

## **Bactericidal action at low doses of a new rifamycin derivative, 3'-hydroxy-5'-(4-isobutyl-1-piperazinyl) benzoxazinorifamycin (KRM-1648) on *Mycobacterium leprae* inoculated into footpads of nude mice**

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*Summary* Among a series of newly-synthesized benzoxazinorifamycins, 2 of the 3'-hydroxy-5'-(4-alkyl-1-piperazinyl) derivatives, named KRM-1648 and KRM-2312, whose respective alkyl residues are isobutyl and isopropyl, were examined for efficacy against nude mouse-model leprosy. KRM-1648 completely inhibited the growth of leprosy bacilli inoculated into nude mouse footpads, even 6 months after the medication had been stopped, when given orally at a daily dose of 0.6 mg/kg, 5 or 6 times weekly, during 3–5 months postinoculation. In comparison, the growth inhibition by KRM-2312 was incomplete under the same conditions, though it was still stronger than that by rifampicin. Complete growth inhibition by KRM-1648 was also observed when it was given orally at a dose of 1 or 3 mg/kg twice weekly during the same period. In contrast, the growth inhibition by rifampicin was only slight at 1 mg/kg and partial at 3 mg/kg under the same condition.

### **Introduction**

Rifampicin is a representative antileprosy drug as well as an antituberculosis drug. To search for a compound with stronger antimycobacterial activity, a series of rifamycin derivatives were synthesized, and as a result several newly-synthesized benzoxazinorifamycins were found to have potent antimycobacterial activities.<sup>1–3</sup> Of these compounds, two 3'-hydroxy-5'-(4-alkyl-1-piperazinyl)benzoxazinorifamycins, KRM-1648 (alkyl residue: isobutyl) and KRM-2312 (alkyl residue: isopropyl) (Figure 1), were selected for further study based on their excellent activities against *Mycobacterium tuberculosis* and *M. avium* complex. In this paper we report the therapeutic activities of KRM-1648 and

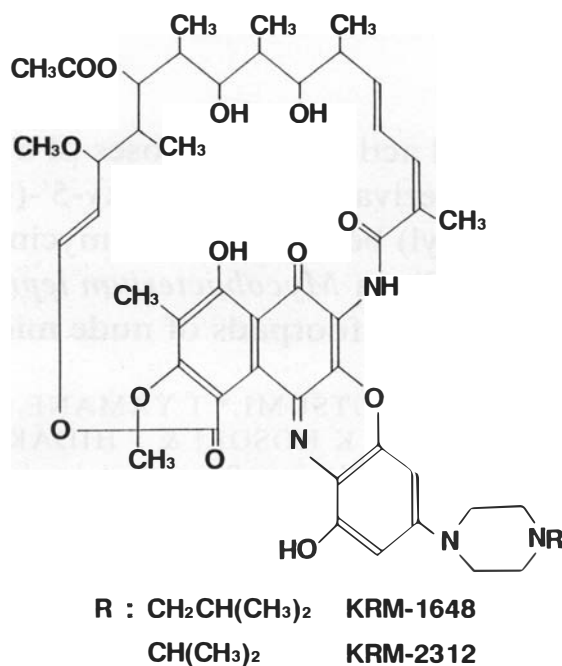


Figure 1. Chemical structures of KRM-1648 and KRM-2312.

KRM-2312 against an *M. leprae* infection model in nude mice, as compared with rifampicin.

## Materials and methods

### DRUG ADMINISTRATION

KRM-1648 and KRM-2312 were synthesized by Biochemical Research Laboratories, the Kaneka Corporation, Takasago, Japan. The rifampicin (RMP) used was Rifadin capsules (1 capsule is equivalent to 150 mg of RMP), purchased from Daiichi Pharmaceutical Co., Ltd, Tokyo, Japan. For the *in vivo* study, all the drugs were aseptically homogenized in sterilized distilled water containing 0.001% Tween 80 and kept at  $-80^{\circ}\text{C}$  until use. Each nude mouse orally received  $\frac{1}{10}$  ml of the drug suspension through a catheter. The treatments followed Shepard's kinetic method.<sup>4</sup> In the 1st experiment, each of the drugs was given continuously 5 or 6 times a week, at a dose of 0.6 mg/kg, and in the 2nd experiment, it was given intermittently twice weekly at doses of 1 and 3 mg/kg.

