Lepr Rev (1995) **66,** 39–47

Inhibition of the multiplication of *Mycobacterium leprae* in nude mice by intermittent administration of a new rifamycin derivative, 3′-hydroxy-5′-(4-isobutyl-1-piperazinyl)benzoxazinorifamycin (KRM-1648) combined with sparfloxacin

M. GIDOH*, G. MATSUKI*, S. TSUTSUMI*§, T. HIDAKA† & S. NAKAMURA‡

**National Institute for Leprosy Research, Aoba-cho 4-2-1, Higashimurayama, Tokyo, Japan; †Kaneka Corporation, Ltd, Hyogo, Japan; and ‡Dainippon Pharmaceutical Company, Ltd, Osaka, Japan*

Accepted for publication 23 September 1994

Summary Inhibition of the multiplication of *Mycobacterium leprae* in the footpads of nude mice by the oral administration of sparfloxacin, a new quinolone, and 3′-hydroxy-5′-(4-isobutyl-1-piperazinyl)benzoxazinorifamycin (KRM-1648), selected from a series of newly synthesized benzoxazinorifamycins, was studied. When the 2 drugs were administered alternately at intervals of 3 or 4 days, (i.e., each drug was administered once weekly), or simultaneously once weekly, between 3 and 5 months after inoculation of nude mice with *M. leprae*, 10 mg sparfloxacin and 0·6 mg KRM-1648 per kg bodyweight were sufficient to prevent multiplication of the organisms. Only partial inhibition of multiplication was achieved by alternate administration of 5 mg sparfloxacin and 0·3 mg KRM-1648 per kg, as was the case for 20 mg sparfloxacin per kg or 1 mg KRM-1648, each drug administered alone once weekly. The addition to these 2 drugs of dapsone, administered in the diet in a concentration of 0·001 g per 100 g, enhanced their effect.

The potential usefulness of multidrug regimens including these compounds is considered.

Introduction

We have previously reported that a new rifamycin derivative, 3′-hydroxy-5′-(4-isobutyl-1-piperazinyl)benzoxazinorifamycin (KRM-1648) (see Figure 1 for structure) entirely prevented the multiplication of *M. leprae* in the footpads of congenitally athymic nu/nu (nude) mice, when the drug was administered twice weekly *per os* in dosages of 1 or 3 mg per kg bodyweight between 3 and 5 months after the animals had been inoculated.[1] We have also reported that administration of sparfloxacin (SPFX) in dosages of 10, 20 or 30 mg per kg by the same schedule partially inhibited the multiplication of the organisms.[2] In this paper, we report the results of several experiments

§ Present address: Aoba-cho 2-29-11, Higashimurayama, Tokyo 189, Japan.

0305-7518/95/066039+09 $01.00

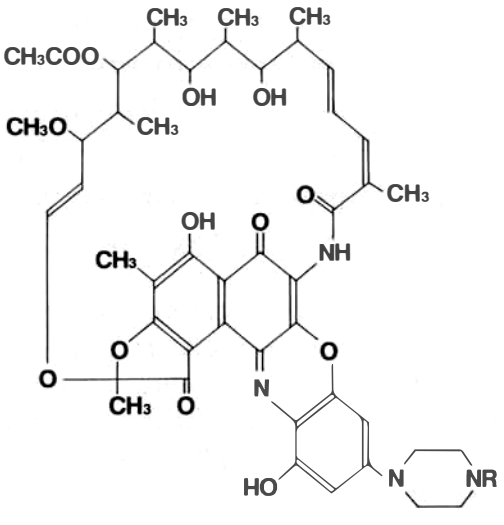

Figure 1. Chemical structure of KRM-1648.

in *M. leprae*-infected nude mice, in which these 2 drugs were administered alternately or simultaneously at weekly intervals.

Materials and methods

KRM-1648 was supplied by the Biochemical Research Laboratories, Kaneka Corporation, Ltd., and SPFX by the Bioscience Research Laboratories, Dainippon Pharmaceutical Co., Ltd. Emulsified suspensions of KRM-1648 and SPFX were prepared and preserved as described previously.[1,2] These 2 drugs were administered through mouse catheters alternately at intervals of 3 or 4 days (each drug was administered once weekly) in experiments 1 and 2, whereas they were administered simultaneously at intervals of 1 week in experiment 3. Dapsone, purchased from Wako Pure Chemicals Co., Ltd., Tokyo, and formulated into heat-stable pellets by Funabashi Nojyo Co., Ltd., Chiba, Japan, was administered incorporated in their diet in a concentration of 0·001 g per 100 g in experiment 4.

