.0 | 118 ± 7 (11) | 216 ± 4 (19) | 131 ± 1 (35) | 63 ± 1 (41) |
| KRM/CFZ | 0.01/0.5 | 84 ± 9 (36) | 111 ± 12 (58) | 34 ± 5 (83) | 18 ± 3 (83) |
| KRM/DDS | 0.01/2.0 | 65 ± 2 (51) | 96 ± 6 (64) | 36 ± 2 (82) | 18 ± 1 (83) |
| KRM/CFZ/DDS | 0.01/0.5/2.0 | 73 ± 5 (45) | 86 ± 3 (68) | 27 ± 1 (87) | 12 ± 0 (89) |

[a] Mean ± standard deviation (N = 2).

× *g* for 5 min. The upper layer was carefully removed. Bacilli were collected by recentrifugation of the upper layer at 1500 × *g* for 15 min. The bacterial suspension was recentrifuged at 150 × *g* for 5 min, and the upper layer was used as an inoculum for experimental infection. Female BALB/c nude mice (5 weeks old) were infected subcutaneously with $1 \times 10^6$ of *M. leprae* into the left hindfoot pad. Drugs were emulsified in 0.1 ml of 2.5% gum arabic–0.1% Tween 80 solution by grinding with a mortar and pestle, and given by gavage once daily six times per week from day 31 to 80. All mice were observed for foot pad swelling. After 360 days the mice were killed, and the number of acid-fast bacilli in the left hindfoot pad was measured according to the method of Shepard ([5]). Statistical calculation was done by using Student's *t* test.

## RESULTS AND DISCUSSION

Table 1 shows *in vitro* anti-*M. leprae* activities of KRM-1648, CFZ, DDS, and their combinations measured by the BACTEC 460 TB System. KRM-1648 (0.01 μg/ml), CFZ (0.5 μg/ml), and DDS (2.0 μg/ml) exhibited a significant anti-*M. leprae* activity, reducing GI values by 78%, 30%, and 35% by day 18, respectively. Combinations of KRM with either CFZ, DDS or both caused a slight increase in the efficacy. Although the increase was not statistically significant, the same tendency was repeatedly found in the other two experiments.

Table 2 shows the therapeutic efficacy of KRM-1648 alone and in combination with CFZ, DDS, or both against *M. leprae* infection induced in mice. *In vivo* anti-*M. leprae* activity of KRM-1648 (0.001 mg/mouse) was intensified by the combined use of other agents, such as CFZ (0.1 mg/mouse) and DDS (0.2 mg/mouse), when compared to the efficacy of each drug alone. Administration of KRM-1648, CFZ, and DDS alone caused a 1.9-, 2.6-, and 2.2-log decrease in the number of leprosy bacilli recovered after 360 days, respectively, when compared to the bacilli in the control mice. The combinations of KRM-1648 + CFZ, KRM-1648 + DDS, and KRM-1648 + CFZ + DDS caused a 3.7-, 3.2-, and 4.4-log decrease, respectively. Although the therapeutic efficacies of KRM-1648 + DDS and KRM-1648 + CFZ + DDS were significantly higher than those of each drug alone ($p < 0.025$), the difference between KRM-1648 + CFZ and CFZ alone was not significant.

These findings demonstrated that *in vivo* anti-*M. leprae* activity of KRM-1648 (0.001 mg/mouse) can be enhanced when combined with other agents, such as CFZ (0.1 mg/mouse) and DDS (0.2 mg/mouse), as compared to the efficacy of each drug alone, although the combined effect of KRM-1648 with CFZ was not significant compared to that of CFZ alone. In separate experiments, KRM-1648 was found to exert the same level of inhibitory activity against bacterial RNA polymerase as that of RMP, thereby indicating the possibility that its perme-

TABLE 2. *Therapeutic efficacy of KRM-1648 (KRM), DDS, or CFZ alone or in combinations against* M. leprae *infection in mice.*[a]

| Agent | Dose (mg/mouse) | No. mice | Log (AFB/FP)[b] | Foot pad thickness[c] (mm) |
|---|---|---|---|---|
| Controls | — | 4 | 9.01 ± 0.74 | 4.95 ± 0.74 |
| KRM | 0.001 | 3 | 7.11 ± 0.33 | 3.03 ± 0.35 |
| CFZ | 0.1 | 4 | 6.37 ± 0.34 | 2.90 ± 0.08 |
| DDS | 0.2 | 4 | 6.79 ± 0.06 | 3.15 ± 0.32 |
| KRM + CFZ | 0.001/0.1 | 3 | 5.30 ± 0.86 | 2.60 ± 0.35 |
| KRM + DDS | 0.001/0.2 | 4 | 5.86 ± 0.33 | 3.23 ± 0.26 |
| KRM + CFZ + DDS | 0.001/0.1/0.2 | 4 | 4.64 ± 0.56 | 2.95 ± 0.12 |

[a] Mice were killed 360 days after infection. In order to get a clear combined effect, drug doses considerably different from those of clinical dosages were used: dose of KRM-1648 was about 200 times less than that of RMP; doses of CFZ and DDS were two to five times larger than clinical doses.