### MICE

BALB/c (nu/nu) female mice, aged 5 weeks, were purchased from Clea Japan Inc., Tokyo, Japan. They were randomly grouped into 10 mice per group (5 mice per cage) in a vinyl isolator (Sanki Scientific Arts Co., Tokyo, Japan) and kept at  $22 \pm 1^{\circ}\text{C}$ , being fed on a

sterilized heat-stable pellet from diet, MB-6E (Funabashi Farm Co., Chiba, Japan), and sterilized drinking water.

#### MYCOBACTERIUM LEPRAE INFECTION

We used the *M. leprae* strain Thai-53, which had been isolated from a subcutaneous leproma of a Thai lepromatous patient in 1980<sup>5</sup> and passaged through the nude mouse footpads 7 or 8 times. Inocula were prepared as follows: several infected swollen footpads were aseptically homogenized with chilled physiological saline (PS), centrifuged at  $330 \times g$  for 3 min at 4°C, and the supernatant was treated with alkali and centrifuged. The bacilli were resuspended in 0.1% Tween 80-containing PS (pH 3)<sup>6</sup> and washed. The washed bacilli were suspended in PS at a cell density of above  $6 \times 10^8$  bacilli/ml. A 0.05 ml-portion was inoculated into each of both hind footpads of nude mice.

#### COUNTING OF ACID-FAST BACILLI

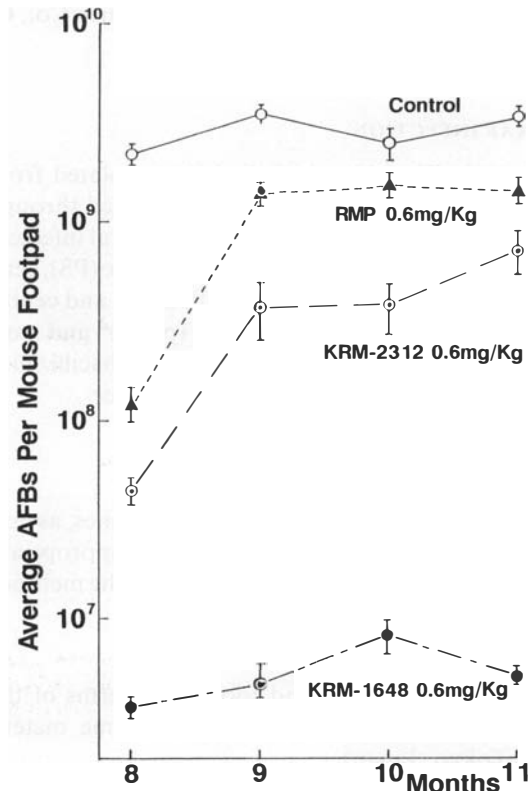
We treated 4 or 6 footpads of 2 or 3 mice taken at specified times, as described above, and the supernatant after centrifugation at  $330 \times g$  was diluted appropriately with PS. Acid-fast bacilli (AFB) were counted in duplication according to the method of Shepard *et al.*<sup>7</sup>

#### DETERMINATION OF HIND FOOTPAD VOLUME

The volume below *malleolus lateralis* of hind footpad in terms of the weight of water displaced by immersion was measured by a digital volume meter, Model MK-550 (Muromachi Kikai Co., Tokyo, Japan).

