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Mode of Action of Lomofungin

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Lomofungin inhibited the growth of some yeasts and mycelial fungi at concentrations between 5 and 10 μg ml. At such concentrations, there was no decrease in endogenous and exogenous oxygen consumption, and even 50 μg of antibiotic per ml caused only slight decreases. The permeation of the cell membrane was changed so that leakage of ninhydrin-positive substances was reduced, and the uptake of ^{14}C-labeled glucose, amino acids, uracil, and thymidine was decreased at concentrations as low as 4 μg/ml. Protein synthesis in whole cells of *Saccharomyces cerevisiae* was reduced 35% at 10 μg/ml. However, the antibiotic did not reduce the incorporation of phenylalanine-*U*-^{14}C into polypeptides with cell-free systems of *Rhizoctonia solani* and *S. cerevisiae*. The synthesis of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) was inhibited even at concentrations of lomofungin of 4 μg/ml. Since RNA synthesis was inhibited at lower concentrations and earlier than DNA synthesis, the primary site of action of the antibiotic appears to be the synthesis of RNA.

Lomofungin is an antibiotic produced by *Streptomyces lomendensis* var. *lomendensis* (1). [This antibiotic was obtained through the courtesy of G. B. Whitfield of the Upjohn Co. and was formerly known as lomondomycin (M. E. Bergey, and L. E. Johnson, U.S. Patent No. 3,359,165, 1967)]. Its molecular weight is 314 and its empirical formula is $C_{15}H_{10}N_2O_6$. The compound is yellow and is decomposed at 320 C; it is soluble in dimethylformamide (727 mg/ml), water at *p*H 9 to 12 (10 mg/ml), and methanol, cyclohexane, and acetone (1 mg/ml). It is active against bacteria, yeast, and fungi.

MATERIALS AND METHODS

The microorganisms used in this study were obtained from the following sources: *Saccharomyces cerevisiae* G.M., Department of Microbiology of the University of Illinois; *S. fragilis* and *S. pastorianus*, Northern Regional Research Laboratory; and *Penicillium oxalicum*, Department of Plant Pathology of the University of Illinois. Cultures were maintained on potato dextrose agar and, for all experiments, were grown with gentle shaking at 26 C in medium GY (glucose, 10 g; yeast extract, 2 g; and distilled water, 1 liter). After sterilization of the medium for 20 min at 121 C, its *p*H was 6.5. Lomofungin solutions were prepared by dissolution of the antibiotic in water that had been adjusted with NaOH to *p*H 9 or 10. Once in solution, the antibiotic could be added to the medium and could remain dissolved at the *p*H of the medium.

To determine the growth inhibitory effect of the antibiotic, the three yeasts were grown in standard assay tubes containing 5 ml of GY medium. The tubes were inoculated with 0.5 ml of a 10-hr culture, and different concentrations of the antibiotic were added immediately after. In other experiments, the yeasts were first allowed to grow for 10 hr and the antibiotic was then added. The optical density (OD) readings for growth determinations were made at 650 nm every 2 hr. *P. oxalicum* was grown in 125-ml Erlenmeyer flasks containing 40 ml of GY medium. These flasks were inoculated with 0.1 ml of a spore suspension made from a 7-day-old agar culture. The lomofungin was added either immediately after inoculation or 2 days after inoculation. Growth was determined on 1- to 15-day-old cultures by drying the mycelia to a constant weight at 80 C in a vacuum oven. Inocula of *S. cerevisiae* were prepared by first growing the yeast in assay tubes containing 5 ml of GY medium to an OD of 0.25 at 650 nm. This culture was added to 500-ml Erlenmeyer flasks containing 100 ml of the medium, and the yeasts were grown for 12 hr with shaking at 26 C. Under these conditions, almost the same yield of yeast cells was obtained from each flask in each experiment.