Female BALB/c *nu/nu* mice, aged 5 weeks, were purchased from Clea Japan Inc., Tokyo. The mice were bred and grouped as described earlier.[1,2]

M. leprae of the Thai 53 strain,[1,2,4] which had been maintained in nude mice through 10 or 11 passages, were employed. Inocula were prepared and the mice infected as described in our earlier reports,[1,2] except that 10^7 organism were inoculated into each hind footpad. Acid-fast bacilli (AFB) were harvested individually (2 hind footpads were pooled), and enumerated by the method of Shepard & McRae.[5] Each time 20 microscopic fields were counted for each of 2 circular smear spots of 1-cm diameter with fields of AFBs comparatively uniformly distributing, as a portion of supernatant from emulsified 2 hind footpads pooled from each animal, and the average count of AFB and the standard deviation (SD) were calculated for 2 or 3 animals.

Results

ALTERNATE ADMINISTRATION OF KRM-1648 AND SPFX

In experiment 1, KRM-1648 was administered *per os* in a dosage of 1 mg per kg bodyweight every Friday, and SPFX was administered *per os* in a dosage of 10 or 20 mg per kg every Monday, from the beginning of the 3rd month until the end of the 5th month after the nude mice had been inoculated with 10^7 *M. leprae* into each hind footpad. As shown in Figure 2, administration of KRM-1648 alone or of SPFX alone in

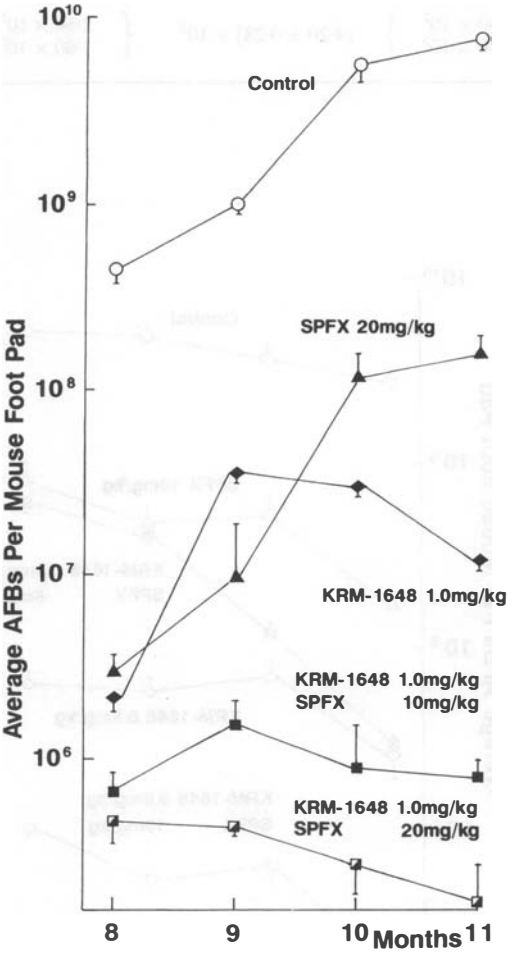

Figure 2. Inhibition of multiplication of *M. leprae* inoculated into nude mouse footpads by alternate treatment with KRM-1648 and sparfloxacin (SPFX) at intervals of 3 or 4 days. Groups of 10 nude mice were infected with *M. leprae*, strain Thai 53 by 1×10^7 bacilli into each of both hind footpads and then treated with oral administration of KRM-1648 and SPFX, alternately at intervals of 3 or 4 days (each drug was administered once weekly), between 3 and 5 months after inoculation. Otherwise, animals were orally treated with KRM-1648 or with SPFX, once weekly. We took 4 or 6 footpads of 2 or 3 mice at the indicated months after inoculation and AFBs in the footpads were counted up.