#### DETERMINATION OF UNCHANGED RMP AND KRM-1648 IN MICE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Plasma (0.2 ml) was loaded onto a Bond Elute Column (C<sub>18</sub> for RMP or C<sub>8</sub> for KRM-1648, Analyt. Internat. Co., Harbor, USA). The column was washed with 3 ml of 30% methanol (RMP) or 30% acetone (KRM-1648) and then the drug was eluted with 2 ml of methanol (RMP) or methanol-ethyl acetate (1:1) (KRM-1648). Each eluate was evaporated to dryness by a centrifugal concentrator (Model VC-960, Taitec Corp. Koshigaya, Japan) and the residue was dissolved into 200 µl of methanol saturated with sodium ascorbate (RMP) or methanol (KRM-1648), and loaded onto a column. For the determination of drug concentration in mouse footpads and organs, 2 ml of 10% liver homogenate (in a phosphate-buffered PS containing sodium ascorbate, pH 7.4 (RMP), acetate-buffered PS, pH 4.0 (KRM-1648)) or an appropriate volume (based on the amount of homogenized spleen or footpads) of 5% tissue homogenate was extracted with a total 20 ml of methanol (RMP) or acetone (KRM-1648), filtered and then the filtrate was evaporated to dryness by a rotary evaporator. The residue was dissolved in 0.5 ml of methanol (RMP, KRM-1648), mixed with 9.5 ml of phosphate-buffered PS and loaded onto the Bond Elute Column C<sub>18</sub> and treated similarly to that of plasma, except that the last dissolution from liver and spleen specimens was done with 400 µl of solvent. Analytical systems for RMP and KRM-1648 were: columns, Shim-pack CLC-ODS, internal diameter 4–6 mm, length 250 mm (Shimadzu Corp.); mobile phases, a mixture of



**Figure 2.** Growth inhibition of leprosy bacilli inoculated into nude mouse footpads by serial treatment with KRM-1648, KRM-2312, or rifampin (RMP). Groups of 10 nude mice were infected with *M. leprae*, strain Thai-53, by inoculating  $3 \times 10^7$  bacilli into each of both hind footpads, followed by oral treatment with KRM-1648, KRM-2312, or RMP, given once a day, 5 or 6 times weekly, between 3 and 5 months postinfection at a daily dose of 0.6 mg/kg. We took 4 or 6 footpads of 2 or 3 mice at the indicated months postinfection, and acid-fast bacilli (AFBs) in the footpads were counted.

a solution A and acetonitrile (1:1) for RMP and (1:2) for KRM-1648, where the solution A (pH 4.4) contained 14.05 g of  $\text{NaClO}_4 \cdot \text{H}_2\text{O}$ , 1.92 g of anhydrous citric acid and 2.94 g of sodium citrate  $\cdot 3\text{H}_2\text{O}$  per litre of deionized water; column temperatures, at 40°C; flow rates (ml/min), 0.5 (RMP) and 1.0 (KRM-1648); column pressures (kg/cm<sup>2</sup>), 52 (RMP) and 72 (KRM-1648); pumps, Tri Rotar Model SR (Japan Spect. Co.); detectors, Model SPD-10A UV-VIS (Shimadzu Corp.) at 475 nm for RMP and Model T-4200 UV-VIS (Hitachi, Ltd) at 643 nm for KRM-1648; injectors, an autoinjector Model SIL-9A (Shimadzu) for RMP and an auto sample processor (Waters) for KRM-1648; data processors, Models Chromatopac C-R3A and C-R4AX (both Shimadzu) for RMP and KRM-1648, respectively. Both the systems were always used in parallel. The peaks of unchanged RMP and KRM-1648 were found at retention times (*T*<sub>rs</sub>, min) of 11.4 and 22.7 with extinction coefficients of  $1.49 \times 10^4$  (at 475 nm) and  $5.93 \times 10^4$  (at 643 nm) and with detection limits (*S/N* = 30) of 8.2 and 9.5 ng/ml, respectively.