[b] Log (acid-fast bacilli/foot pad), mean ± S.D.) is indicated. There were statistically significant differences between the controls (none) and each of test drug regimens ($p < 0.025$; Student's $t$ test), between KRM-1648 + DDS and either KRM-1648 or DDS alone ($p < 0.025$), between KRM-1648 + CFZ + DDS and either KRM-1648, DDS or CFZ alone ($p < 0.025$), and between KRM-1648 + CFZ + DDS and KRM-1648 + DDS ($p < 0.025$). There were no significant differences in the other combinations.

[c] Thickness of the infected foot pads (mean ± S.D.) is indicated.

ability into the cell membrane was much improved from that of RMP (Fujii, *et al.,* manuscript in preparation). Although more detailed studies using larger numbers of mice for each regimen are still needed to give a conclusion, the present data suggest that KRM-1648 would be effective in multidrug regimens for the clinical control of bacilliferous leprosy patients. KRM-1648 possessing much more potent antileprosy activity *in vivo* ([7]) may be preferable to RMP in multidrug regimens for clinical control of leprosy patients, given that KRM-1648 has comparable toxicity (Hidaka, T., *et al.,* personal communication) and pharmacokinetics ([9, 11]). Although plasma and liver levels of KRM were 10- to 50-fold lower than those of RMP, its lung and spleen levels were equal to or higher than those of RMP ([11]). In addition, KRM-1648 showed a prolonged elimination from organs including the lungs, liver, spleen, and kidneys when compared to rifabutin, another new rifamycin derivative.

In the present study, the dose of KRM-1648 was much lower than the equivalent clinical dosages because KRM-1648 caused complete inhibition of *in vivo* growth of *M. leprae* when given to mice at doses equivalent to the clinical dosages for rifamycins ([3]). This might cause some artificial features of the combined efficacy of this drug with other antileprosy agents. Therefore, it is necessary to carry out another experiment using clinical dosages of KRM-1648 in an *M. leprae* infection model where chemotherapy is given to infected animals possessing sufficiently high levels of bacterial loads in the foot pads.

In our previous study ([6]), it was found that the *in vivo* antileprosy activity of ofloxacin (OFLX) (3 mg/mouse) was significantly improved by combination with either RMP (0.01 mg/mouse) or DDS (0.2 mg/mouse), as compared to the efficacy of each drug alone. In one experiment, OFLX alone, RMP alone, and OFLX + RMP decreased the number of leprosy bacilli recovered after 365 days by 1.6-, 2.0-, and 3.3-log units, respectively. In another experiment, OFLX alone, DDS alone, and OFLX + DDS decreased the number of leprosy bacilli recovered after 350 days by 0.9-, 1.0-, and 1.8-log units, respectively. Therefore, it seems that some new quinolones with appreciable *in vivo* anti-*M. leprae* activity, such as OFLX and sparfloxacin, may be useful in multidrug regimens containing a rifamycin for treating leprosy patients.

As previously reported ([4, 8, 9]), KRM-1648 possesses much more potent *in vitro* and *in vivo* antimycobacterial activities than RMP. In addition, we observed significantly higher *in vivo* antileprosy activity of KRM-1648 compared to that of RMP ([7]). Therefore, it is possible that KRM-1648 may exert a better therapeutic efficacy against the *M. leprae* infection when used in MDT instead of

RMP. The present study indicated that KRM-1648 displayed a combined therapeutic efficacy with DDS and CFZ, but this study did not make a direct comparison of the efficacies of KRM-1648 and RMP. Therefore, further detailed *in vivo* studies are needed both to evaluate the activity of KRM in combination with other antileprosy drugs and to compare its efficacy with that of RMP before clinical application of this drug for treatment of leprosy patients.


