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

IV. MECHANISM OF ACTION

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(Received for publication August 12, 1978)

Rapamycin, an antifungal antibiotic produced by *Streptomyces hygroscopicus* showed a strong candicidal activity, which could not be reversed by sterols. It had no effect on efflux of K^+, Pi or U.V. absorbing materials and cell permeability of *Candida albicans*. Thus, in its action it differs from the polyenes. Mechanism of action of rapamycin appears to be different from many known antifungal agents. In *C. albicans*, rapamycin at the minimum growth inhibitory concentration inhibited: 1) phosphate incorporation into nucleic acids, 2) acetate incorporation into lipids and 3) substrate respiration of amino acids. The effect on amino acid metabolism was expressed as inhibition of oxidative deamination. At low concentrations rapamycin caused degradation of P^{32}-labeled intracellular macromolecules. Inhibition of threonine incorporation into cell wall and leucine incorporation into cellular protein was observed at relatively higher concentrations of rapamycin. The antibiotic showed no effect on cell-free protein synthesizing systems of *Escherichia coli*, rat liver and *C. albicans* and in the mitochondrial enzyme systems. Whether the lethal action of rapamycin on *C. albicans* is primarily due to one of the above effects or is the result of combined effect on some of these biosynthetic parameters remains to be established.

Isolation and characterization of rapamycin, a new antifungal antibiotic, has been reported from this laboratory[1,2]. The antibiotic is highly active against *Candida* species, especially *C. albicans*. Its *in vitro* and *in vivo* activity has also been reported[3]. In the present paper we are presenting the results of our studies on the mechanism of action of rapamycin on *C. albicans*.

Materials and Methods

Chemicals

L-Leucine-C^{14} (U) with a specific activity of 280 mCi/mmole; L-leucine-4,5-H^3, 30.8 Ci/mmole; adenine-8-C^{14}, 57 mCi/mole; uracil-2-C^{14}, 56.8 mCi/mmole; D-glucosamine hydrochloride-1-C^{14}, 56.5 mCi/mole; N-acetyl-D-glucosamine-1-C^{14}, 41.7 mCi/mmole; D-glucose-C^{14} (U), 196 mCi/mmole; orthophosphoric acid [P^{32}], D-mannose-1-C^{14}, 53 mCi/mole; L-threonine-C^{14} (U), 199 mCi/mmole; L-phenylalanine-C^{14} (U), 410 mCi/mmole; L-aspartic acid-C^{14} (U), 204 mCi/mole; L-glutamic acid-C^{14} (U), 244 mCi/mole and L-proline-C^{14} (U), 255 mCi/mole, were obtained from New England Nuclear. Mevalonic acid-2-H^3, 1 mCi/mole, was prepared from DL-mevalonic acid lactone-2-H^3 obtained from Amersham and Searle. L-Leucine-C^{14} (U) and L-leucine-4,5-H^3 were diluted with "cold" leucine to give a 1 mM, 5 mCi/mmole solution. Uracil-2-C^{14} was diluted with carrier substrate to give a 1 mM, 5 mCi/mmole solution. Uridine-2-C^{14} was diluted with carrier substrate to 1 mM, 0.5 mCi/mM. Other radioisotopes were used without further dilutions with carrier substrates. Aculeacin, a new antifungal antibiotic was kindly supplied by Toyo Jozo Co., Tokyo, Japan.

Rapamycin

Solutions of rapamycin were prepared fresh every day. A stock solution of rapamycin was prepared in methanol (2 mg/ml). A working solution was prepared by diluting 1 volume of the stock solution with 1 volume of water. Further dilutions were made with 50% methanol. The final

concentration of methanol in the assay mixtures was kept lower than or equal to 1% (v/v), and the same amount of methanol was added to the controls containing no antibiotic. Concentration of rapamycin in different test systems is indicated in the text.

Organisms and Growth Media

Most of our studies were done with *C. albicans* AY F-598. For some experiments *Saccharomyces cerevisiae* AY F-190 and *Candida stellatoidea* AY F-682, kindly supplied by Dr. KAPICA of McGill University, Montreal, were also used. The organisms were maintained on SABOURAUD dextrose agar slants. Routinely, the organisms were grown overnight (17~18 hours) at 30°C in shake cultures in Bacto-SABOURAUD dextrose broth (SABOURAUD broth) or a medium containing 2% glucose and 1% yeast extract (GYE). Two synthetic media were used: (1) a modified KESSLER and NICKERSON's medium[4]; (2) a modified HAYDUCK solution containing 8% sucrose, 0.1% K_2HPO_4, 0.001% Bacto-casamino acids, and 0.032% $MgSO_4 \cdot 7H_2O$.

Minimum Inhibitory Concentration (MIC)

Standard serial broth-dilution assay (SABOURAUD broth) was used to determine MIC. The MIC was read after 48 hours of incubation at 37°C.

Growth Curve and Viable Count

Effect of rapamycin on the growth of *C. albicans, S. cerevisiae* and *C. stellatoidea* was followed by determining the absorbancy of cultures at 660 nm (A_{660}).

For viable count determination, samples removed at 1-hour intervals were diluted with 1% peptone and plated on SABOURAUD dextrose agar. After 48 hours incubation at 37°C, the colonies were counted.

Incorporation of Radioactive Precursors

Cells grown to the exponential phase in GYE or SABOURAUD broth were collected by centrifugation and suspended in a medium containing 0.4% glucose and 0.2% yeast extract (GYE dil.) to the desired concentration. The assay mixture contained per ml: 0.46 ml GYE dil., 20 μl antibiotic solution and 0.5 ml cell suspension. After incubation for 15 minutes at 30°C, 20 μl of the labeled precursor were added. The amount of cells (expressed in mg dry weight) and the radioisotope varied according to the isotope studied. The reaction tubes were incubated at 30°C with shaking and the reaction was terminated by the addition of 1 volume of ice-cold 10% trichloroacetic acid. The insolubles were collected on Millipore filters (0.45 mμ). The dried filters were placed in vials containing 15 ml of a toluene base scintillation counting solution and radioactivity was counted in a Packard liquid scintillation counter, model 2375. Methods used for the incorporation of specific precursors were as follows:

Leucine, Uracil and Uridine

Protein and RNA syntheses were monitored by determining the incorporation of labeled L-leucine, uracil (or uridine) respectively, into cold trichloroacetic acid insoluble materials. Each 1 ml reaction mixture contained 1.0 mg of cells and 0.1 μCi L-leucine-C^{14} (U), 0.1 μCi uracil-2-C^{14} or 0.01 μCi uridine-2-C^{14}.