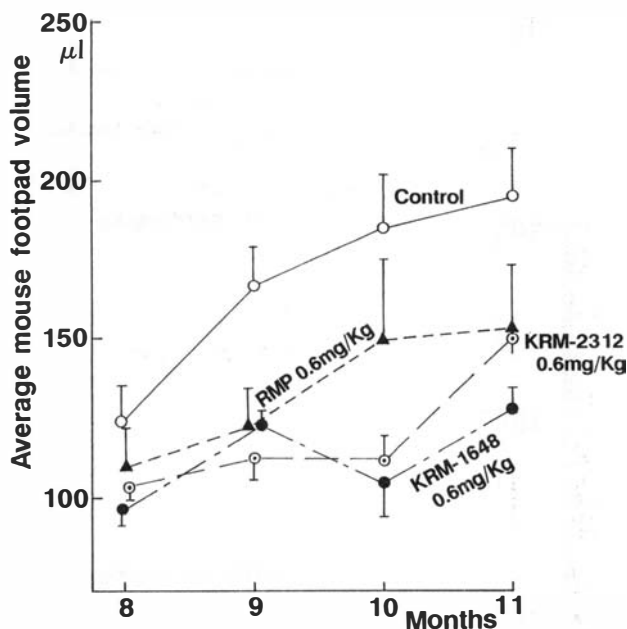


Figure 3. Suppression of footpad swelling of nude mice infected with *M. leprae* by serial treatment with KRM-1648, KRM-2312, or RMP. Mouse footpad volumes were measured in the mice used in the experiment shown in Figure 2. Each point indicates a mean value of 4 or 6 footpads of 2 or 3 mice, and the bar is the standard error.

## Results

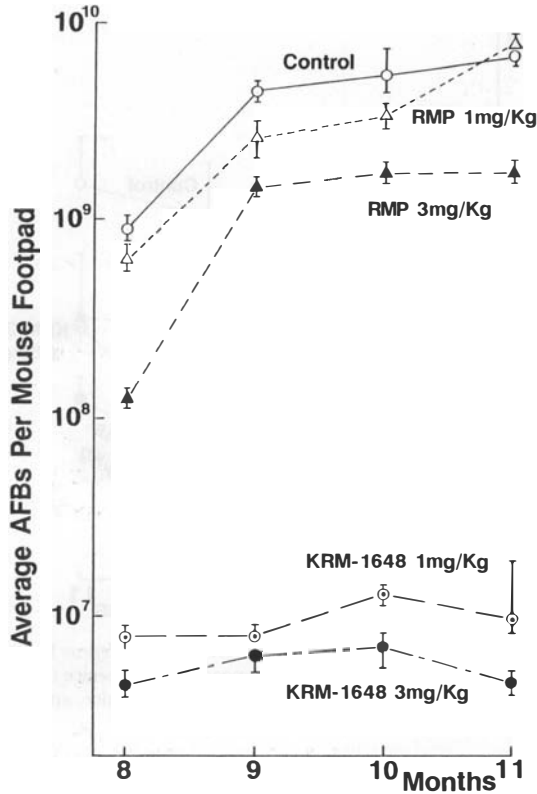
### *Efficacy with serial treatment*

The results are shown in Figures 2 and 3. In the control nude mice inoculated with  $3 \times 10^7$  leprosy bacilli per footpad, the average number of AFB had already reached above the  $10^9$  level at 8 months after inoculation and it remained at that level until the 11th month.

When the treatment with drugs at a dose of 0.6 mg/kg was performed 5 or 6 times weekly from the 3rd to the 5th months, after inoculation, KRM-1648 was the most active antibiotic: it reduced the average numbers of AFB to a  $10^6$  level, which was less than the inoculated number of AFB, between 8 and 11 months after inoculation, indicating that KRM-1648 inhibited the multiplication of leprosy bacilli in the nude mouse footpads. KRM-2312 and rifampin (RMP) were also effective in reducing the number of AFB. However, the activity of KRM-2312 seemed to be somewhat greater than that of RMP. In both groups, a gradual increase in the numbers of AFB was observed between 8 and 11 months after inoculation.

In the control group, the footpad volume increased to nearly double between 8 and 11 months after inoculation.

All the examined drugs suppressed the swelling of footpads, and a significantly stronger suppression in the KRM-1648 group than in the other 2 treated groups was seen at the 11th month (versus RMP group,  $P < 0.01$ ; versus KRM-2312 group,  $P < 0.001$ ).