## SUMMARY

In this study, the *in vitro* and *in vivo* anti-*Mycobacterium leprae* activity of the newly developed benzoxazinorifamycin, KRM-1648, in combination with clofazimine (CFZ) or dapsone (DDS) was evaluated. *In vitro* anti-*M. leprae* activities of KRM-1648, CFZ, and DDS along with their combinations were measured by the BACTEC 460 TB System. KRM-1648 (0.01 μg/ml), CFZ (0.5 μg/ml), and DDS (2.0 μg/ml) exhibited a significant anti-*M. leprae* activity, reducing growth index (GI) values by 78%, 30%, and 35% by day 18, respectively. Combinations of KRM-1648 with either CFZ or DDS, or both caused only a slight increase in the efficacy. BALB/c nude mice infected subcutaneously with $1 \times 10^6$ of *M. leprae* Thai-53 strain and test drugs were given to mice by gavage once daily six times per week for up to 50 days, from day 31 to day 80. Animals were observed for the growth of organisms in the hindfoot pad during the 12 months following infection. KRM-1648 given at the dose of 0.001 mg/mouse exhibited potent antileprosy activity. KRM-1648 exhibited a significant combined effect with either CFZ or DDS, or both against *M. leprae* infection, except that there was no significant difference in efficacy between KRM-1648 + CFZ and CFZ alone. Furthermore, the efficacy was most increased in the three-drug regimen KRM-1648 + CFZ + DDS.

## RESUMEN

En este estudio se evaluó *in vivo* e *in vitro,* la actividad anti-*Mycobacterium leprae* de la droga benzoxazinorifamicina (KRM-1648) administrada sola, o combinada con clofazimina (CFZ) o con dapsona (DDS). La actividad de esta droga y sus combinaciones se midió por el sistema BACTEC 460 TB. Hacia el día 18, la KRM-1648 (0.01 μg/ml), la CFZ (0.5 μg/ml) y la DDS (2.0 μg/ml), exhibieron una significante actividad anti-*M. leprae* al reducir su índice de crecimiento en un 78%, un 30%, y un 35%, respectivamente. La combinación de KRM-1648 con CFZ, con DDS, o con ambas drogas, condujo sólo a un ligero incremento de su eficacia. Para los estudios *in vivo,* se inyectaron ratones BALB/c desnudos con 1 × 106 *M. leprae* de la cepa Thai-53 por la vía subcutánea y 31 días después se trataron con las drogas administradas junto con la dieta, una vez al día, 6 veces por semana, hasta completar 50 días. Los animales se examinaron para establecer la multiplicación de los bacilos en las almohadillas plantares durante los 12 meses siguientes a la infección. La droga KRM-1648 administrada a la dosis de 0.001 mg por ratón exhibió una potente actividad antileprosa. Combinada con CFZ, con DDS, o con ambas, la droga exhibió un significante efecto antileproso combinado, excepto que no hubo ninguna diferencia significativa entre la eficacia de la mezcla KRM-1648 + CFZ y la eficacia de la CFZ sola. La eficiencia antileprosa se incrementó en forma máxima cuando se administraron las 3 drogas juntas.

## RÉSUMÉ

Dans cette étude, on a évalué l'activité anti-*Mycobacterium leprae in vitro* et *in vivo* du nouveau composé qu'est la benzoxazinorifamycine, le KRM-1648, en combinaison avec la clofazimine (CFZ) ou la dapsone (DDS). Les activités *in vitro* du KRM-1648, du CFZ et de la DDS, ainsi que de leurs combinaisons ont été mesurées par le système BACTEC 460 TB. Le KRM-1648 (0,01 μg/ml), le CFZ (0, 5 μg/ml) et la dapsone (2,0 μg/ml) ont montré une activité anti-*M. leprae* significative, réduisant les valeurs de l'indice de croissance (IC) de respectivement 78%, 30% et 35% au dix-huitième jour. Les combinaisons du KRM-1648 avec le CFZ, la DDS ou les deux n'ont entraîné qu'une légère augmentation de l'efficacité. Des souris nues BALB/c ont été infectées par voie sous-cutanée avec $1 \times 10^6$ *M. leprae* de la souche Thai-53, et les substances testées leur ont été données par gavage une fois par jour et six jours par semaine jusqu'à 50 jours, du jour 31 au jour 80. On a observé pour la croissance des organismes dans le coussinet des pattes postérieures des animaux durant les 12 mois qui ont suivi l'infection. Le KRM-1648 à la dose de 0,001 mg/souris a montré une puissante activité anti-lépreuse. Le KRM-1648 a montré un effet combiné significatif avec le CFZ, la DDS, ou les deux, vis-à-vis de l'infection à *M. leprae,* si ce n'est qu'il n'y avait pas de différence significative d'efficacité entre le KRM-1648 + CFZ et le CFZ seul. De plus, l'efficacité était maximale pour le régime comprenant les trois substances KRM-1648 + CFZ + DDS.


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