Studies on respiration were carried out at 26 C by using standard manometric techniques (11). The flasks contained a total of 3.2 ml of the fluid; the body of the cell held 1 ml of 0.2 M glucose and 0.3 ml of 0.1 M magnesium chloride. A suspension of the yeast containing 5 mg (dry weight) of cells was added to the side arm. An 0.2-ml amount of 20% potassium hydroxide was placed into the center well. Lomofungin was used in amounts appropriate to give the desired concentrations, and then distilled water was used to bring all flasks to the same volume.

Since the antibiotic produced a strong red color in

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medium, the uptake of the antibiotic by the cells was determined by measuring the disappearance of color from the medium. The lomofungin was added to 12-hr cultures of *S. cerevisiae*, and, after 4 hr, the cultures were centrifuged and the optical density of the medium was measured at 550 nm. Control flasks contained only medium and lomofungin but no yeast. For measuring the uptake by dead cells, the yeast cultures were kept in boiling water for 10 min before the addition of the antibiotic. Studies on the leakage of ninhydrin-positive metabolites were done on centrifuged cells that had been washed four times with water (9). Portions of cells were suspended in 100 ml of sterile distilled water containing the antibiotic.

The uptake of compounds from the medium was determined by adding radioactive compounds to the growth medium. Twelve-hour-old cells were centrifuged and suspended in 100 ml of fresh medium, and one of the following was added: amino acid-*U*-^{14}C mixture, 3.3 μg (5 μc); uracil-2-^{14}C, 34 μg (2 μc); thymidine-*methyl*-^{3}H, 0.9 μg (4 μc). Lomofungin was added to the medium immediately after the radioactive materials. Samples were taken at different time intervals and centrifuged. An 0.5-ml amount of the supernatant liquid was added to counting vials, and then 15 ml of dioxane scintillation solution [dioxane, 1,250 ml; ethyl cellosolve, 250 ml; 2,5-diphenyloxazole (PPO), 15 g; 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP), 0.75 g; naphthalene, 75 g.] was also added. Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer.

To determine the effect of the antibiotic on the synthesis of protein, ribonucleic acid (RNA), and deoxyribonucleic (DNA), 12-hr-old cells were centrifuged and suspended in 100 ml of fresh medium. A 5-μc amount of ^{14}C-amino acid mixture was added for experiments on protein synthesis, 2 μc of ^{14}C-uracil was added for RNA synthesis, and 0.9 μc of thymidine-*methyl*-^{3}H was added for DNA synthesis. The antibiotic was added immediately after the radioactive material, and the cells were incubated for various periods of time. They were harvested by centrifugation, washed three times with distilled water, and fractionated in the following manner. The yeast was suspended in 10% trichloroacetic acid for 2 hr at 4 C and centrifuged; the pellet was placed in ethyl alcohol-ether (3:1) for 30 min at room temperature. The suspension was then centrifuged, and the pellet was resuspended in acetone-ether (1:1) for 30 min. This was followed by a centrifugation and suspension in ether for 30 min, centrifugation and suspension in 10% trichloroacetic acid for 2 hr at 90 C, and then a final centrifugation. The supernatant fraction, or the hot trichloroacetic acid-soluble fraction, was used to determine the incorporation of ^{14}C-uracil and thymidine-*methyl*-^{3}H into RNA and DNA, respectively. The pellet was hydrolyzed with 6 N HCl for 4 hr at 150 C. After centrifugation, the supernatant portion was used to determine the incorporation of ^{14}C-amino acid mixture into protein. Both the hot trichloroacetic acid fraction and the protein fraction were neutralized; 0.5 ml was added to the counting vials with 15 ml of dioxane scintillation solution and counted in a liquid scintillation counter. RNA was determined separately by the orcinol test (8) with yeast RNA as a standard. DNA was measured by the diphenylamine test (3) with salmon sperm DNA as a standard. Protein was determined by the ninhydrin method with casein hydrolysate as standard.