Adenine-8-C^{14} and $H_3P^{32}O_4$

Each 2 ml reaction mixture contained: cells, 4.0 mg, and $H_3P^{32}O_4$, 2 μCi, or adenine-8-C^{14}, 1.0 μCi. After 1-hour incubation, the reaction was terminated by the addition of 2 ml of 10% trichloroacetic acid. The acid insolubles were fractionated into RNA and DNA by a modified SCHMIDT-THANNHAUSER method, according to KENNELL[5].

C^{14}-Labeled Mannose, Glucosamine, Glucose, N-Acetylglucosamine

Each 1 ml assay consisted of 1.0 mg cells and 0.1 μCi radioisotope. Incorporation into the whole cell was followed by determining the amount of label incorporated into the entire acid (5% TCA) insoluble fraction collected on Millipore filter (0.45 mμ). The cell wall fraction was separated by the PARK-HANCOCK method[6]. In determining the incorporation of labeled glucose into the glucan and mannan fractions of the cell wall, the assay volume was increased to 10 ml and wall polysaccharides were extracted according to ELORZA and SENTANDREU[5].

Sodium Acetate-1-C^{14}, Methionine-Methyl-C^{14} and Mevalonate-2-H^3

The assay mixture contained cells, 2 mg/ml and the radioisotope 0.02 μCi/ml. To isolate the total lipids the acid-insolubles were extracted according to IWATA *et al.*[8] Fractionation of total lipids into free fatty acid (FFA), non-saponifiable fraction (NSF) and sterol fraction was done according to the method described by NOMURA *et al.*[9]

Rat Liver Homogenate

Rat liver homogenate was prepared according to BUCHER and MCGARRAHAN[10]. The 5,000 $\times g$ supernatant of the homogenate was used as the enzyme system. The reaction mixture contained: the supernatant, 2 ml; NADH, 1 μM; NADP, 1 μM; ATP, 2.5 μM; glucose-6-phosphate, 2 μM; and mevalonate-2-H^3, 1 μM (0.25 μCi), in a total volume of 2.5 ml. After incubation at 37°C for 1 hour the reaction was terminated by the addition of 5 pellets of KOH (final KOH concentration approximately 3 M). Cholesterol fraction was obtained according to HUMBER *et al.*[11].

Cellular Uptake of Labeled Substrates

Exponentially growing cells of *C. albicans* were collected by centrifugation and suspended in fresh GYE dil. medium. The assay mixture containing 1 mg/ml of cell and 0.2 μCi/ml of C^{14} substrate was shaken at 30°C. Samples taken at various time intervals were chilled, the cells collected on Millipore filters and washed with ice-cold saline. The radioactivity of the cell pellet was a measure of the uptake of the substrate by the cells.

Glycolysis and Respiration

Conventional manometric methods were employed. Cells grown overnight in GYE were washed with 0.15 M NaCl and suspended in 0.067 M phosphate buffer, pH 7.0.

Aminoacid Metabolism

A. Decarboxylation: *C. albicans* was grown overnight in a medium containing 3% glucose, 1% sodium glutamate and 1% peptone, pH 5.5. Decarboxylase activity of washed cells was determined by the Warburg manometric technique described by SUGAWARA[12].

B. Transamination: *C. albicans* was grown aerobically overnight in SABOURAUD broth, centrifuged and washed three times with 0.15 M NaCl. Cell extracts were prepared according to SUGAWARA[12] and assayed for glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic-transaminase (GPT) activities following the method described by LKB Instruments Inc.[13].

C. Oxidative Deamination: Washed *C. albicans* cells suspended in 0.05 M sodium phosphate, pH 7.5 were used as a source of enzyme. The reaction was carried out in Warburg flasks essentially according to SUGAWARA[12]. The reaction was stopped by the addition of trichloroacetic acid. Samples were centrifuged and total ammonia content in the supernatants was determined by an automated system using the Nessler reagent, described by MORGAN *et al.*[14].

Hemolytic Action

The method used was essentially the same as described by KINSKY *et al.*[15].

Sorbose Retention

The method described by CIRILLO *et al.*[16] was followed.

Leakage of Cellular Constituents

Cells in early logarithmic phase in SABOURAUD broth were washed and suspended in 0.06 M Tris-succinate buffer, pH 7.5 containing 0.12 M glucose. The antibiotic was added and the mixture shaken at 30°C. Samples taken at 0 minute and after 60 minutes incubation were centrifuged at 4°C, and the supernatant was assayed for its K^+ and inorganic phosphate (Pi) content (extracellular). The cell pellet was then extracted with boiling water and the supernatant assayed for K^+ and Pi (intracellular). Potassium was determined by flame photometry using an Instrument Laboratory Inc. flame photometer model 343. Phosphorus was determined by a modification of the method described by KRAML[17] for determination of phospholipid phosphorus. To determine the leakage of UV absorbing materials, assays were set up as described above, except that the absorbance of the super-

natants at 260 and 280 nm was determined.

Stability of Macromolecules During the Candicidal Action of Rapamycin

Cells were pre-labeled with $H_3P^{32}O_4$, leucine-C^{14} (U), uracil-2-C^{14} or threonine-C^{14} (U) and the stability of the C^{14}-labeled macromolecules in the cells was determined by the method described by IWATA *et al.*[8].

Protein Synthesis in Cell-free Systems

A. *E. coli*

E. coli Q-13 early log-phase cells, obtained from General Biochemicals, were used to prepare cell-free extract according to NIRENBERG[18]. The dialysed S-30 fraction (without preincubation) was used as the cell-free preparation in the assays for poly U directed protein synthesis according to NIRENBERG[18]. After 30 minutes incubation, 0.1 ml of the reaction mixture was applied to Schleicher and Schuell No 895-E filter paper 25-mm discs and C^{14}-aminoacid incorporation determined according to BOLLUM[19].

B. *C. albicans*

Cells extract was prepared according to SUGAWARA[12]. The extract was centrifuged at $10,000 \times g$ for 10 minutes, then at $20,000 \times g$ for 20 minutes and again at $20,000 \times g$ for 30 minutes, discarding the pellet after each centrifugation. For the incorporation studies, the complete reaction mixture contained: 50.0 mM Tris-HCl, pH 7.5, 3.5 mM magnesium acetate, 7.0 mM potassium chloride, 0.5 mM spermine tetrahydrochloride, 0.05 mM dithiothreitol, 0.15 mM GTP, 0.5 mM ATP, 5 mM phosphoenol pyruvate (PEP), phenylalanine-C^{14} (U) 0.1 μc, 100 μg polyuridylic acid (Poly U), 26.4 μg pyruvate kinase and enzyme extract (1 mg protein) in a final volume of 0.5 ml. After incubation at 30°C for 30 minutes, 1 volume of 10% trichloroacetic acid was added to terminate the reaction. Tubes were placed in a boiling water bath for 15 minutes. After cooling, the hot trichloroacetic acid insoluble fraction was collected on Millipore filter.

