Antifungal Activities of Rapamycin and Its Derivatives, Prolylrapamycin, 32-Desmethylrapamycin, and 32-Desmethoxyrapamycin

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The antifungal agent rapamycin is highly effective in inhibiting growth of yeast and mold strains. This study demonstrates that in liquid medium, rapamycin is more active than its derivatives (prolylrapamycin, 32-desmethylrapamycin, 32-desmethoxyrapamycin) against *Candida albicans*, *Saccharomyces cerevisiae*, and *Fusarium oxysporum*. All the rapamycins were more active than amphotericin B. Although four other molds were not inhibited in liquid medium, they were very sensitive to rapamycin and its derivatives when tested on agar. The latter assay showed that rapamycin is the most active and 32-desmethylrapamycin is more active than prolylrapamycin and 32-desmethoxyrapamycin. The conclusion of this study is that rapamycin is the most active antifungal agent of the compounds examined. The unexpected finding of high activity of rapamycin and its derivatives against filamentous fungi when assayed by the agar diffusion assay suggests that rapamycin or a derivative may hold promise for chemotherapy against pathogenic molds as well as yeasts.

Rapamycin, a secondary metabolite produced by *Streptomyces hygroscopicus*, was first isolated from soil samples collected on Rapa-nui (Easter Island)[7]. Rapamycin has a distinctive structure as a 31-membered lactone ring consisting of reactive regions such as a retroaldol site, and a tricarbonyl array with an amide, ketone, and hemiketal[6]. Rapamycin production by aerobic fermentation is sometimes accompanied by the production of derivatives: prolylrapamycin[3], desmethylrapamycin[1], and desmethoxyrapamycin[5]. The chemical structures of rapamycin and its derivatives are given in Figure 1.

Rapamycin is structurally similar to FK506, another *Streptomyces* metabolite with potent immunosuppressive activities[2]. The structural similarity between these two macrocyclic polyketides led to intense studies of rapamycin for immunosuppressive activities. Although these two polyketides inhibit immune responses in different manners, both are very active in inhibiting T-cell activation[2].

Although it is a powerful immunosuppressant[4], rapamycin was initially discovered as an antifungal agent[7]. However there have been very few studies on antifungal activities of rapamycin and its derivatives[1,5]. Furthermore, there has yet to be a comparative antifungal study under identical conditions of rapamycin and three of its derivatives: prolylrapamycin, 32-desmethylrapamycin†, and 32-desmethoxyrapamycin†. Such a study is of importance for the future application of rapamycin or its derivatives in human medicine[2]. The present work simultaneously compares these four compounds against two yeast strains and five mold strains.

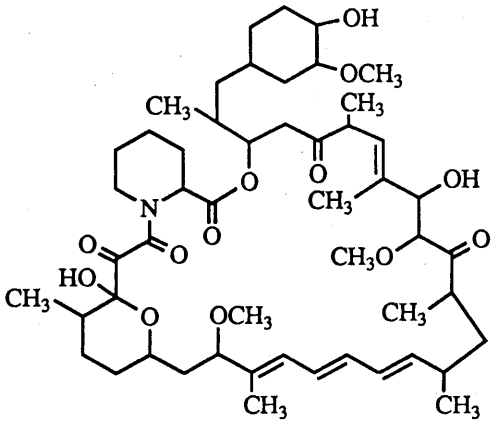
Materials and Methods

Fungal Strains Used and Initial Concentrations

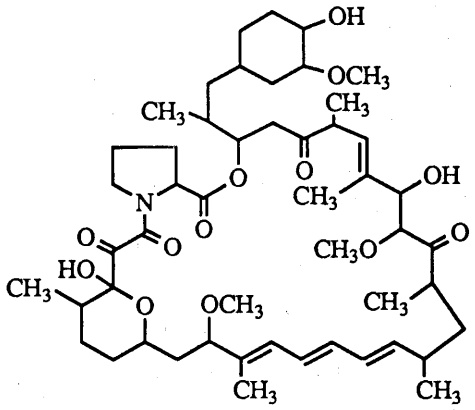
Two yeast strains: *Candida albicans* ATCC 11651 and *Saccharomyces cerevisiae*, and five mold strains: *Aspergillus flavus* ATCC 10124, *Aspergillus fumigatus* KM

† 32-Desmethylrapamycin was previously called 27-*O*-desmethylrapamycin[1] and 32-desmethoxyrapamycin was previously called 29-desmethoxyrapamycin[5]; the rapamycin numbering system has changed several times.