Table 1. Number of AFBs detected at 9 and 11 months after inoculation, in the 4 footpads of 2 mice, respectively, belonging to the 2 groups shown in Figure 2

	Number of acid-fast bacilli detected			
	9 months		11 months	
Group	AFB/FP	(Mean ± SD)	AFB/FP	(Mean ± SD)
Control	$1{\cdot}12 \times 10^9$ $0{\cdot}89 \times 10^9$	$(1{\cdot}01 \pm 0{\cdot}16) \times 10^9$	$7{\cdot}85 \times 10^9$ $7{\cdot}51 \times 10^9$	$(7{\cdot}68 \pm 0{\cdot}24) \times 10^9$
KRM 1 mg/kg+ SPFX 10 mg/kg	$1{\cdot}89 \times 10^6$ $1{\cdot}14 \times 10^6$	$(1{\cdot}52 \pm 0{\cdot}53) \times 10^6$	$8{\cdot}48 \times 10^5$ $7{\cdot}35 \times 10^5$	$(7{\cdot}88 \pm 0{\cdot}74) \times 10^5$
KRM 1 mg/kg+ SPFX 20 mg/kg	$4{\cdot}00 \times 10^5$ $4{\cdot}40 \times 10^5$	$(4{\cdot}20 \pm 0{\cdot}28) \times 10^5$	$2{\cdot}60 \times 10^5$ $1{\cdot}00 \times 10^5$	$(1{\cdot}80 \pm 1{\cdot}13) \times 10^5$

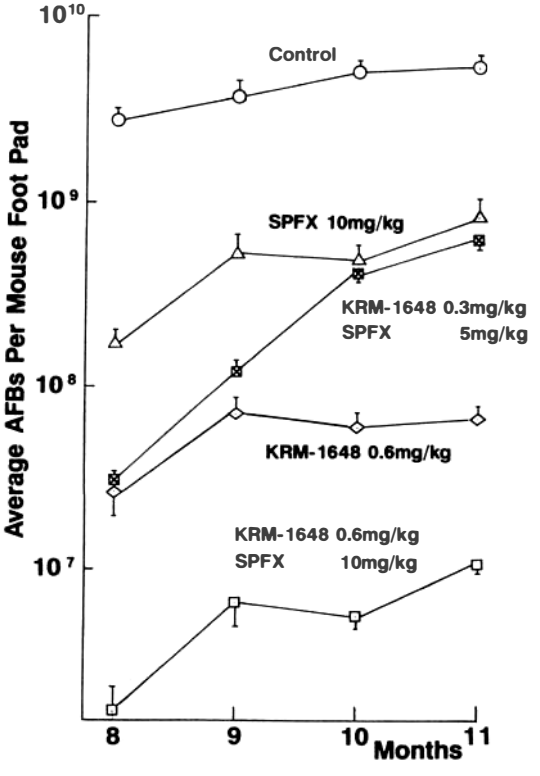

Figure 3. Inhibition of multiplication of *M. leprae* inoculated into nude mouse footpads by alternate treatment with low doses of KRM-1648 and SPFX at intervals of 3 or 4 days. Grouping of nude mice, inoculation of *M. leprae*, frequency of dose and counting of AFBs were performed according to the methods shown in the legend to Figure 2.

the higher dosage only partially inhibited multiplication of the *M. leprae*. On the other hand, alternate administration of these 2 drugs entirely prevented multiplication of the organisms, even when SPFX was administered in the lower dosage. As for 2 groups in Figure 2 in which detected AFBs were always below the number of *M. leprae* inoculated into each foot pad, the results are demonstrated by number of AFBs at 9 and 11 months after inoculation, as shown in Table 1.

In experiment 2, the efficacy was tested of the drugs administered in smaller dosages by the same schedule. As shown in Figure 3, weekly administration of SPFX in the dosage of 10 mg per kg only partially inhibited the multiplication of the *M. leprae*, as may also have been the case for KRM-1648 administered in a weekly dosage of 0·6 mg per kg. Similarly, the alternate administration of 5 mg SPFX and 0·3 mg KRM-1648 per kg was only partially inhibitory, whereas alternate administration of 10 mg SPFX and 0·6 mg KRM-1648 entirely prevented the multiplication of the organisms.

SIMULTANEOUS ADMINISTRATION OF KRM-1648 AND SPFX

In experiment 3, the 2 drugs were administered simultaneously on 12 occasions 1 week