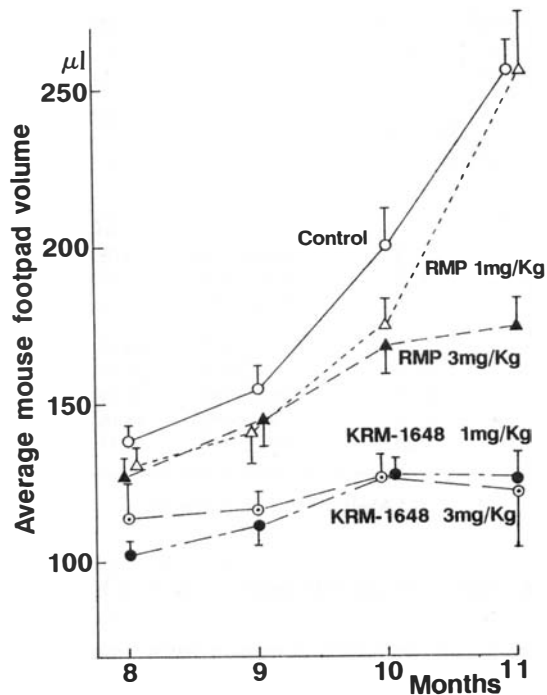


**Figure 4.** Growth inhibition of leprosy bacilli inoculated into nude mouse footpads by intermittent treatment with KRM-1648 or RMP. Groups of 10 nude mice were infected with *M. leprae*, strain Thai-53, by inoculating  $3 \times 10^7$  bacilli into each of both hind footpads, followed by oral treatment with KRM-1648 or RMP, given once a day, twice weekly, at a dose of 1 or 3 mg/kg, between 3 and 5 months postinfection. We took 4 or 6 footpads of 2 or 3 mice at the indicated months postinfection, and AFBs in the footpads were counted.

*Efficacy with intermittent treatment*

The activity of KRM-1648 against nude mouse-model leprosy in comparison with that of RMP was examined by intermittent dosing with a 2- or 3-day interval. Nude mice inoculated with  $3 \times 10^7$  leprosy bacilli per footpad were administered orally with KRM-1648 or RMP at a dose of 1 or 3 mg/kg, twice weekly between 3 and 5 months after inoculation. As shown in Figure 4, the average number of AFB in the control group reached nearly  $10^9$  at 8 months after inoculation and increased gradually thereafter at every specified time. In contrast, the average numbers of AFB in both the KRM-1648 1-mg/kg and 3-mg/kg groups were below the inoculated number of AFB throughout the specified times, demonstrating that KRM-1648 inhibited the multiplication of leprosy bacilli also by the intermittent dosing. The growth inhibition observed in RMP groups was only partial, even at the higher dose of 3 mg/kg, and the inhibition was slight at 1 mg/kg.

The average footpad volumes at each specified time are shown in Figure 5. It shows that while KRM-1648 completely suppressed the swelling of footpads, the suppression by



**Figure 5.** Suppression of footpad swelling of nude mice infected with *M. leprae* by intermittent treatment with KRM-1648 or RMP. Mouse footpad volumes were measured in the mice used in the experiment shown in Figure 4. Each point indicates a mean value of 4 or 6 footpads of 2 or 3 mice, and the bar is the standard error.

RMP was weak. In this experiment, the degree of the swelling suppression by a drug was well correlated with the inhibition of bacillary growth by the drug.

All these results indicated that the bactericidal action of KRM-1648 was more potent than that of RMP on leprosy bacilli inoculated into nude mouse footpads.

## Discussion

The importance given to rifampicin for the treatment of leprosy followed the report by Rees *et al.*<sup>8</sup> on the bactericidal action of the drug at a minimal inhibitory dose of 5 mg/kg on *Mycobacterium leprae* inoculated into footpads of normal mice, and the demonstration by Shepard *et al.*<sup>9</sup> that *M. leprae* was eradicated from the footpads of mice given intermittently 2 doses of 25 mg/kg body weight.

