RAPAMYCIN (AY-22,989), A NEW ANTIFUNGAL ANTIBIOTIC

III. *IN VITRO* AND *IN VIVO* EVALUATION

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(Received for publication March 11, 1978)

The activity of rapamycin, a new anti-*Candida* antibiotic, was not affected by pH values between 6 and 8; at pH 4, however, activity was abolished. The MIC of rapamycin did not vary drastically with the size of inoculum: a ten-fold dilution of the inoculum reduced the MIC only two-fold. Serum binding was extensive. Serum levels obtained in mice were higher on subcutaneous injection than with oral administration. Dogs absorbed rapamycin after oral administration.

Rapamycin cured systemic candidosis in mice: PD_{50} s. c. was 9.5 mg/kg; PD_{50} p. o. was 11 mg/kg. In the same experimental infections amphotericin B and nystatin exhibited PD_{50} values of <0.25 mg and $>4,000$ units/kg respectively. Rapamycin and amphotericin B, administered at 1, 4 and 24 hours after infection, gave approximately the same percent survival after 30 days of observation. When the above treatment was extended by an additional daily treatment for 6 days, rapamycin by the subcutaneous route yielded a higher percentage of survival than either rapamycin or amphotericin B, administered orally, after a 30-day observation period. Vaginal candidosis in female rats was treated efficiently (91% cure) by rapamycin administered orally. No increase of resistance of *C. albicans* was observed during treatment.

Rapamycin is an antifungal antibiotic produced by *Streptomyces hygroscopicus* NRRL 5491[10]. It was isolated in pure, crystalline form and found to be mainly active against *Candida* species; *C. albicans* is the most sensitive species[6]. It has no activity against bacteria, *Trichomonas vaginalis, T. foetus*, and *Protheca segbwema* (pathogenic alga); it is moderately active against filamentous fungi, including some Dermatophytes, and weakly active against dimorphic fungi. Acute toxicity is low[7]: LD_{50} in mice is 597 (i. p.) and $>2,500$ mg/kg (p. o.); LD_{50} in rats is $>1,600$ (i. p.), 40 (i. v.), and $>1,600$ mg/kg (p. o.).

The present study deals with the influence of pH, serum and inoculum size on rapamycin activity, its bioavailability in mice and dogs, and the protection it affords to mice and rats against both systemic and vaginal candidoses. Its *in vivo* activity is compared to that of amphotericin B[3,8] and nystatin[4]. A method is described for studying the effect of antifungal agents on vaginal candidosis in rats.

Materials and Methods

Test antibiotics

Nystatin and amphotericin B were generously provided by E. R. Squibb and Sons, Ltd., Montreal, Que. Pure rapamycin was prepared as previously reported[6].

Candida albicans strains

C. albicans strain AY F-598 is the test organism for rapamycin assay. *C. albicans* ATCC 11,651 (AY F-634) was used to produce systemic infections in mice and vaginal infections in rats.

Culture media and inocula

Candidal strains were maintained as lyophilized cultures. Cultures were grown on BBL-SABOURAUD

Dextrose Agar (Baltimore Biological Laboratory, Inc., Baltimore, Maryland) at 37°C for 18 hours, then transferred to BBL-SABOURAUD Liquid Broth (modified) and incubated under the same conditions. These liquid cultures were diluted ten-fold, and the resulting diluted suspensions served as inocula for SABOURAUD Liquid Broth used for MIC (minimum inhibitory concentration) determinations, and BBL-Nystatin Assay Agar (Antibiotic Medium No. 12) for serum assay of rapamycin[1].

MIC determination

The minimum inhibitory concentration was determined by the conventional two-fold serial broth dilution method. Culture tubes containing 10 ml of the medium were inoculated with 0.1 ml of the inoculum described in the preceding section, and incubated at 37°C for 7 days. Tubes were examined for visible turbidity at 2 and 7 days. To determine the influence of pH on MIC, HCl or NaOH was added to SABOURAUD Liquid Broth before sterilization to give pH values of 4, 6, 7, and 8 at the time of inoculation. MIC's were also determined in the presence of 5% sterile horse serum added to SABOURAUD Liquid Broth before inoculation. The effect of inoculum size on MIC was studied using an inoculum prepared as above, and adjusted by plate counts to contain 52×10^6 cells/ml; MIC's were read at 48 hours, then each tube was subcultured on SABOURAUD Dextrose Agar plates to determine the minimum fungicidal concentration (MFC).