Mitochondrial Protein Synthesis

C. albicans cells grown overnight in the medium described by KIM and BEATTIE[20] were collected and spheroplasts prepared according to CABIB[21]. The washed spheroplasts obtained from 1 g of cells were gently homogenized in 3 ml of 0.01 M Tris-HCl pH 7.4 containing 0.25 M mannitol and 0.001 M EDTA. The pellet between $1,000 \times g$ and $10,000 \times g$ was collected as the mitochondrial fraction. The mitochondria were washed twice with 0.01 M Tris-HCl buffer pH 7.4 containing 0.25 M mannitol and 0.001 M EDTA and suspended in 0.5 ml of the same buffer. For the incorporation studies, the reaction mixture was according to LAMB *et al.*[22]. After 20 minutes incubation at 30°C the reaction was terminated by addition of 1 ml of 10% trichloroacetic acid. The samples were placed in a boiling water bath for 20 minutes. After cooling, the precipitate was collected by Millipore filtration and the radioactivity counted.

Protein was determined by the method of LOWRY *et al.*[23], using bovine serum albumin as a standard.

Results

Effect of Rapamycin on Growth of Yeast

Minimum inhibitory concentration of rapamycin against *C. albicans* and *C. stellatoidea* in SABOURAUD broth was 0.032 μg/ml; against *S. cerevisiae* the MIC was >10.0 μg/ml.

As monitored by A_{660} measurements, rapamycin at concentrations up to 1.0 μg/ml did not inhibit growth of *C. albicans*, for the first hour after its addition. At rapamycin concentrations of $0.005 \sim 1.0$ μg/ml, growth inhibition was apparent after 90 minutes (Fig. 1). However, within $1 \sim 2$ hours, rapamycin-treated cells had suffered a considerable loss in viability (Fig. 2). At concentrations greater than 0.02 μg/ml A_{660} of cultures started to decrease after $3 \sim 4$ hours and after $5 \sim 6$ hours the A_{660} was lower than the initial A_{660} at the start of the experiment, indicating lysis of cells. *C.*

Fig. 1. Effect of rapamycin on the growth of *C. albicans*.

Cultures were incubated at 30°C on a rotary shaker (240 rev./minutes) and the A_{660} determined at 1-hour intervals.

Concentrations of rapamycin used are indicated in the figure.

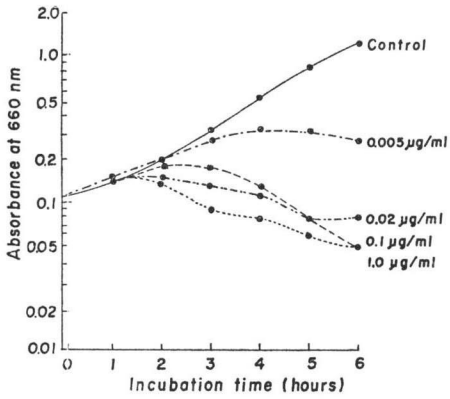


Fig. 2. Effect of rapamycin on the viability of *C. albicans*.

At 1-hour intervals, samples were removed from a growing culture. Samples were diluted with 1% peptone and then they were plated on SABOURAUD dextrose agar. After incubation at 37°C for 48 hours, the colonies were counted.

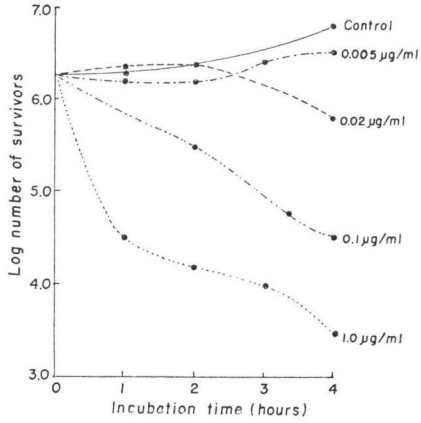


Table 1. Effect of rapamycin on growth of *C. albicans*, *S. cerevisiae* and *C. stellatoidea*

Organism	A_{660}					
	Control		Rapamycin 1.0 μg/ml		Rapamycin 5.0 μg/ml	
	0 hour	24 hours	0 hour	24 hours	0 hour	24 hours
C. albicans	0.28	>6.0	0.29	0.16	0.295	0.16
S. cerevisiae	1.26	8.2	1.33	8.0	1.26	7.8*
C. stellatoidea	0.13	2.4	0.12	0.12	0.12	0.08

Medium: GYE

Temperature of incubation: 30°C.

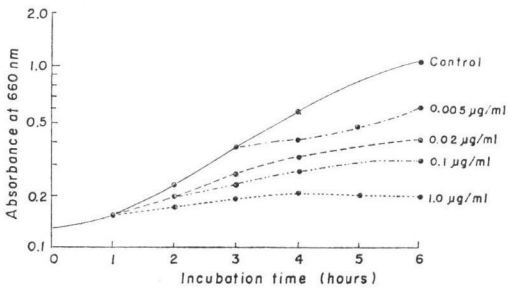
* Same A_{660} readings were obtained at rapamycin concentration of 25 μg/ml.

albicans cells did not recover from this growth inhibitory effect as shown by absorbancy readings taken after 24 hours (Table 1).

Rapamycin is somewhat less effective in inhibiting growth of *C. stellatoidea* (Fig. 3). The decrease in A_{660} observed with *C. albicans* after 2~3 hours of exposure to rapamycin (> 0.02 μg/ml) was not observed with *C. stellatoidea* even after 6 hours. Lysis of rapamycin-treated *C. stellatoidea* cells was observed after 24 hours (Table 1).

Fig. 3. Effect of rapamycin on the growth of *C. stellatoidea*.

Concentrations of rapamycin used are indicated in the figure.