Since then we, like many others, have attempted to develop derivatives with still greater antimycobacterial activity than rifampicin. Our first objective was to administer and assess the fate and the antileprosy activity of the metabolites of rifampicin (<sup>14</sup>C-labelled) we had synthesized from [3-<sup>14</sup>CH=] RMP. All the metabolites were found to be more rapidly excreted and their tissue levels lower than rifampicin. In particular the rifampicin 1,4-quinone metabolite was inactive against the *M. leprae* footpad infection in mice fed 0.0005% in their diet, while rifampicin was very active at this dose.<sup>10</sup> However, it seemed reasonable to conclude that protection of rifampicin from 1,4-oxidation, as well

**Table 1.** Distributions of unchanged KRM-1648 and RMP in BALB/c (nu/nu) female mice after a single oral administration of 10 mg/kg

Drug	Time after administration (hr)	Concentration ( $\mu\text{g/ml}$ or g)		
		Plasma	Liver	Spleen
KRM-1648	1	0.01 $\pm$ 0.01*	3.13 $\pm$ 0.71	1.38 $\pm$ 0.62
	3	0.05 $\pm$ 0.00	3.40 $\pm$ 0.31	2.19 $\pm$ 0.36
	5	0.29 $\pm$ 0.02	5.08 $\pm$ 0.94	5.45 $\pm$ 1.05
	8	0.18 $\pm$ 0.05	3.48 $\pm$ 0.42	5.56 $\pm$ 1.39
	24	0.01 $\pm$ 0.01	0.21 $\pm$ 0.10	0.18 $\pm$ 0.12
RMP	1	9.37 $\pm$ 0.45	49.88 $\pm$ 2.65	3.85 $\pm$ 0.30
	3	7.38 $\pm$ 0.26	43.15 $\pm$ 0.39	2.61 $\pm$ 0.11
	5	7.62 $\pm$ 0.65	51.89 $\pm$ 3.71	2.90 $\pm$ 0.10
	8	7.01 $\pm$ 0.67	48.16 $\pm$ 0.91	1.97 $\pm$ 0.16
	24	0.42 $\pm$ 0.01	6.15 $\pm$ 0.53	0.19 $\pm$ 0.01

\* Mean  $\pm$  SEM of 3 animals.

as from desacetylation, might enhance its activity, at least by prolonging the tissue half-life.

Therefore, in addition to the more common approach used for developing more potent rifampicin analogues by modifying the 4-methyl-1-piperazinyl moiety,<sup>11</sup> more stable rifampicins were developed by protecting the ring 4-hydroxy group from oxidation. Unfortunately this modification, as exemplified by rifabutin,<sup>12</sup> and in spite of improved antimycobacterial activity *in vitro* and pharmacokinetics,<sup>13-15</sup> failed to be of clinical use in leprosy.<sup>16</sup>

In addition to the potent activity of KRM-1648 against *M. leprae* in the footpads of nude mice inoculated with  $3 \times 10^7$  bacilli/footpad, both KRM-1648 and KRM 2312 showed potent and mutually comparable *in vitro*<sup>17,18</sup> and *in vivo* activities<sup>19</sup> against cultivable mycobacteria, such as *Mycobacterium avium* complex infection in female beige mice, and their therapeutic effects were superior to those of rifampicin and rifabutin.

Before considering pilot trials of KRM-1648 in man extensive pharmacokinetic studies are planned. In one such experiment, carried out by the Kaneka Corp. using ddY male mice, it was found that the plasma concentration ratio (%) of KRM-1648/rifampicin was only between 1.9 and 22.7 at specified time points during the 24 hr following a single oral dose of 20 mg/kg body weight. However, at 48 hr, KRM-1648 retained a plasma concentration 11 times higher than that of rifampicin and from which its plasma half-life was presumed to be at least longer than 8 hr. According to a report by Bruna *et al.*,<sup>20</sup> the plasma half-life of rifabutin in mice after a single oral dose of 50 mg/kg body weight is about 8 hr. The results also showed that, despite a far lower hepatic concentration of KRM-1648 than that of rifampicin, the splenic concentration of KRM-1648 was higher at every determination time throughout 24 hr post administration (data to be published elsewhere by the Kaneka Corp.).