Bioavailability study

Swiss albino Wistar male mice (25~30 g) were administered micronized rapamycin as a suspension in 5% acacia; the suspension was given by gavage (p. o.) or by subcutaneous injection. Mice, in groups of three, were bled by cardiac puncture at 0, 1, 2, 3, and 4 hours; therefore, the sera of three animals were combined to constitute one bleeding.

A cross-over design was used in the dog (10 kg each) study. On day 0, one dog received 250 mg and the other dog 500 mg of rapamycin contained in a gelatin capsule. After one week rest, the same doses were reversed in the same dogs. Blood was sampled from the jugular vein in the neck at 0, 1, 2, 4 and 6 hours after rapamycin administration, allowed to clot, and the serum collected for assay.

Sera were assayed on the day of bleeding by a modification of the agar diffusion method of BENNETT *et al.*[1] with *C. albicans* AY F-598 as the test organism and Nystatin Assay Agar as the assay medium. The liquefied assay medium (200 ml) was inoculated with 0.25 ml of a ten-fold dilution of a 18-hour broth culture of the test organism. Standard solutions of rapamycin were prepared in appropriate serum from a stock solution containing 100 μg of rapamycin per ml (in methanol) to give final concentrations of 5, 2.5, 1.25, 0.625 and 0.3125 μg/ml of serum. These standard sera were pipetted in quadruplicate into agar wells according to a randomized arrangement. Serum samples, undiluted, were similarly distributed. All plates were then incubated at 37°C for 18 hours. Zones of inhibition were measured, averaged and the average of each standard plotted against rapamycin concentration on semi-log graph paper to draw the standard curve. Concentration of the unknowns was obtained by interpolation from the standard curve.

Protection against systemic candidosis

Preliminary experiments had shown that the intravenous injection of $3 \sim 7 \times 10^6$ cells of *C. albicans* ATCC 11,651 killed untreated Swiss albino Wistar male mice (25~30 g) within 48 hours. In subsequent studies, the infecting dose ranged from 5 to 7×10^6 cells.

The treatment consisted of three doses, administered either orally or subcutaneously at 1, 4 and 24 hours after infection. In one study, after the initial three treatments, a single daily treatment was continued for 6 days. Deaths were recorded, and survivors kept under observation for 7, 18 and 30 days, depending on the study. Infected, non-treated, as well as non-infected, treated mice served as controls for mortality and drug toxicity. Each treatment group comprised at least 10 mice. PD_{50} was calculated according to REED and MUENCH[5].

Rapamycin was administered as a suspension in 5% acacia. Mycostatin (nystatin) and Fungizone (amphotericin B) were administered as the commercial products according to the recommendations of the manufacturer.

Protection against vaginal candidosis

Sprague-Dawley female rats (150 g) were infected intravaginally by means of specially prepared

airfoam sponge plugs previously contaminated with *C. albicans* ATCC 11,651. Sterile cylindrical airfoam sponge plugs (7/16″ in length × 5/16″ in diameter) were placed in 125-ml Erlenmeyer flasks (25 plugs/flask) containing 75 ml of SABOURAUD Liquid Broth inoculated with *C. albicans* ATCC 11,651. Flasks were incubated without agitation at 37°C for 18 hours. After incubation, the plugs were removed to sterile Petri plates and the broth culture centrifuged to recover the candidal cells which were then resuspended in 7.5 ml of sterile 10% glucose solution. Each plug was saturated with 0.2 ml of this suspension. One infected plug was inserted and remained in the rat vagina for the duration of the experiment. Presence of candidal cells was ascertained by microscopic smear examination and cultures.

The treatment consisted of a single oral dose of rapamycin (50 mg/kg) or Mycostatin (40,000 units/kg), administered daily for 6 days. The course of infection was monitored by vaginal swab cultures taken on days 1, 4 and 6 of treatment, and on days 2 and 3 after termination of therapy. The amount of candidal growth was estimated semi-quantitatively on a scale from 0 (no growth) to 4+ (heavy growth). The response to treatment was evaluated by the decrease of number of animals having vaginal cultures with moderate (3+) and heavy (4+) growth as compared to untreated controls.

Results

Effect of pH, Serum and Inoculum Size

At pH 6 and 7, the MIC of rapamycin against *C. albicans* was <0.02 μg/ml as read after 2 and 7 days of incubation in SABOURAUD liquid broth. At pH 4, the MIC increased to >10 μg/ml; at pH 8, it was found to be <0.02 μg/ml after 2 days of incubation, but 0.16 μg/ml after 7 days of incubation.