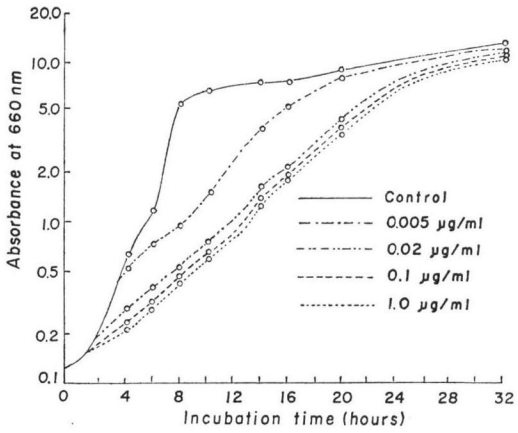


Rapamycin (≥0.02 μg/ml) inhibited the growth of *S. cerevisiae* after 2~3 hours, however, cessation of growth was not observed even after 6 hours (Fig. 4), and viable count indicated no

significant loss of viability. After 24 hours, the absorbancy of rapamycin containing cultures of *S. cerevisiae* was approximately the same as that of the control (Table 1 and Fig. 4). This indicated that rapamycin is not fungicidal for *S. cerevisiae* but affects its generation time. Cells grown for 24 hours in the presence of rapamycin (0.1 ~ 5 μg/ml) were thoroughly washed and the washed cells used to inoculate fresh GYE medium. The effect of rapamycin on the growth of the rapamycin-treated cells was identical to that observed above (Fig. 4). After 24-hour incubation in GYE medium some degradation of rapamycin was (approximately 50% at 1.0 and 5.0 μg/ml) observed but this would not account for its lack of fungicidal action. Therefore, the effect of rapamycin (or the lack of fungicidal effect) on the growth of *S. cerevisiae* cannot be explained by development of resistance or degradation of the antibiotic.

Fig. 4. Effect of rapamycin on the growth of *Saccharomyces cerevisiae*.

Concentrations of rapamycin used are indicated in the figure.



The growth inhibitory effect of rapamycin was greatly influenced by the growth medium used. *C. albicans* grew slowly in 0.05 M potassium phosphate buffer, pH 7.0, 0.05 M Tris-HCl buffer, pH 7.0, or 0.05 M N-Tris (hydroxy-methyl) methyl-2-aminoethane sulfonic acid, each buffer supplemented with 50 mM glucose. In KESSLER and NICKERSON's medium (synthetic medium), the growth rate was approximately half of that in GYE. Rapamycin did not significantly affect the growth of *C. ablicans* in any of these media.

Reversing Effect of Vitamins, Aminoacids, Purines, Pyrimidines, Fatty Acids and Sterols

The possible reversal of candicidal activity of rapamycin by fatty acid, sterols and other compounds was examined by the cylinder cup agar diffusion method. Each portion of 8 ml of GYE agar or SABOURAUD agar containing 20 μg/ml of the test compound was inoculated with 0.1 ml of overnight growth of *C. albicans* and poured into a 15 × 100 mm Petri dish. Stainless steel cylinders, 7 mm in diameter, were placed on the surface of agar, and filled with rapamycin, 0.1, 0.25, 0.5 or 1.0 μg/ml. Plates were incubated at 37°C for 48 hours and the zone of inhibition measured. None of the following compounds caused reversal of rapamycin activity: Vitamin A, vitamin D, thiamine, riboflavin, pyridoxine, ascorbic acid, calcium pantothenate, nicotinic acid, para-aminobenzoic acid, folic acid, L-arginine, L-homoserine, DL-leucine, L-lysine, L-methionine, L-phenylalanine, L-threonine, L-tryptophan, L-tyrosine, DL-valine, adenine, guanine, cytosine, uracil, thymine, myristic acid, ergosterol, palmitic acid, stearic acid, oleic acid and linoleic acid. Ergosterol which often reverses the activity of polyenic antifungal antibiotics, slightly increased the zone of inhibition around rapamycin.

Effect of Rapamycin on Anaerobic Glycolysis and Respiratory Metabolism of *C. albicans*

Rapamycin at 10 μg/ml did not affect anaerobic CO_2 evolution of *C. albicans*. Aerobic, endogenous respiration and utilization of glucose by intact cells of *C. albicans* were also not affected by

Table 2. Effect of rapamycin on the respiration of aminoacids.

Substrate	No rapamycin	Rapamycin, 0.02 μg/ml		Rapamycin, 0.1 μg/ml	
	O_2 uptake (μl)	O_2 uptake (μl)	Inhibition (%)	O_2 uptake (μl)	Inhibition (%)
L-Alanine	217	192	12	199	8
L-Arginine	95	70	26	45	53
L-Glutamic acid	233	136	42	103	56
L-Proline	272	200	27	69	75
L-Asparagine	122	97	20	69	43
L-Aspartic acid	142	96	32	56	61
L-Glycine	—	48	—	55	—
L-Glutamine	129	117	9	81	37
L-Threonine	90	—	—	73	19
L-Leucine	17	—	—	72	0

The main compartment of a Warburg flask contained 15 mg (wet weight) cells, 40 μmoles substrate and 0.67 M phosphate buffer pH 7.0; 0.1 ml 20% KOH in the centre well; oxygen uptake in 2 hours at 28°C.

rapamycin up to 10 μg/ml. Oxygen uptake of *C. albicans* cells with pyruvate, acetate, oxaloacetate, isocitrate, citrate, ketoglutarate or succinate was not significantly affected by 1.0 μg/ml rapamycin; at higher antibiotic concentrations (10 μg/ml) oxygen uptake in 2 hours with acetate, pyruvate, or oxaloacetate was inhibited by 80%, 30% and 45% respectively. These results indicate that rapamycin does not act primarily on the electron transport system or the respiratory energy-yielding system of *C. albicans*.

Rapamycin (0.02 and 0.1 μg/ml) inhibited substrate respiration of amino acids tested; the most pronounced effect was observed with proline (Table 2).

Effect of Rapamycin on Amino Acid Metabolism

Glutamic acid decarboxylase activity of *C. albicans* even when grown on glutamic acid, was low and was unaffected by rapamycin 5.0 μg/ml. Rapamycin at 1.0 μg/ml had no effect on a com-

Table 3. Effect of rapamycin on oxidative deamination of glutamic and aspartic acids with *C. albicans*.

Substrate	Rapamycin μg/ml	NH_3 liberated		O_2 uptake	
		mμ moles/ml	% of control	μl	% of control
Endogenous	0	135		18	
L-Glutamic acid	0	556	100	128	100
	0.02	476	86	131	102
	0.05	106	19	69	54
	0.5	70	13	65	51
L-Aspartic acid	0	823	100	115	100
	0.02	653	79	98	85
	0.05	406	49	68	59
	0.1	176	21		
	0.5	165	20	40	35

Incubation: 2 hours at 28°C.

Table 4. Effect of rapamycin and amphotericin B on the uptake of labeled substrates.

Substrate	Uptake of labeled substrate-DPM				
	Control No antibiotic	Rapamycin 0.1 μg/ml	Rapamycin 1.0 μg/ml	Rapamycin 10.0 μg/ml	Amphotericin B 2.5 μg/ml
Na-acetate-1-C^{14}	16,707	17,262	17,556	12,707	—
L-Leucine-C^{14} (U)	11,090	8,790	7,320	10,323	—
Uracil-2-C^{14}	7,073	8,950	10,350	5,413	—
L-Aspartic acid-C^{14} (U)	8,065	11,766	10,991	12,127	—
L-Threonine-C^{14} (U)	43,691	34,503	38,435	37,479	13,080
L-Glutamic acid-C^{14} (U)	11,337	15,494	18,894	17,824	—
L-Proline-C^{14} (U)	15,281	14,898	19,354	20,684	9,485

mercial *E. coli* glutamic acid decarboxylase.