The concentrations of KRM-1648 and rifampicin in BALB/c (nu/nu) female mice were also examined. As shown in the Table 1, after a single oral dose of 10 mg/kg body weight, the splenic concentration of KRM-1648 was again observed to be higher than that of rifampicin, though, in this case, only at 5 and 8 hr postadministration. The dosage was



half that given to ddY male mice, and so an accurate comparison at 24 hr between these 2 experiments cannot be made. But, in general, the retention of KRM-1648 seemed to be shorter than that observed in the normal mouse experiment on ddY mice.

Although the pharmacokinetic characteristics are not consistent with the extremely potent activity of KRM-1648, further studies will be carried out, particularly on the unexpected and sustained levels of KRM-1648 in spleen tissues.

In the meantime, the new rifampicin derivative, KRM-1648, has all the potentials of an even more powerful antileprosy drug when compared to rifampicin, and which can be given intermittently.

### Acknowledgment

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**Action bactéricide à faibles doses d'un nouveau dérivé de la rifamycine, 3'-hydroxy-5'-(4-isobutyl-1-pipérazinyl)benzoxazinorifamycine (KRM-1648) sur *Mycobacterium leprae* inoculé dans la plante due pied de la souris "nude".**

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*Résumé* Sur une série de benzoxazinorifamycines récemment synthétisés, nous avons examiné, sur la souris "nude" utilisée comme modèle, l'action anti-lépreuse de 2 des dérivés 3'-hydroxy-5'-(4-alkyl-1-piperazinyl) appelés KRM-1648 et KRM-2312, dont les résidus alkyl sont respectivement isobutyl et isopropyl. En prises orales à la dose journalière de 0,6mg/kg, 5 ou 6 fois par semaine, pendant les 3 à 5 mois suivant l'inoculation, KRM-1648 a complètement inhibé la croissance des bacilles lépreux dans la plante du pied de la souris "nude", jusqu'à 6 mois après l'arrêt du traitement. En comparaison, l'inhibition de croissance obtenue avec KRM-2312 dans les mêmes conditions a été incomplète, bien que plus forte que celle obtenue avec la rifampine. L'inhibition de croissance complète obtenue avec KRM-1648 a été également observée aux doses orales de 1 et 3 mg/kg deux fois par semaine pendant la même période. Par contre, l'inhibition de croissance obtenue avec la rifampicine a été faible seulement à la dose de 1 mg/kg et partielle avec 3 mg/kg dans les mêmes conditions.

**Acción bactericida a dosis bajas de un nuevo derivado de la Rifamicina, 3'-hidroxi-5-(4-isobutil-1-piperazinil)-benzoxazinorifamicina (KRM-1648), sobre *Mycobacterium leprae* inoculada en la almohadilla plantar de ratones desnudos**

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*Resumen* Se examinaron 2 de los derivados de 3'-hidroxi-5'-(4-alkilo-1-piperazinilo) con un residuo alquílico de isobutilo o isopropilo con los nombres KRM-1648 y KRM-2312 respectivamente, de una serie de benzoxazinorifamicinas recientemente sintetizadas, para eficacia contra la lepra en un modelo de ratón desnudo. La KRM-1648 inhibió por completo el crecimiento de los bacilos de la lepra inoculados en la almohadilla plantar de ratones desnudos, mismo 6 meses después de discontinuar la medicación, cuando se la administraba por vía oral a una dosis diaria de 0,6 mg/kg, 5 o 6 veces por semana, durante los meses 3 a 5 después de la inoculación. En comparación, bajo las mismas condiciones, la inhibición del crecimiento por KM-2312 fue incompleta, aunque el efecto era más marcado que con la rifampicina. Se observó una inhibición total del crecimiento mediante la KRM-1648 cuando se administraba en una dosis oral de 1 a 3 mg/kg dos veces por semana durante el mismo periodo. Vuelta en cambio, la inhibición de crecimiento por la rifampicina fue solamente leve a 1 mg/kg y parcial a 3 mg/kg, bajo las mismas condiciones.