In the presence of 5% horse serum, the MIC of rapamycin was >10 μg/ml as compared to <0.02 μg/ml in the absence of serum; observation was at 2 and 7 days of incubation in SABOURAUD liquid broth.

The MIC value of rapamycin against *C. albicans* was not affected by inoculum sizes between 10 and 50×10^5 cells/ml. For an inoculum of 5.2×10^6 cells/ml or less, a two-fold decrease in MIC was noted. The MFC remained constant at 0.05 μg/ml with all inoculum sizes tested.

Bioavailability in Mice, Rats and Dogs

Rapamycin was administered to male mice at a dose of 15 mg/kg. Results are illustrated in Fig. 1. Serum levels were higher for the subcutaneous than for the oral route of administration. Distinct

Fig. 1. Average serum concentration of rapamycin in mice after oral or subcutaneous administration of 15 mg/kg.

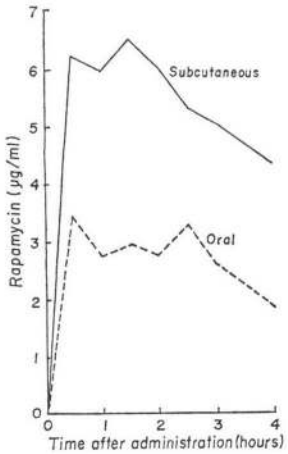
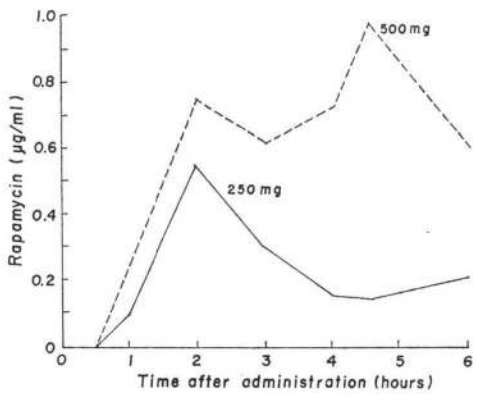


Fig. 2. Average serum concentration of rapamycin in dogs after oral administration of 250 and 500 mg/dog: two-dog cross-over experiment.



peaks were observed in both curves. Rapamycin was not eliminated very rapidly from blood; 4 hours after subcutaneous or oral administration, the concentration of rapamycin still present in blood was 4.3 and 1.85 μg/ml respectively; these values are well above the MIC and MFC of rapamycin against *C. albicans* ATCC 11,651.

Rapamycin was administered orally to two dogs in a cross-over study at two doses, 250 and 500 mg/dog. The results are illustrated in Fig. 2. For the lower dose a single peak of 0.55 μg/ml was obtained at 2 hours; the concentration then decreased to reach a minimum at about 4 hours, after which it increased slightly to 0.21 μg/ml at 6 hours, probably toward a second peak. For the higher dose two peaks were observed as in mice, one peak of 0.75 μg/ml at 2 hours, and another peak of 0.98 μg/ml at 4.5 hours. At 6 hours the concentration was 0.61 μg/ml.

In summary, subcutaneous or oral administration of rapamycin to mice and dogs led to rapid absorption and yielded blood serum concentrations well above the MIC and MFC values of the antibiotic against *C. albicans* ATCC 11,651.

Protection against Systemic Candidosis

Rapamycin was compared to amphotericin B and nystatin in protecting mice against a systemic infection with *C. albicans* ATCC 11,651. Antibiotics were administered orally 1, 4 and 24 hours after an intravenous injection of 6.5×10^6 candidal cells. The results are tabulated in Table 1. The PD_{50}'s for rapamycin, amphotericin B and nystatin were respectively 11 mg/kg, <0.25 mg/kg and $>4{,}000$ units/kg. When the antibiotics were administered subcutaneously (Table 2) the results were essentially the same; PD_{50} of rapamycin was slightly lower (9.5 mg/kg) for subcutaneous than for oral (11 mg/kg) administration, which corroborates the results of bioavailability studies (Fig. 1). STEINBERG *et al.*[8]

Table 1. Protective effect of rapamycin, amphotericin B and nystatin administered orally to mice systemically infected with *C. albicans* ATCC 11,651

Antibiotic	Dose (mg/kg)[a]	Survival (%)[b]	PD_{50} (mg/kg)[c]
Rapamycin	0	0	11.0
	7.5	12.5	
	10.0	30.0	
	12.5	63.1	
	15.0	86.3	
	20.0	100.0	
Amphotericin B	0	0	<0.25
	0.25	72.7	
	0.50	94.7	
	1.00	96.4	
Nystatin (unit/kg)	0	0	>4,000 (units/kg)
	2,000	7.1	
	3,000	14.2	
	4,000	30.7	

[a] Treatment: 1, 4 and 24 hours after infection.
[b] Observation at 7 days.
[c] Infectious dose: 6.5×10^6 cells.