Effect of rapamycin on glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvate transaminase (GPT) of *C. albicans* was studied using a cell-free preparation of *C. albicans*. Rapamycin, 5.0 μg/ml, did not inhibit GOT or GPT.

Oxidative deamination of glutamic and aspartic acids with whole cells of *C. albicans* was strongly inhibited by rapamycin; at 0.05 ~ 0.1 μg/ml, ammonia liberation was reduced by about 80% (Table 3).

Effect of Rapamycin on the Uptake of Labeled Substrates by *C. albicans*

Rapamycin at concentrations manyfold greater than MIC, had very little effect on the uptake of labeled acetate, L-leucine, uracil, L-aspartate, L-glutamate, L-threonine and L-proline by *C. albicans* (Table 4). Low levels of amphotericin B, under similar conditions, strongly inhibited the uptake of most of these substrates (Table 4).

Effect of Rapamycin on Incorporation of Various Radioactive Precursors into Major Cellular Constituents

Rapamycin inhibited the incorporation of L-leucine-C^{14} (U) and uridine-2-C^{14} by *C. albicans* and the incorporation of L-leucine-C^{14} (U) and uracil-2-C^{14} by *C. stellatoidea*, when the assay medium was GYE-dil. In *C. albicans*, rapamycin inhibited incorporation of L-leucine-C^{14} (U) and uridine-2-C^{14} more strongly than the incorporation of uracil-2-C^{14}. The effective dose for 50% inhibition of incorporation (ED_{50}) was 0.6 μg/ml for leucine-C^{14} (U), 0.35 μg/ml for uridine-2-C^{14} and > 5.0 μg/ml for uracil-2-C^{14}. The inability of rapamycin to inhibit uracil incorporation at lower concentrations may be partly due to low uptake and incorporation of uracil-2-C^{14} (0.5 ~ 1% incorporation of uracil-2-C^{14} compared to 50 ~ 60% incorporation of uridine-2-C^{14}). In *C. stellatoidea*, ED_{50} for radioactive L-leucine and uracil incorporation was 0.15 and 0.05 μg/ml respectively. In *S. cerevisiae*, incorporation of uridine-2-C^{14} and of uracil-2-C^{14} was strongly inhibited; ED_{50} for uridine-2-C^{14} was 0.05 μg/ml and for uracil 0.02 μg/ml (Figs. 5, 6 and 7). L-Leucine-C^{14} (U) incorporation was inhibited the least; ED_{50} was 0.8 μg/ml. When the cells were suspended in phosphate or Tris-HCl buffer containing glucose or modified HAYDUCK solution, rapamycin had no effect on protein and RNA synthesis in *C. albicans, C. stellatoidea* or *S. cerevisiae*. Rapid growth, in a complete medium, seemed to be essential for the inhibitory effect of rapamycin.

C. albicans did not incorporate thymidine-2-C^{14}, thymine-2-C^{14} or thymidine-methyl-H^3. Therefore, synthesis of DNA by *C. albicans* could not be followed by using any of these precursors.

Fig. 5. Effect of rapamycin on the incorporation of leucine-C^{14} (U).

Each 1 ml mixture contained 1.0 mg cells and 0.1 μCi L-leucine-C^{14} (U); assay mixtures were incubated at 30°C for 1 hour.

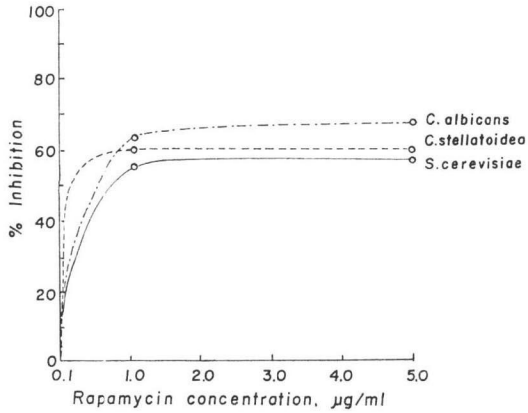
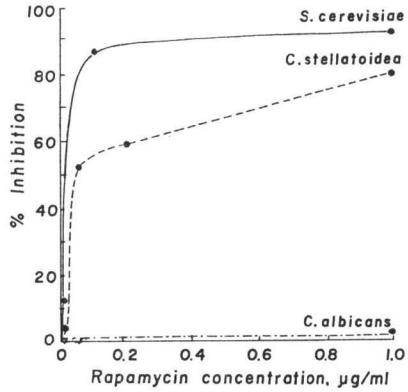


Fig. 6. Effect of rapamycin on the incorporation of uracil-2-C^{14}.

Each 1 ml mixture contained 1.0 mg cells and 0.1 μCi uracil-2-C^{14}. Incubation 1 hour at 30°C.



Effect of rapamycin on nucleic acid synthesis was investigated by monitoring the cellular incorporation of adenine-8-C^{14} and P^{32}-phosphoric acid and by fractionation of the trichloroacetic acid-insoluble fraction into DNA and RNA. In control cultures, the incorporation of adenine-8-C^{14} into RNA was manyfold greater than its incorporation into DNA. Rapamycin had a stronger inhibitory effect on the incorporation of adenine-8-C^{14} into RNA than on the incorporation of adenine-8-C^{14} into DNA. Incorporation of phosphate [P^{32}] into RNA and DNA was strongly inhibited by rapamycin: 49 and 29 % inhibition respectively by 0.02 μg/ml

Fig. 7. Effect of rapamycin on the incorporation of uridine-2-C^{14}.

Each 1 ml mixture contained 1 mg cells and 0.01 μCi uridine-2-C^{14}.

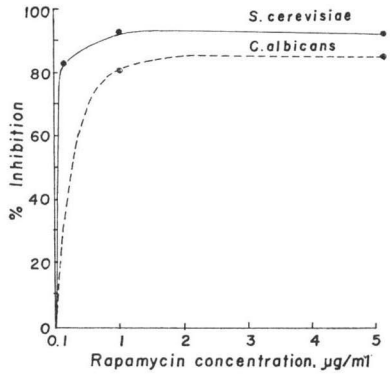


Table 5. Effect of rapamycin on incorporation of adenine-8-C^{14} and $H_3P^{32}O_4$ into the RNA and DNA of *Candida albicans*.