For protein synthesis in cell-free systems from *S. cerevisiae*, the inoculum was grown in tubes for only 6 hr and then in the flasks for another 6 hr. The cells were harvested by centrifugation, washed three times with distilled water, and suspended at a ratio of 1:1 (w/v) in buffer A [tris(hydroxymethyl)aminomethane (Tris), *p*H 7.8, 0.2 M; sucrose, 0.4 M; magnesium acetate, 0.01 M; potassium chloride, 0.06 M; mercaptoethanol, 0.02 M; reduced glutathione, 0.003 M; spermine, 40 μg/ml]. The cells were broken in a French pressure cell (18,000 to 22,000 lb/in^2) at 0 C. Whole cells and cellular debris were removed by centrifugation at 20,000 $\times$ *g* for 30 min, and the liquid portion was recentrifuged at 105,000 $\times$ *g* for 3 hr in a Beckman L2-65 ultracentrifuge at 4 C. The resulting supernatant fluid (S-105) was passed through a Sephadex G-25 column previously equilibrated with buffer B (Tris, *p*H 7.8, 0.02 M; magnesium acetate, 0.01 M; potassium chloride, 0.06 M; mercaptoethanol, 0.02 M; reduced glutathionine, 0.003 M; spermine, 40 μg/ml; ethylenediaminetetraacetic acid, 10^{-4} M). The ribosomes (P-105) were suspended in buffer B and centrifuged at 10,000 $\times$ *g* for 10 min, and the supernatant fluid was centrifuged at 105,000 $\times$ *g* for 150 min; these ribosomes were suspended in a small volume of buffer B. Ribosomes and supernatant fluid were kept in liquid nitrogen until used. The protein in the supernatant fluid was determined by the method of Lowry et al. (7). RNA in the ribosomes was calculated on the basis of 1 mg of ribosomal RNA per ml having an optical density at 260 nm of 25.

The following incubation mixture (0.5 ml) was used for protein synthesis: Tris buffer (*p*H 7.8), 50 μmoles; reduced glutathione, 0.5 μmole; magnesium acetate, 5 μmoles; ammonium chloride, 25 μmoles; mercaptoethanol, 7.5 μmoles; pyruvate kinase, 10 μmoles; adenosine triphosphate, 1 μmole; phosphoenolpyruvate, 2.5 μmoles; guanosine triphosphate, 0.1 μmole; spermine, 20 μg; phenylalanine-*U*-^{14}C, 0.3 μc; each of the remaining 19 (unlabeled) amino acids, 0.005 μmole; yeast soluble RNA, 200 μg; polyuridylic acid, 60 μg; P-105, 200 μg; and S-105, 300 μg. The reaction mixtures were incubated in glass tubes on a Dubnoff incubator at 25 C for 45 min with gentle shaking. The reaction was stopped by the addition of trichloroacetic acid to a final concentration of 8%. After heating in boiling water for 10 min, the tubes were cooled in an ice bath, filtered through a cellulose acetate type HA filter (Millipore Corp., Bedford, Mass.) with 0.45-μm pores, and washed three times with 7% trichloroacetic acid. The filters were dried, placed in counting vials containing 15 ml of counting solution (PPO, 5 g; POPOP, 0.3 g; toluene, 1 liter), and counted.

RESULTS

Growth of fungi was readily inhibited by lomofungin, although the concentrations re-

quired varied with the species. The yeasts *S. cerevisiae*, *S. fragilis*, and *S. pastorianus* were inhibited 90 and 100% by 5 and 10 μg of antibiotic per ml, respectively. *P. oxalicum* was inhibited only 60% by the lower concentration but almost completely by the higher concentration. The effect of the antibiotic was very rapid, and growth was prevented even when the lomofungin was added to cultures of *S. cerevisiae* in the middle of the log phase of growth (Fig. 1).

This cessation of growth was not caused by a decrease in aerobic respiration since neither endogenous nor exogenous oxygen consumption was greatly reduced at concentrations that completely prevented growth (Fig. 2). There was no apparent reduction in exogenous respiration of *S. cerevisiae* at 5 or 10 μg/ml and only a slight reduction at 50 μg/ml. Endogenous respiration was similarly resistant to lomofungin.