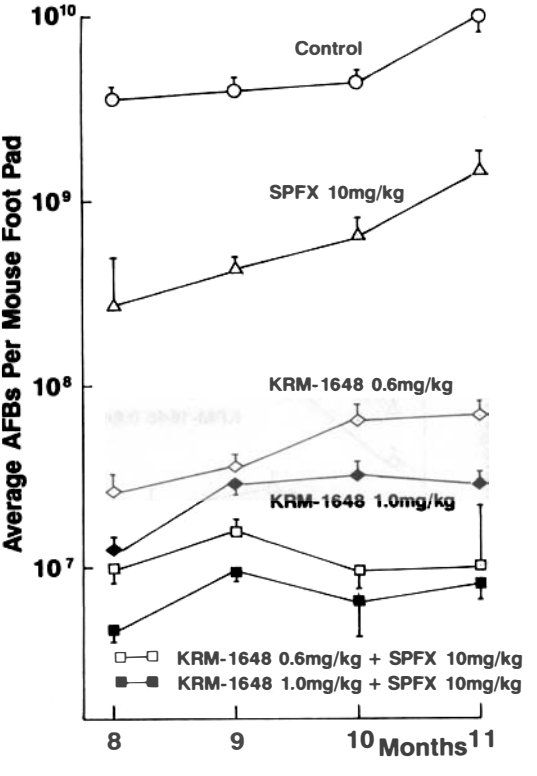

Figure 4. Inhibition of multiplication of *M. leprae* inoculated into nude mice footpads with simultaneous and once-weekly administration of KRM-1648 and SPFX. Grouping of nude mice, inoculation of *M. leprae* and counting of AFBs were performed according to the methods shown in the legend to Figure 2.

apart. As shown in Figure 4, the combination of 10 mg SPFX per kg with KRM-1648, the latter drug administered in a dosage of 0·6 or 1·0 mg per kg, entirely prevented multiplication of the organisms, as had been the case when the 2 drugs were administered alternately.

COMBINATION OF KRM-1648 AND SPFX WITH DAPSONE

In the final experiment, 2 groups of mice were administered dapsone in their diet at a concentration of 0·001 g per 100 g for 3 months, from the beginning of the 3rd month to the end of the 5th month after inoculation. In addition, 0·6 mg KRM-1648 and 10 mg SPFX were administered simultaneously once weekly to 1 of the groups from the start till the end of dapsone administration. Administration of these 3 drugs alone only partially inhibited the multiplication of the *M. leprae*, whereas the simultaneous administration of KRM-1648 and SPFX entirely prevented their multiplication. The addition of dapsone appears to have enhanced the efficacy of the 2-drug combination.

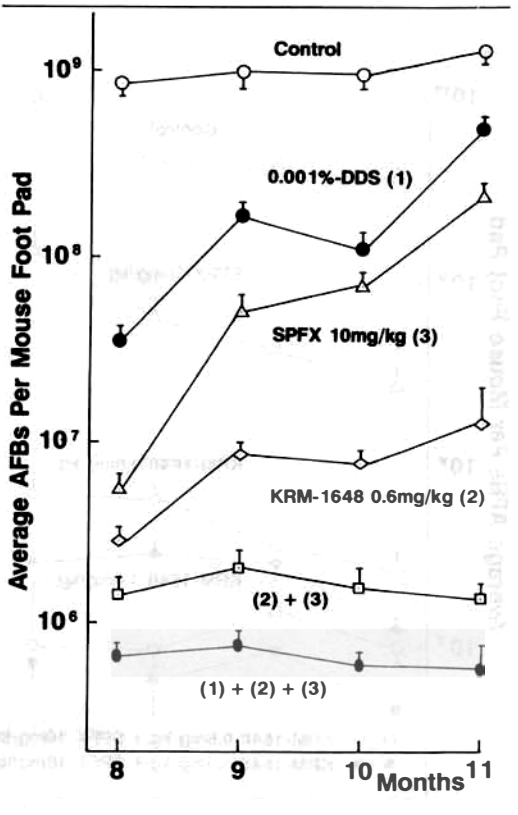

Figure 5. Inhibition of multiplication of *M. leprae* inoculated into nude mice footpads with simultaneous and once-weekly administration of KRM-1648 and SPFX, or with that further combined with dapsone. Grouping of nude mice, inoculation of *M. leprae* and counting of AFBs were performed according to the methods shown in the legend to Figure 2. Dapsone was administered between 3 and 5 months after inoculation through the diet prepared by containing 0·001 g dapsone per 100 g.