Rapamycin concentration μg/ml	Adenine-8-C^{14}				$H_3P^{32}O_4$			
	Incorporation-dpm		% Inhibition		Incorporation-dpm		% Inhibition	
	RNA	DNA	RNA	DNA	RNA	DNA	RNA	DNA
0	726,491	39,934	—	—	166,686	18,512	—	—
0.02	629,475	46,028	13.4	−15.26	84,666	13,269	49.2	29.4
0.1	281,948	27,600	61.2	30.9	35,640	3,795	78.7	79.5
1.0	128,025	15,353	82.4	61.6	16,170	1,953	90.8	89.5
10.0	129,098	13,628	82.7	65.9	18,195	2,472	91.8	86.9

Each 2 ml reaction mixture contained: cells, 40 mg and $H_3P^{32}O_4$, 2 μCi or adenine-8-C^{14}, 1 μCi; incubation 1 hour at 30°C.

Table 6. Effect of rapamycin on the incorporation of labeled mannose, glucosamine, N-acetylglucosamine or glucose into *C. albicans*.

Labeled precursor	Incorporation-DPM (1 hour)			
	No rapamycin	Rapamycin 0.1 μg/ml	Rapamycin 1.0 μg/ml	Rapamycin 5.0 μg/ml
Mannose	1,558	1,482	915	891
Glucosamine	4,327	—	5,155	4,860
Glucose	6,856	6,223	5,683	5,923
N-Acetylglucosamine	9,508	10,917	11,153	9,836

Each 1 ml assay contained 1.0 mg cells and 0.1 μCi radioisotope.

Table 7. Effect of rapamycin and aculeacin on the incorporation of glucose-C^{14} (U) into the glucan and mannan of *C. albicans*.

Antibiotic μg/ml		Incorporation-dpm		% Inhibition	
		Mannan	Glucan	Mannan	Glucan
No antibiotic		1,248	25,251	—	—
Rapamycin	1.0	672	22,428	46	11
	5.0	511	22,174	59	12
Aculeacin	1.0	231	2,035	82	92
	5.0	431	3,790	66	85

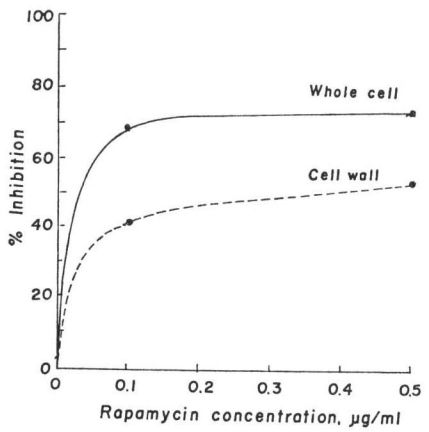
Incubation 1 hour at 30°C.

(Table 5).

Rapamycin up to 5 μg/ml did not inhibit incorporation of labeled glucosamine or N-acetylglucosamine into the whole cell; incorporation of glucose was slightly inhibited (Table 6). When the cell wall was fractionated into the glucan and mannan fractions, rapamycin was shown to inhibit the incorporation of glucose into mannan. Incorporation of glucose into glucan was weakly inhibited (Table 7). This is in contrast to aculeacin which affects glucan synthesis rather than mannan synthesis[24]. Incorporation of mannose was inhibited at 1.0 μg/ml but not at 0.1 μg/ml. Incorporation of threonine into the whole cell or cell wall fraction was inhibited by rapamycin; 50% inhibition was obtained at rapamycin concentration of 0.65 μg/ml for incorporation into the whole cell and 3.5 μg/ml for incorporation into the cell wall fraction (Fig. 8).

Fig. 8. Effect of rapamycin on the incorporation of threonine into the whole cell and into the cell wall of *Candida albicans*.

Each reaction mixture contained 1.0 mg cells, 0.1 μCi L-threonine-C^{14} (U). Incorporation into the whole cell was determined by measuring the amount of L-threonine-C^{14}(U) incorporated into the entire 5% TCA insoluble fraction which was collected on Millipore filter. The cell wall fraction was separated by the PARK-HANCOCK method[6].



Incorporation of Na-acetate-1-C^{14}

At 0.02 μg/ml, rapamycin showed 30% inhibition of the incorporation of Na-acetate-1-C^{14} into

Table 8. Effect of rapamycin on the incorporation of Na-acetate-1-C^{14} into the total lipid fraction.

Rapamycin concentration μg/ml	*Candida albicans*		*Saccharomyces cerevisiae*		*Candida stellatoidea*	
	dpm	% Inhibition	dpm	% Inhibition	dpm	% Inhibition
No rapamycin	89,734	—	21,584	—	68,870	—
0.02	61,101	32	—	—	34,936	49
0.1	50,477	44	20,193	6	27,757	60
1.0	46,834	48	17,500	19	18,037	74
10.0	40,380	55	17,780	18	18,557	73

Incubation 1 hour at 30°C.

Table 9. Effect of rapamycin on the incorporation of Na-acetate-1-C^{14} into the total lipid fraction.

Rapamycin concentration μg/ml	*Candida albicans*			*Candida stellatoidea*		
	FFA	NSF	Sterol	FFA	NSF	Sterol
0.02	20.0	39.2	38.0	28.0	33.4	38.6
0.1	27.8	47.9	57.3	57.3	56.3	68.2
1.0	70.8	56.1	69.6	66.4	67.6	79.2

Incubation 1 hour at 30°C.

the total lipid fraction of *C. albicans*. At 0.10 μg/ml, there was about 40% inhibition. Increasing the antibiotic concentration 100-fold (10 μg/ml) caused only a 10% increase in inhibition, *i.e.* to about 50% inhibition. Similar results were obtained when sodium acetate-2-C^{14} was used as a precursor. *C. stellatoidea* was more sensitive to rapamycin with respect to inhibition of acetate-1-C^{14} incorporation. Again, increasing the antibiotic concentration 100-fold did not increase the inhibition significantly. *S. cerevisiae* was the least sensitive of the three organisms (Table 8).

When the total lipid fraction was fractionated into 3 separate fractions *viz.* free fatty acids (FFA), non-saponifiable fraction (NSF), and sterol fraction, the inhibitory effect of rapamycin on the incorporation of acetate-1-C^{14}, into all three fractions was observed. In *C. albicans*, the incorporation of acetate into FFA fraction was inhibited the least. The degree of inhibition of incorporation of acetate-1-C^{14} into the fractions in *C. stellatoidea* was more or less equal (Table 9).

Incorporation of Methionine-Methyl-C^{14}

Rapamycin at 0.1 μg/ml showed 21% inhibition of the incorporation of methionine-methyl-C^{14} into the total lipid fraction of *C. albicans*; at 1.0 μg/ml there was 40% inhibition. Increasing the antibiotic concentration 10-fold did not result in an increase of inhibition (Table 10). When the cells were treated with 20% (w/v) KOH and sterols extracted with petroleum ether no inhibition of incorporation of methionine-methyl-C^{14} into sterol fraction was observed.