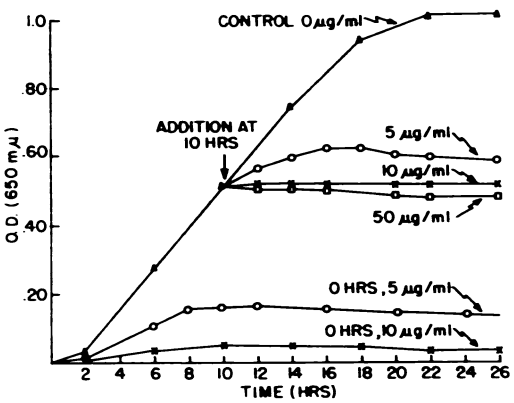

FIG. 1. *Effect of lomofungin on the growth of S. cerevisiae.*

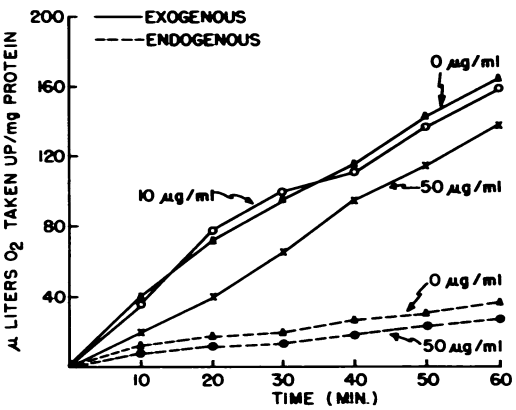

FIG. 2. *Effect of lomofungin on respiration of S. cerevisiae.*

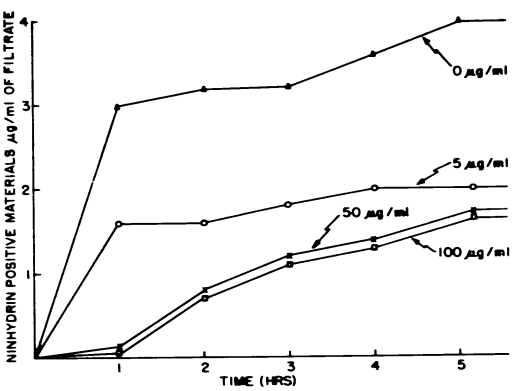

FIG. 3. *Effect of lomofungin on leakage of amino acids from S. cerevisiae.*

The antibiotic affected the permeability properties of the cell in two ways. First, it decreased the leakage of ninhydrin-positive substances from the yeast. A 5-μg amount of lomofungin per ml reduced the normal leakage 50%, but higher concentrations did not reduce it much more and merely delayed the time at which leakage occurred (Fig. 3). The second effect was to reduce the uptake of materials from the medium (Table 1). For labeled amino acids, glucose, uracil, and thymidine, there was a decreased uptake of these compounds from the medium as the antibiotic concentration was increased. The uptake of metabolites from the medium decreased in the presence of lomofungin as the time of incubation increased. The uptake of amino acids and glucose was less sensitive than that of uracil or thymidine at all concentrations of the antibiotic. Thymidine uptake was the most sensitive, and, after 10 min of incubation, the inhibition was 70, 80, 90, and 95% at concentrations of lomofungin of 4, 10, 20 and 50 μg/ml, respectively. Even the lowest concentration, 4 μg/ml, inhibited the uptake of thymidine 90% within 30 min. Uracil uptake also was markedly inhibited, 38 and 55% at concentrations of 4 and 10 μg, respectively, and higher concentrations of antibiotic resulted in even greater inhibition.

Lomofungin caused a reduction in protein synthesis in *S. cerevisiae* as measured by the incorporation of ^{14}C-labeled mixtures of amino acids (Table 2). At concentrations of antibiotic that were minimal for preventing growth, 10 μg/ml for 60 min, the inhibition was 35% but at higher levels even reached 60%. At levels above 10 μg/ml, there was no increase in the per cent inhibition with longer incubation times.