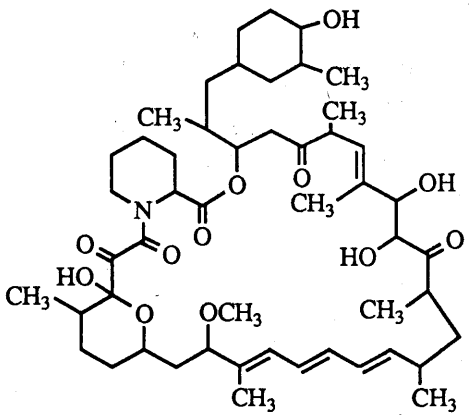
Fig. 1. Structures of rapamycin and its derivatives.



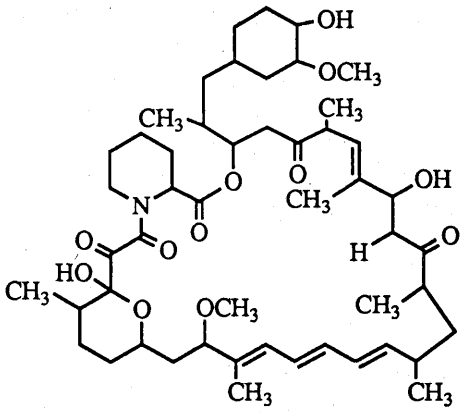
Rapamycin
(Isomer B)



Prolylrapamycin



Desmethylrapamycin
(WAY-125286)



Desmethoxyrapamycin
(WAY-24688)

8001, *Aspergillus niger*, *Fusarium oxysporum* ATCC 48112, and *Penicillium* sp. were used to test the antifungal activities of rapamycin and its derivatives. The concentrations of cells as colony forming units (CFU) per ml were established by plating of the cell/spore suspensions. Each suspension was diluted 10^{-4}, 10^{-5} and 10^{-6} and plated in duplicate on 15 ml YM 1.8% agar plates for CFU counting. YM 1.8% agar was prepared in double distilled water, per liter, as follows: glucose, 10 g; yeast extract (Difco Laboratories, Detroit, MI), 3 g; malt extract (Difco), 3 g; Bacto peptone (Difco), 5 g; agar, 18 g. The plates were incubated for 2 days at 30°C and counted.

MIC of Rapamycin and Its Derivatives in Liquid Medium

The antimicrobial activities of rapamycin and its derivatives were determined on the seven strains in liquid culture. For comparison, amphotericin B, a known polyene antifungal drug used in antifungal chemotherapy, was also employed. Each drug was tested in solution at concentrations from 0.0625 to 64 μg/ml on each yeast and mold strain. The minimal inhibitory concentration (MIC) of each drug in the liquid bioassay is defined as the lowest drug concentration that restricts colony formation to the initial count of CFUs/ml. The strains were diluted with Sabouraud medium (glucose 40 g, Bacto-peptone 10 g, per liter of double-distilled water) to a concentration of 10^5 CFU/ml.

For yeast strains, each 12 × 75 mm glass culture tube received 180 ml of yeast suspension and 20 μl of drug at the various concentrations. The solutions of drugs had been prepared by serial dilution with 50% methanol-100 mM MES buffer (pH 6.0). Control tubes (no drug)

were plated out at zero time on 15 ml YM 1.8% agar plates. The tubes, covered with polyethylene plugs, were incubated at 30°C for 2 days before being plated. All plates were incubated for 2 days at 30°C. Various dilutions were made from each tube and plated at 0.1 ml per plate. Selected plates containing 10~100 colonies were used to calculate MICs.