Incorporation of Mevalonic Acid-2-H^3

C. albicans, C. stellatoidea and *S. cerevisiae* did not incorporate mevalonic acid-2-H^3; therefore it was not possible to determine the effect of rapamycin on such incorporation. On the other hand, mevalonic acid is incorporated by rat liver homogenates; rapamycin at 1.0 μg/ml had no significant effect on the incorporation of mevalonic acid-2-H^3 into the entire lipid fraction or into the cholesterol fraction of rat liver homogenate (Table 11).

Table 10. Effect of rapamycin on the incorporation of methionine-methyl-C^{14} into the total lipid fraction of *Candida albicans* F-598.

Rapamycin concentration μg/ml	Incorporation-dpm	% Inhibition
0	10,753	—
0.02	10,131	5.8
0.1	8,464	21.3
1.0	6,443	40.1
5.0	6,258	42.0

Incubation 1 hour at 30°C.

Table 11. Effect of rapamycin on the incorporation of mevalonic acid-2-H^3 by rat liver homogenate.

Rapamycin concentration μg/ml	Incorporation dpm	
	Into whole lipid fraction	Into cholesterol fraction
0	10,997	894
0.02	11,349	—
0.1	11,257	—
1.0	8,608	729

Incubation 1 hour at 30°C.

Effect of Rapamycin on Protein Synthesis in Cell-free and Mitochondrial Systems

Rapamycin (2 μg/ml) did not inhibit polyU-directed C^{14}-phenylalanine incorporation into protein by cell-free extracts of *E. coli* and *C. albicans*. Protein synthesis by *C. albicans* mitochondria was also unaffected by rapamycin (Table 12).

Hemolytic Action

Rapamycin, unlike most polyenes such as amphotericin B or candicidin, did not induce lysis of rat erythrocytes (Fig. 9).

Effect on Sorbose Retention

L-Sorbose is transported across yeast membrane by a facilitated diffusion process. With untreated organism, sorbose enters the cell and equilibrates with the external medium; washing with cold saline results in negligible loss of sorbose. Nystatin and filipin damaged the cell

Fig. 9. Effect of rapamycin, amphotericin B, and candicidin on the lysis of rat erythrocytes.

Washed rat erythrocytes were suspended in 0.9% NaCl buffered with 0.02 M potassium phosphate, pH 6.7 and antibiotic added. After incubation at 37°C, samples were centrifuged and the A_{550} of the supernatant was determined.

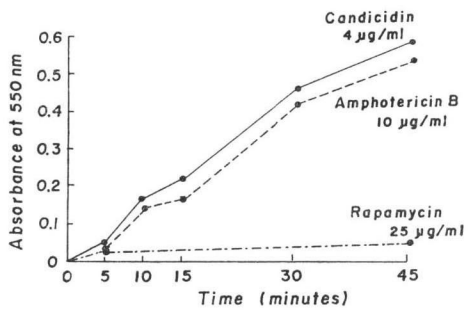


Table 12. Effect of rapamycin on protein synthesis by cell-free extracts and mitochondrial system.

System	Rapamycin concentration μg/ml	Amino acid incorporation dpm/mg protein
E. coli Q_{13} cell-free extract[a]		
Complete system	0	6,725
〃 〃	2	7,193
〃 〃	10	6,460
〃 〃	20	6,347
-Poly U	0	797
C. albicans cell-free extract[b]		
Complete system	0	2,787
〃 〃	10	2,707
〃 〃	25	2,626
-Poly U	0	158
-ATP, PEP, pyruvate kinase	0	88
C. albicans mitochondria[c]		
Complete system	0	181
〃 〃	0.1	188
〃 〃	1.0	198
〃 〃	10.0	170

[a] Reaction volume: 0.25 ml; reaction time: 30 minutes.

[b] Reaction volume: 0.5 ml; reaction time: 30 minutes.

[c] Reaction volume: 1.0 ml; reaction time: 20 minutes.

membrane and prevented the retention of sorbose resulting in leakage of sorbose during washing with saline (CIRILLO *et al.*[16]). Our observations confirmed this inhibition of sorbose retention in *C. albicans* by nystatin. However, rapamycin, even at 25 μg/ml, had no effect on sorbose retention of *C. albicans* (Table 13).

Table 13. Effect of rapamycin and polyenes on sorbose retention.

Compound	Sorbose content μg/ml	
	S. cerevisiae	*C. albicans*
Control	25.0	23.5
Amphotericin B 10 μg/ml	1.0	1.0
Nystatin 25 μg/ml	1.0	1.0
Rapamycin 25 μg/ml	24.5	23.5
Candicidin 10 μg/ml	7.5	7.0

Leakage of Cellular Constituents

Unlike most polyenes, rapamycin did not cause leakage of K^+ or inorganic phosphate (Pi) in *C. albicans*. Log-phase cells were washed and suspended in Tris-succinate buffer; rapamycin was added to 10 μg/ml. Exposure of *C. albicans* cells to rapamycin for 4 hours did not enhance extracellular K^+, Pi, ninhydrin positive materials or UV (260 and 280 nm) absorbing materials. Similar results were obtained when the above experiments were repeated using a suspending medium consisting of 0.4% glucose and 0.2% yeast extract. Under the same conditions nystatin and amphotericin B caused considerable leakage of K^+, Pi and UV absorbing materials (Table 14).

Table 14. Effect of rapamycin, nystatin and amphotericin B on the leakage of K^+, Pi, and UV absorbing materials from *C. albicans*.

Antibiotic added	Extracellular K^+ mEq/l		Extracellular Pi μg/ml		Extracellular A 260		Extracellular A 280	
	0 min	60 min	0 min	60 min	0 min	120 min	0 min	120 min
None	<0.1	0.09	0.19	0.19	0.17	0.90	0.105	0.50
Nystatin 25 μg/ml	<0.1	0.82	0.19	0.69	0.23	1.80	0.20	0.79
Amphotericin B 10 μg/ml	0.11	0.84	0.19	0.94	0.22	1.79	0.14	0.65
Rapamycin 10 μg/ml	<0.1	0.09	0.19	0.19	0.19	0.78	0.115	0.43

Degradation of Intracellular Macromolecules

C. albicans cells were extensively labeled with P^{32} by growing the organism overnight in the presence of P^{32}-orthophosphoric acid. Washed labeled cells were suspended in 0.4% glucose, 0.2% yeast extract and rapamycin was added. For up to 60 minutes of exposure to rapamycin, no leakage of total cellular phosphate was observed. On longer exposure to rapamycin (0.02~1.0 μg/ml) leakage of total radioactivity was observed. Leakage of cold acid-precipitable radioactivity was observed within 60 minutes after addition of rapamycin at concentrations as low as 0.02 μg/ml. The extent of leakage was dependent on the concentration of rapamycin (Figs. 10a and 10b). These results suggest that rapamycin at levels approaching MIC caused degradation of intracellular macromolecular components such as nucleic acids. Under similar conditions cells prelabeled with leucine-C^{14} (U), uracil-2-C^{14} or threonine-C^{14} (U) did not show any leakage of radioactivity.