The antibiotic, however, had no inhibitory effect on the incorporation of ^{14}C-phenylalanine

TABLE 1. *Effect of lomofungin on the uptake of substances from the medium by S. cerevisiae*

Concn (μg/ml)	Per cent inhibition of uptake															
	10 min				20 min				30 min				60 min			
	AA[a]	GL	UR	TH	AA	GL	UR	TH	AA	GL	UR	TH	AA	GL	UR	TH
4	0	0	24	70	0	0	36	85	16	10	38	90	48	30	52	90
10	18	16	59	80	24	20	52	90	19	26	55	90	50	45	75	90
20	30	28	50	90	43	35	55	95	50	43	72	95	58	63	77	95
50	30	39	75	95	49	48	75	95	60	54	85	95	72	70	90	95

[a] AA, amino acids; GL, glucose; UR, uracil; TH, thymidine.

TABLE 2. *Effect of lomofungin on protein synthesis[a] in whole cells of S. cerevisiae*

Concn (μg/ml)	Incubation time (min)			
	10	20	30	60
0	18,366[b]	23,640	74,053	71,160
4	21,547[c]	26,296[c]	64,885	58,444
10	13,834	16,563	51,850	46,151
20	9,401	11,960	32,476	34,089
50	7,741	9,203	27,122	28,404

[a] A mixture of ^{14}C-labeled amino acids was used as precursors for protein synthesis.

[b] Data expressed as disintegrations per minute per milligram of protein.

[c] Increased incorporation.

TABLE 3. *Effect of lomofungin on protein synthesis in cell-free systems of S. cerevisiae*

Incubation system[a]	Counts/min	Inhibition
		%
Complete, 45 min	27,185	
Complete, 0 min	103	
+Puromycin, 100 μg/ml	16,260	40
+Streptomycin, 100 μg/ml	32,015	0
+Lomofungin, 50 μg/ml	27,200	0
+Lomofungin, 100 μg/ml	26,980	0
+Ribonuclease	813	97
−Polyuridylic acid	904	96
−S-105	976	95
−R-105	3,354	88
−Energy	102	99

[a] S-105, supernatant fluid; P105 ribosome, pellet after centrifugation at 105,000 × *g*.

into protein by cell-free systems of *Rhizoctonia solani* or *S. cerevisiae*. The data for the *S. cerevisiae* system are given in Table 3. Lomofungin concentrations of 100 μg/ml failed to decrease the incorporation of the amino acid, although under the same conditions puromycin inhibited it 40%. That the cell-free system was operating normally was also shown by the results obtained when various components were omitted; under such conditions, there was almost no significant radioactivity in the protein fraction.

The synthesis of DNA and RNA by *S. cerevisiae* was readily inhibited by the antibiotic. The incorporation of thymidine-*methyl*-^{3}H was inhibited 65% by 4 μg of lomofungin per ml at the end of 60 min and even more at higher concentrations (Table 4). At the same time, the incorporation of ^{14}C-uracil was inhibited 82% by 4 μg/ml. The greater inhibition of RNA synthesis occurred at all incubation periods from 10 to 60 min. At 10 min, for example, DNA was inhibited 40% and RNA was inhibited 76%. At 50 μg of antibiotic per ml, RNA inhibition after 1 hr was 93%. The inhibitory effects of very low concentrations of lomofungin indicated the difference in the sensitivity between these two nucleic acid-synthesizing systems even more clearly (Table 5). At 10 min, RNA synthesis was reduced 38% at 1 μg/ml, whereas DNA synthesis was inhibited at 3 μg/ml and then only by 16%. At 4 μg/ml, the reduction in the synthesis of RNA and DNA was 78 and 35%, respectively.

The removal of lomofungin from the medium by *S. cerevisiae* did not appear to be a vital phenomenon because similar amounts per unit weight of cells were absorbed by living and dead cells (Table 6). The greater the concentration of antibiotic that was present in the suspension, the more antibiotic that was absorbed. However, the per cent uptake decreased with increasing concentration of antibiotic.