Table 2. Protective effect of rapamycin, amphotericin B and nystatin administered subcutaneously to mice systemically infected with *C. albicans* ATCC 11,651

Antibiotic	Dose (mg/kg)[a]	Survival (%)[b]	PD_{50} (mg/kg)[c]
Rapamycin	0	0	9.5
	7.5	22.7	
	10.0	52.9	
	12.5	90.0	
	15.0	96.5	
	20.0	97.3	
Amphotericin B	0	0	<0.25
	0.25	81.8	
	0.50	95.0	
	1.00	96.2	
Nystatin (units/kg)	0	0	>4,000 (units/kg)
	2,000	0	
	3,000	0	
	4,000	0	

[a] Treatment: 1, 4 and 24 hours after infection.
[b] Observation at 7 days.
[c] Infectious dose: 7×10^6 cells.

Table 3. Protective effect of rapamycin (20 mg/kg) and amphotericin B (1 mg/kg) administered orally or subcutaneously to mice systemically infected with *C. albicans* ATCC 11,651

	Antibiotic and route of administration	Percent survival			
		Treatment A		Treatment B	
		18 days	30 days[a]	18 days	30 days[b]
Infected	Rapamycin, oral	46.6	33.3	93.3	26.6
	Rapamycin, s. c.	93.3	46.6	86.6	66.6
	Amphotericin B, oral	93.3	40.0	53.3	33.6
Non-infected control	Rapamycin, oral	100	100	93.3	93.3
	Rapamycin, s. c.	100	100	93.3	93.3
	Amphotericin B, oral	ND	ND	100.0	100.0

[a] Treatment A: 1, 4 and 24 hours after infection. Infectious dose: 3.15×10^6 cells.
[b] Treatment B: 1, 4 and 24 hours, and 2, 3, 4, 5, 6 and 7 days after infection.
Infectious dose: 5.0×10^6 cells.
ND: not determined.

reported the PD_{50} of amphotericin B in experimental *C. albicans* infection of mice to be <0.55 mg/kg, *per os*, and <0.32 mg/kg, subcutaneously.

In another study, rapamycin *per os* and subcutaneously, or amphotericin B, *per os*, were given at the doses that were shown, in the previous experiments, to give almost complete protection to mice systemically infected with *C. albicans*, *i. e.* 20 mg rapamycin/kg and 1 mg amphotericin B/kg, given at 1, 4 and 24 hours after infection. Animals were observed for 18 and 30 days (Treatment A). The results are presented in the first and second columns of Table 3. The highest percentage of deaths occurred between day 7 (Tables 1 and 2) and day 18 (Table 3). It would appear that rapamycin controlled the infection for a period of 7 days with a subsequent mortality of 53% between days 7 and 18. Rapamycin, given subcutaneously, and amphotericin B, *per os*, protected 93.3% of the mice for 18 days (Table 3). The survival rate at 30 days were essentially the same for rapamycin, given orally or subcutaneously, and amphotericin B, given orally.

In a parallel study, after the initial three treatments, a single daily dose was administered for 6 days and the animals were observed for 18 and 30 days (Treatment B). The results are reported in the third and fourth columns of Table 3. The additional daily treatment (for 6 days) with rapamycin given orally extended the percent survival rate from 7 days to 18 days. The daily treatment with rapamycin administered subcutaneously did not significantly affect the percentage of survivors over the initial treatment after 18 days of observation. After 30 days of observation, the percent survival was higher with subcutaneous treatment than with oral treatment, but not much higher than the initial subcutaneous treatment (Treatment A). The percentage of survivors with amphotericin B after daily treatment and 18 days of observation was significantly lower than that of Treatment A; this may be due to the combination of drug toxicity and infection. After 30 days of observation, the percentage of survivors with amphotericin B (Treatment A) was not significantly different. In conclusion, we would suggest that in the *in vivo* evaluation of an anti-*Candida* agent, irregardless of treatment, the test animals should be kept for 30 days after infection, and the survivors observed after 7, 18 and 30 days.