Discussion

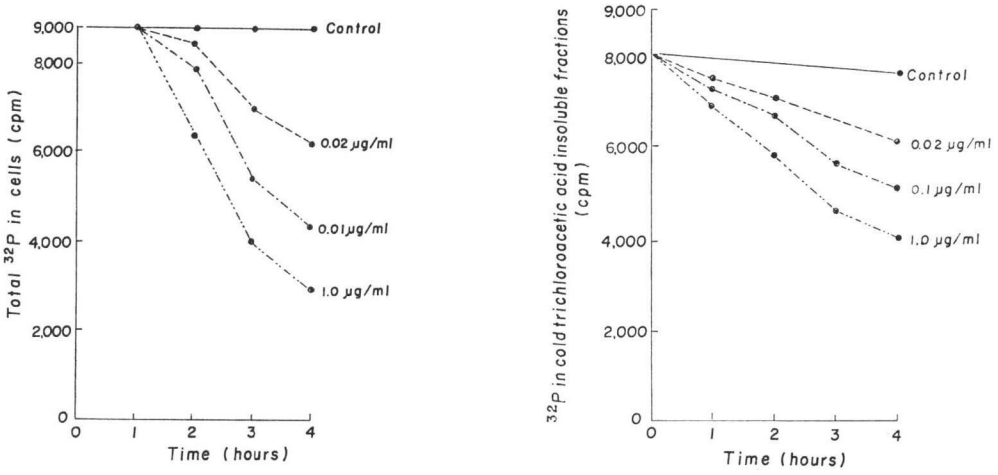
Rapamycin at low concentrations showed a strong candicidal activity, but, unlike the polyenes, this activity could not be reversed by sterols. Rapamycin (up to 10 μg/ml) did not cause hemolysis

Fig. 10. Effect of rapamycin in the degradation of intracellular macromolecules.

Cells extensively labeled with P^{32} were suspended in fresh medium and rapamycin was added. Duplicate samples were taken at 1 hour intervals.

A. Total radioactivity - samples passed through 0.45 mμ Millipore filter and washed with cold water.

B. Cold trichloroacetic acid (TCA) precipitable radioactivity. Sample precipitated with 1 volume cold 10% TCA, and the precipitate was collected on Millipore filter (0.45 mμ) and washed with cold TCA followed by cold ethanol.



of mammalian erythrocytes, had no effect on the efflux of K^+, Pi or UV absorbing materials and did not influence sorbose retention in cells of *C. albicans*. In its action, therefore, rapamycin differs from polyenes. Most polyenes are known to bind the sterols on the cytoplasmic membrane of yeast and cause abnormality in cell permeability[15,16] resulting in leakage of essential cell constituents.

Rapamycin is not an inhibitor of glycolytic and respiratory systems. At concentrations manyfold higher than the MIC, rapamycin had no effect on endogenous or exogenous respiration of *C. albicans* with glucose or other intermediates of the tricarboxylic acid cycle.

Rapamycin weakly inhibited incorporation of labeled leucine into cellular protein. It has no effect on protein synthesis by cell-free preparations of *C. albicans, E. coli* or rat liver and mitochondrial preparations of *C. albicans*. This indicated that the primary action of rapamycin on *C. albicans* is not on the synthesis of protein.

Rapamycin resembles azalomycin F[12] in inhibiting generally the substrate respiration of amino acids by *C. albicans*. The effect on amino acid metabolism was expressed as inhibition of oxidative deamination. Azalomycin, however, differs from rapamycin in that it (like polyenes) causes leakage of 260 and 280 nm absorbing materials from cells of *C. albicans* and also causes lysis of rabbit erythrocytes. Azalomycin F inhibited uptake of substrates especially some amino acid into cells, whereas rapamycin had no effect on the uptake of any of the radioactive precursors investigated.

Rapamycin inhibited incorporation of threonine into cell wall fraction of *C. albicans*. It inhibited incorporation of glucose into mannan by *C. albicans* (46% at 1 μg/ml); synthesis of glucan is only weakly affected. Aculeacin[24], a new antifungal antibiotic, has been reported to affect glucan synthesis but not mannan synthesis. Since the inhibition of threonine incorporation into cell wall and inhibition of glucan synthesis were observed at rapamycin concentrations much higher than its MIC, cell wall synthesis would not seem to be the primary site of action of rapamycin.

At low concentrations (0.02~0.1 μg/ml) rapamycin inhibited incorporation of adenine and phosphate into RNA and DNA, the inhibition of RNA being more pronounced. Relatively weaker effect of rapamycin on uridine incorporation into RNA is difficult to explain. The percent incorporation of uracil incorporation is low and that can partly explain the weak inhibition by rapamycin of its incorporation into RNA. Rapamycin at MIC levels also caused degradation of P^{32} labeled intracellular

macromolecules, presumably nucleic acids.

Rapamycin inhibited incorporation of acetate into lipids and in this respect resembled cerulenin[9]. However, the antifungal activity of cerulenin can be reversed by ergocalciferol. Rapamycin also showed inhibition of incorporation of methyl-C^{14}-methionine into total lipids, but the incorporation into the sterol fraction was not affected. Therefore, rapamycin is not (unlike sinefungin[25], a new antibiotic) a specific inhibitor of methyl transferase.

Mechanism of action of rapamycin appears to be different from many known antifungal antibiotics. In *C. albicans*, at concentrations approaching the MIC, rapamycin caused: (1) inhibition of nucleic acid synthesis; (2) degradation of P^{32}-labeled cellular macromolecules presumable nucleic acids; (3) inhibition of amino acid metabolism and (4) inhibition of lipid synthesis. Its effect on cell wall synthesis and protein synthesis was relatively weaker. Whether the lethal action of rapamycin on *C. albicans* is primarily due to one of the above effects or is the result of a combination of some of these biosynthetic parameters remains to be established.

Acknowledgement

We wish to thank Dr. S. N. SEHGAL for a generous supply of rapamycin, Mrs. A. SIDOROWICZ and Dr. H. BAKER for MIC determinations and Miss M. REEVES for secretarial assistance.

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