DISCUSSION

The primary action of lomofungin is probably on the synthesis of RNA since RNA formation was inhibited at lower concentrations than DNA, and, when equal concentrations of antibiotic were used, a greater inhibition of RNA than DNA occurred. In these respects, the action of lomofungin resembles that of daunamycin (1)

TABLE 4. *Effect of lomofungin on DNA and RNA synthesis[a] by S. cerevisiae*

Concn (μg/ml)	DNA or RNA at various incubation times (disintegrations per min per mg)							
	10 min		20 min		30 min		60 min	
	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA
0	4,540	33,278	6,760	47,444	11,909	56,833	25,828	66,368
4	2,724	8,143	3,216	11,750	4,760	11,200	9,030	13,785
10	1,980	6,612	2,345	9,432	4,738	8,402	7,740	9,974
20	1,665	6,641	2,144	7,991	3,332	7,288	6,450	6,628
50	1,575	5,280	2,278	7,069	2,618	7,200	5,160	4,624

[a] Thymidine-*methyl*-^{3}H was the precursor for DNA, and uracil-2-^{14}C was the precursor for RNA.

TABLE 5. *Effect of lomofungin, after incubation for 10 min, on nucleic acid synthesis[a] in S. cerevisiae*

Concn (μg/ml)	Disintegrations per min per mg of RNA	Inhibition	Disintegrations per min per mg of DNA	Inhibition
		%		%
0	30,361		4,852	
1	18,816	38	4,831	0
2	10,019	67	5,003	0
3	8,197	73	4,075	16
4	6,679	78	2,753	35

[a] Uracil-2-^{14}C and thymidine-*methyl*-^{3}H were precursors for RNA and DNA, respectively.

TABLE 6. *Uptake of lomofungin by cells of S. cerevisiae*

Concn (μg/ml)	Per cent uptake	
	Living cells	Dead cells
5	80	75
10	65	65
20	68	60
50	50	45

and nogalomycin (2) which inhibit both DNA and RNA synthesis but differs from that of chromomycin A_3 (12) and olivomycin (4) which do not inhibit DNA synthesis. The action of lomofungin also differs from those antibiotics that are primarily active in DNA complexing (5). However, one cannot yet entirely rule out a direct interference by lomofungin with DNA synthesis. An inhibition of RNA formation may be caused either by an interference with the RNA polymerase system, as in the case of olivomycin and pluramycin A (10), or by combining with RNA directly. The inhibition of both RNA and of DNA, such as occurred with lomofungin, may be caused by a reaction of the antibiotic with template DNA which would block the replication processes, but this is unlikely because the synthesis of RNA was more sensitive than the synthesis of DNA.

The reduction in protein synthesis from amino acids does not seem to be a direct effect of lomofungin. The amount of inhibition always was less than that for RNA and occurred later during the incubation period. Furthermore, cell-free protein synthesizing systems from two fungi were not affected by the antibiotic. In whole cells, the reduced incorporation of phenylalanine into protein could be ascribed to a deficiency in the RNA that is needed for protein synthesis.

No evidence is available to explain the effect of lomofungin on the permeability characteristics of the cell. It is not a general breakdown of these membrane systems, such as occurs with polyene antibiotics (6), since the effect was specific and the uptake of uracil and thymidine was inhibited much more rapidly than that of amino acids or glucose. Nor was this breakdown in mechanism for the uptake of nutrients by yeast cells caused by a deficiency in the general respiratory energy-generating systems, because, at growth inhibitory concentrations, there was no significant reduction of aerobic or anaerobic respiration. One cannot rule out the possibility that the inhibition of RNA or DNA might also account for change in permeability. Nucleic acids have been found in the cell membranes of yeast and might be needed for the synthesis of the protein components of these membranes; however, any relationship between the presence of nucleic acids and the permeability properties of the cell has not been investigated.

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