For testing the mold strains, drug solutions were prepared in 100% methanol. Each culture tube was filled with 15 ml of drug solution and the methanol was evaporated off by incubation of the tubes loosely capped at 37°C for 30~60 minutes. After evaporating the methanol completely, 150 μl of mold suspension was added into each test tube. Incubation of the mold cultures was at 30°C for 3 days. Plating was done as above and selected plates containing 10~30 colonies were used to calculate MICs.

Bioassay of Antimicrobial Activities on Agar

The antimicrobial activities of rapamycin and its derivatives were measured based on inhibition zone diameters in the paper disk-agar diffusion method. The inhibitory effect of various concentrations of rapamycin *vs.* the diameter of the respective inhibition zones was measured for each of the fungal strains. For each yeast strain, 200 μl of yeast suspension was seeded into 100 ml of warm YM 0.8% agar. (Instead of the YM 1.8% agar used for CFU counting, a lower agar concentration was used here to facilitate drug diffusion in the agar medium.) The volume was 15 ml and 20 ml per plate for *C. albicans* and *S. cerevisiae* respectively. For each mold strain, 630 μl of mold suspension was seeded into 100 ml of warm YM 0.8% agar. The plating volume was 15 ml for all mold strains.

Rapamycin was serially diluted in 100% methanol to the following concentrations: 20, 10, 5, 2.5, and 1.25 μg/ml. A 15 ml volume of each concentration was deposited on paper disks (1/4", Schleicher & Schuell Filter Paper, Keene, NH) and allowed to evaporate for 5 minutes. The rapamycin derivatives were similarly tested at 10 or 20 μg/ml. The paper discs were then loaded onto plates, one in each quadrant of a plate. The plates, containing the drug-laden paper discs, were placed in the cold room for 30 minutes to allow for drug diffusion, and then incubated at 30°C for 2 and 3 days for yeast and mold strains, respectively. The diameter of each inhibition zone was measured and plotted against the logarithm of the drug concentration added to the disk. Extrapolation down to 7 mm zone diameter revealed the MIC value since the diameter of the disk is 6.35 mm. The rapamycin derivatives were examined at 10 or 20 μg/ml on disks and zone sized compared to those observed with a series of concentrations of rapamycin. The relative antifungal activity of each derivative was calculated by dividing the concentration of rapamycin required to produce a particular zone diameter by the concentration of derivative giving the same zone diameter.

Results

MIC of Rapamycin, Its Derivatives, and Amphotericin B in Liquid Culture

The MICs of each drug tested on the seven fungal strains are listed in Table 1. In the liquid culture bioassay, both *C. albicans* and *S. cerevisiae* were inhibited by a very low concentration of rapamycin (MIC < 0.0625 μg/ml for both strains). With one exception (prolylrapamycin, 1.0 μg/ml), the derivatives of rapamycin were able to inhibit growth of the yeast strains at low con-

Table 1. MIC of drugs tested for antifungal activity in liquid culture.

Microorganism[a]	MIC (μg/ml)[b]				
	Rapamycin	Prolyl-rapamycin	Desmethoxy-rapamycin	Desmethyl-rapamycin	Amphotericin B
Candida albicans ATCC 11651	<0.0625	1.0	0.25	0.125	1.0
Saccharomyces cerevisiae	<0.0625	0.125	0.125	0.125	1.0
Aspergillus flavus ATCC 10124	>64	>64	>64	>64	32
Aspergillus fumigatus KM 8001	>64	>64	>64	>64	32
Aspergillus niger	>64	>64	>64	>64	8
Fusarium oxysporum ATCC 48112	0.25	2.0	4.0	0.5	16
Penicillium sp.	>64	>64	>64	>64	8

[a] Fungal suspensions were prepared to 10^5 CFU/ml.
[b] The MIC was obtained after 2 and 3 days of incubation, for yeast and mold strains respectively.

centrations (0.125 to 0.25 μg/ml) in comparison with the reference drug amphotericin B (MIC 1.0 μg/ml for both yeast strains).