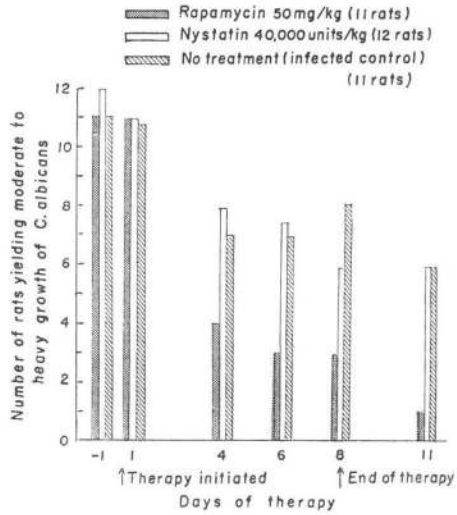
In the course of these experiments *C. albicans* was regularly reisolated from animals treated with rapamycin; the cultures showed no increase in MIC, an indication that the resistance of candidal cells

did not develop during treatment.

Protection against Vaginal Candidosis

A number of female rats were infected intravaginally by means of the sponge plug technique described in Materials and Methods and 34 proved to carry the infection (Fig. 3, day-1). They were grouped as follows: group A (11 rats) received no treatment (control); group B (12 rats) was treated with Mycostatin; group C (11 rats) was treated with rapamycin. Oral treatments started on day 0 and lasted for 8 days. All animals were examined on days 1, 4, 6, 8 and 11 (3 days after cessation of therapy) after the beginning of therapy. The histogram in Fig. 3 reports the number of animals having vaginal cultures with moderate (3+) and heavy (4+) growth of *C. albicans*. At the end of therapy (day 8) the cure rates were: 6 of 12 (50%) for Mycostatin, 8 of 11 (73%) for rapamycin, and 3 of 11 (27%) for spontaneous cure. Three days after cessation of treatment (day 11) no change was recorded for Mycostatin, but 10 of the 11 rats (91%) treated with rapamycin were cured.

Fig. 3. Protective effect of rapamycin and nystatin against vaginal candidosis in rats.



Histological examination of infected vaginas and normal vaginas in which sterile plugs had been inserted (non-infected, non-treated control) showed some degree of irritation caused by these plugs, but the purulent exudate was present only in the vaginas containing infected plugs.

Discussion

It was previously reported[6] that rapamycin is more active than candicidin and nystatin against clinical isolates of *C. albicans*; the MIC's obtained with candicidin and nystatin were within the range reported by DROUHET[2]. It was also reported that rapamycin was more active than amphotericin B against eight species of *Candida*; only *C. pseudotropicalis* was more sensitive to amphotericin B.

In the present study, rapamycin was found not to be affected by pH between 6 and 8, but at pH 4 there was complete loss of activity. The MIC and MFC of rapamycin against *C. albicans* were not influenced by the size of inoculum. In the presence of 5% horse serum, the antibiotic lost its *in vitro* activity possibly due to the binding of rapamycin to serum components. This binding must be reversible, since bioavailability data in mice and dogs demonstrated that rapamycin is present in the blood at a level well exceeding its MIC against *C. albicans* (Figs. 1 and 2), and was sufficient to eradicate severe systemic candidosis in mice (Tables 1 and 2).

In the *in vivo* studies rapamycin, given orally or subcutaneously, was compared to amphotericin B, an antibiotic effective in the treatment of systemic candidosis. The dose selected was based on an initial study in which both antibiotics yielded 96.2~100% survival after 7 days of observation.

Rapamycin at 20 mg/kg, *per os* and subcutaneously, and amphotericin B at 1 mg/kg, orally, administered at 1, 4 and 24 hours after infection gave approximately the same percent survival after a 30-day observation period (Treatment A). When the above treatment was extended by an additional daily treatment for 6 days (Treatment B), the percent survivals for rapamycin and amphotericin B given orally were similar, but subcutaneous administration of rapamycin gave higher survival after 30 days of observation. Rapamycin was also found very active orally in the treatment of an experimental vaginal candidosis in rats. The high *in vivo* activity of rapamycin, combined with its low acute toxicity (lower

than that of amphotericin B[9]) and good oral absorption, makes it a possible candidate for the treatment of vaginal candidosis, as well as acute and chronic systemic candidosis.

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