Table 2. Number of AFBs detected at 9 and 11 months after inoculation, in the 4 footpads of 2 mice, respectively, belonging to the 3 groups shown in Figure 5

	Number of acid-fast bacilli detected			
	9 months		11 months	
Group	AFB/FP	(Mean ± SD)	AFB/FP	(Mean ± SD)
Control	$9{\cdot}35 \times 10^8$ $10{\cdot}29 \times 10^8$	$(9{\cdot}82 \pm 0{\cdot}66) \times 10^8$	$1{\cdot}22 \times 10^9$ $1{\cdot}27 \times 10^9$	$(1{\cdot}25 \pm 0{\cdot}04) \times 10^9$
KRM-1648 0·6 mg/kg (2)	8.19×10^6 $8{\cdot}61 \times 10^6$	$(8{\cdot}40 \pm 0{\cdot}30) \times 10^6$	$1{\cdot}72 \times 10^7$ $0{\cdot}85 \times 10^7$	$(1{\cdot}29 \pm 0{\cdot}62) \times 10^7$
(2) +SPFX 10 mg/kg (3)	$2{\cdot}21 \times 10^6$ $1{\cdot}79 \times 10^6$	$(2{\cdot}00 \pm 0{\cdot}30) \times 10^6$	$1{\cdot}47 \times 10^6$ $1{\cdot}21 \times 10^6$	$(1{\cdot}34 \pm 0{\cdot}18) \times 10^6$
(2) + (3)+ 0·001%-DDS (1)	$6{\cdot}30 \times 10^5$ $8{\cdot}40 \times 10^5$	$(7{\cdot}35 \pm 1{\cdot}48) \times 10^5$	$6{\cdot}83 \times 10^5$ $4{\cdot}20 \times 10^5$	$(5{\cdot}52 \pm 1{\cdot}86) \times 10^5$

The results of 3 groups shown in Figure 5, in which multiplication of *M. leprae* was suppressed to a level comparable to or below the number of the organisms inoculated into each footpad, are demonstrated by the detected number of AFB in Table 2.

Discussion

The inhibitory effect on multiplication of *M. leprae* by sparfloxacin and KRM-1648 administered alternately at intervals of 3 or 4 days or simultaneously at intervals of 1 week were determined using an *M. leprae* infection model in nude mouse footpads. Findings demonstrated that 10 mg of sparfloxacin and 0·6 mg of KRM-1648 per kg bodyweight were sufficient to prevent entirely the multiplication of *M. leprae* in mouse footpads in both the dosing methods. Nevertheless, when the 2 agents were combined, administration of 5 mg of the former and 0·3 mg of the latter per kg of bodyweight at 1-week intervals proved to be insufficient. Review of the findings of our experiments suggests that the alternative administration of 20 mg of sparfloxacin and 1 mg of KRM-1648 per kg of bodyweight at intervals of 3 or 4 days (1st experiment) and the simultaneous administration of 10 mg of sparfloxacin with 0·6 mg of KRM-1648 per kg of bodyweight combined with dapsone (4th experiment) seem to be the 2 most potent regimens.

An adverse effect of rifamycin analogues is the toxicity to hepatic enzymes[7] and blood cells at high dosages.[8] Repeated use of high-dose rifabutin or rifampicin induces predominancy of extrahepatic metabolism, which appears to reduce AUC.[9] For this reason, lower dosage and frequency of administration are best for rifamycin analogues. KRM-1648 is useful not only because it can be administered in low doses but also because it may achieve economical inhibition of the multiplication of *M. leprae*.

Regarding the selection of a companion drug in multidrug therapy (MDT), Banerjee *et al.*[10] reported a 100-fold reduction in the viability of *M. leprae* in nude mouse footpads achieved with the combined administration of high-dose ofloxacin and rifabutin compared that with administration of either drug alone, suggesting the possibility of

synergism between this new quinolone, sparfloxacin and this new rifamycin derivative, KRM-1648. In fact, a co-operative effect of sparfloxacin in combination with KRM-1648 was detected in the 1st experiment.

Yoder *et al.*[11] reported the cross-resistance of rifabutin to rifampicin in a strain of rifampicin-resistant *M. leprae* in nude mice. Saito *et al.*[12] found strain-specific cross-resistance of KRM-1648 to rifampicin in various rifampicin-resistant mycobacterial strains. Partial cross-resistance of sparfloxacin and ofloxacin in the treatment of *M. tuberculosis in vitro* has also been reported.[13] These findings remind us of the importance of completely eradicating *M. leprae* as rapidly as possible with strong MDT.

In the 4th experiment, dapsone was tested in combination with KRM-1648 and sparfloxacin. Pronounced inhibition of multiplication as evidenced by reduction even in the number of nonsolid bacilli was observed. However, if the amount of dapsone contained in the 0·001%-DDS diet is converted to the amount in the diet of an adult weighing 60 kg, it corresponds to approximately 72–150 mg daily. Although the tissue concentrations of KRM-1648 are higher in the spleen than in the liver,[1] while those of sparfloxacin[14] and dapsone are the opposite, a devised administration of dapsone to decrease the metabolic load on the host due to this multidrug regimen can be expected for clinical use, particularly because both KRM-1648 and sparfloxacin are long-acting substances, but also because dapsone exhibits slow clearance.