For the mold strains tested, only *F. oxysporum* was inhibited in liquid medium by rapamycin (MIC 0.25 μg/ml) and its derivatives (MIC 0.5~4.0 μg/ml), all more active than amphotericin B (MIC 16 μg/ml). With the other four mold strains, *A. flavus*, *A. fumigatus*, *A. niger* and *Penicillium* sp., rapamycin and its derivatives were unable to inhibit fungal growth in liquid medium even at high concentrations (>64 μg/ml) as compared with lower inhibitory concentrations of amphotericin B (8~32 μg/ml).

Relative Antifungal Activities of Rapamycin and Its Derivatives on Agar

The activity of rapamycin is shown in Table 2. Excellent activity was observed against all the fungi. For each fungal strain, the relative activity of each derivative as compared to rapamycin is summarized in Table 3. No derivative was more active than rapamycin against any fungus tested.

Table 2. MICs of rapamycin against fungi by paper disk-agar diffusion assay.

Microorganism	MIC[a] (μg/ml)
Candida albicans ATCC 11651	0.10
Saccharomyces cerevisiae	0.05
Aspergillus flavus ATCC 10124	0.027
Aspergillus fumigatus KM 8001	0.003
Aspergillus niger	0.007
Fusarium oxysporum ATCC48112	0.82
Penicillium sp.	0.007

[a] That concentration of rapamycin that produces a zone diameter of 7.0 mm when added at 15 μl to a paper disk of 6.35 mm diameter.

Discussion

Studies on the antifungal properties of rapamycin and its derivatives are very important for the following reasons: (i) Immunosuppressed patients are more susceptible to fungal infections than the normal population; (ii) as stated by HIGH[1], rapamycin is known to have excellent anti-*Candida* properties but its activity against molds is difficult to assess from available literature; (iii) a derivative with high antifungal activity and low immunosuppressive activities might be developed as an effective antifungal agent.

It is quite interesting that the activities of rapamycin and its derivatives in liquid medium are revealed predominantly against the yeasts, not the molds. In zone assays done on agar, however, activities against the filamentous fungi were very high. We do not know the reason for this phenomenon but the results are encouraging for the eventual use of rapamycin or a derivative against both pathogenic yeasts and molds. We found none of the three derivatives to be as active as rapamycin.

Although the MIC values of the three rapamycin derivatives against *C. albicans* differed considerably in liquid culture (Table 1), activities of rapamycin derivatives on agar medium were approximately the same (Table 3). All derivatives were about 30% as effective as rapamycin on *C. albicans*. Against *S. cerevisiae* on agar, 32-desmethoxyrapamycin and 32-desmethylrapamycin were quite potent, *i.e.* 84% and 69% as active as rapamycin respectively while prolylrapamycin was less active (36%).

Table 3. Relative antifungal activities of rapamycin *vs.* its derivatives by paper disk-agar diffusion assay.

Microorganism	Relative antifungal activities			
	Rapamycin	Prolylrapamycin	Desmethoxy-rapamycin	Desmethyl-rapamycin
Candida albicans ATCC 11651	1.00	0.32	0.33	0.32
Saccharomyces cerevisiae	1.00	0.36	0.84	0.69
Aspergillus flavus ATCC 10124	1.00	0.28	0.15	<0.10
Aspergillus fumigatus KM 8001	1.00	0.64	0.76	0.96
Aspergillus niger	1.00	0.20	0.39	0.75
Fusarium oxysporum ATCC 48112	1.00	<0.06	0.09	0.20
Penicillium sp.	1.00	0.23	0.17	0.78

F. oxysporum was the only mold inhibited in liquid culture. In agar medium, the activities of the rapamycin derivatives against *F. oxysporum* were 20% or less than that of rapamycin. Of the three derivatives, 32-desmethylrapamycin was the most active in both liquid and solid media.

In the agar medium bioassay, all the molds were inhibited. The activities of the three derivatives varied between $<10\%$ to 96% of that of rapamycin. No one derivative was outstanding against all five molds. 32-Desmethylrapamycin was the most active of the three rapamycin derivatives against the molds with the exception of *A. flavus*.

Acknowledgments

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