Recently, Franzblau *et al.* (personal communication) examined the clinical effect of sparfloxacin on 9 lepromatous cases using assay of PGL-1 antigen, determination of killing of leprosy bacilli with the mouse footpad method, and a radiorespirometry. Daily treatment with 200 mg sparfloxacin for 12 weeks was found to be sufficiently effective. This dosage of sparfloxacin corresponds at most to only 20–40% of those used for nude mice in the present study.

Given these findings, studies on rapid and complete eradication of *M. leprae* with a minimal number of combined administration of KRM-1648 and sparfloxacin with or without dapsone will be continued using the *M. leprae* infection model in nude mice as described here.

Acknowledgment

We are grateful to Mr T. Fujimiya of the National Institute for Leprosy Research for his support to this study through his environmental control for breeding nude mice.

References

[1] Gidoh M, Tsutsumi S, Yamane T, Yamashita K, Hosoe K, Hidaka T. 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. *Lepr Rev*, 1992; **63:** 319–28.

[2] Gidoh M, Tsutsumi, S. Activity of sparfloxacin against *Mycobacterium leprae* inoculated into footpads of nude mice. *Lepr Rev*, 1992; **63:** 108–16.

[3] Shepard CC. A kinetic method for the study of activity of drugs against *Mycobacterium leprae* in mice. *Int J Lepr*, 1967; **35:** 429–35.

[4] Mori T, Miyata Y, Kohsaka K, Makino M. Respiration in *Mycobacterium leprae. Int J Lepr*, 1985; **53:** 600–9.

[5] Shepard CC, McRae DH. A method for counting acid-fast bacteria. *Int J Lepr*, 1968; **36:** 78–82.

[6] Yamane T, Hashizume T, Yamashita K, Nonishi E, Hosoe K, Hidaka T, Watanabe K, Kawaharada H, Yamamoto T, Kuze F. Synthesis and biological activity of 3′-hydroxy-5′-aminobenzoxazinorifamycin derivatives. *Chem Pharm Bull*, 1993; **41:** 148–55.

[7] Perucca E, Grimaldi R, Frigo GM, Sardi A, Monig H, Ohnhaus EE. Comparative effects of rifabutin and rifampicin on hepatic microsomal enzyme activity in normal subjects. *Eur J Clin Pharmacol*, 1988; **34:** 595–9.

[8] Burnett PK, Ameer B, Hoang V, Phifer W. Rifampin-associated thrombocytopenia secondary to poor compliance. *Drug Intelligence & Clin Pharm*, 1989; **23:** 382–4.

[9] Strolin Benedetti M, Efthymiopoulos C, Sassella D, Moro E, Repetto M. Autoinduction of rifabutin metabolism in man. *Xenobiotica*, 1990; **20:** 1113–19.

[10] Banerjee DK, McDermott-Lancaster RD. An experimental study to evaluate the bactericidal activity of ofloxacin against an established *Mycobacterium leprae* infection. *Int J Lepr*, 1992; **60:** 410–5.

[11] Yoder LJ, Jacobson RR, Hastings RC. The activity of rifabutin against *Mycobacterium leprae*. *Lepr Rev*, 1991; **62:** 280–7.

[12] Saito H, Tomioka H, Sato K, Emori M, Yamane T, Yamashita K, Hosoe K, Hidaka T. *In vivo* antimycobacterial activities of newly synthesized benzoxazinorifamycins. *Antimicrob Agents Chemother*, 1991; **35:** 542–7.

[13] Kibata M, Nagara J. *In vitro* activities of newly developed quinolones, fleroxacin, lomefloxacin and sparfloxacin against *Mycobacterium tuberculosis*. *Kekkaku*, 1991; **66:** 429–31.

[14] Nakamura S, Kurobe N, Ohue T, Hashimoto M, Shimizu M. Pharmacokinetics of a novel quinolone, AT-4140, in animals. *Antimicrob Agents Chemother*, 1990; **34:** 89–93.

[15] Chan GP, Garcia-Ignacio BY, Chavez VE, Livelo JB, Jimenez CL, Parrilla MR, Franzblau SG. Clinical trial of sparfloxacin for lepromatous leprosy. *Antimicrob Agents Chemother*, 1994; **38:** 61–5.