ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1973, p. 723-728

Vol. 3, No. 6
Printed in U.S.A.

Lomofungin, an Inhibitor of Deoxyribonucleic Acid-Dependent Ribonucleic Acid Polymerases

F. R. CANO, S.-C. KUO, AND J. O. LAMPEN

Institute of Microbiology, Rutgers University, The State University of New Jersey, New Brunswick, New Jersey 08903

Received for publication 5 January 1973

Lomofungin, an antibiotic active against fungi, yeasts, and bacteria, was found to be a potent inhibitor of purified *Escherichia coli* deoxyribonucleic acid (DNA)-dependent ribonucleic acid (RNA) polymerase. It prevents RNA synthesis by a direct interaction with the polymerase and not with the template or substrate; chain elongation is halted promptly. Three DNA-dependent RNA polymerases isolated from *Saccharomyces* strain 1016 were also sensitive to the antibiotic. Lomofungin does not appear to react generally with proteins, as bovine serum albumin did not prevent the inhibitory effect and numerous other enzymes were not affected by lomofungin.

Lomofungin has a broad antibacterial and antifungal spectrum, being active against gram-positive and gram-negative bacteria, yeasts, and other fungi (10). The only published information on its mode of action was obtained by Gottlieb and Nicolas (8), who studied its effect on *Saccharomyces cerevisiae* and suggested that the primary site of action is the synthesis of ribonucleic acid (RNA). In the accompanying paper (11) we showed that lomofungin markedly and rapidly inhibits RNA synthesis by yeast protoplasts. This inhibition occurs prior to any substantial reduction in protein synthesis.

To clarify the mode of action of lomofungin and determine its value as a tool for study of the role of RNA synthesis in the control of enzyme synthesis and secretion by yeast, we tested the antibiotic against isolated deoxyribonucleic acid (DNA)-dependent RNA polymerases (nucleoside triphosphate; RNA nucleotidyl transferase, EC 2.7.7.6). This communication demonstrates the inhibition by lomofungin of DNA-dependent RNA polymerases from both *Escherichia coli* and *Saccharomyces* strain 1016.

MATERIALS AND METHODS

Preparation of yeast cellular extract. *Saccharomyces* strain 1016 and the general growth conditions used are described in the accompanying paper (11). Cells were transferred from a stock culture to 200 ml of 0.3% Difco yeast extract-0.5% Bactopeptone-0.2% glucose medium, pH 6.8, and incubated for 15 h at 30 C on a reciprocating shaker. The culture was then added to 2 liters of the same medium. After 4 to 5 h of incubation, approximately 8 g (wet weight) of exponential-phase cells was harvested by centrifugation, washed in 30 ml of 50 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.95, 5 mM $MgCl_2$, 0.1 mM disodium ethylenediaminetetraacetate (EDTA), 0.5 mM dithioerythritol, 15% glycerol, and 1 mM phenylmethylsulfonylfluoride, and resuspended in 10 ml of the same buffer. The remainder of the procedure closely resembled that of Ponta, Ponta, and Wintersberger (18). The cells were disrupted in a Braun homogenizer and sonically treated for two 15-s intervals in an MSE model 3000 Sonifier at maximal power. Ammonium sulfate was added to a concentration of 0.3 M and sonic treatment was repeated. After 10 ml of the same buffer had been added, the sample was centrifuged for 20 min at 39,000 × *g*. The supernatant fluid was then centrifuged for 60 min at 150,000 × *g*. RNA polymerase, still in solution, was precipitated by ammonium sulfate at a final concentration of 3.2 M. The precipitate was collected by centrifugation for 30 min at 39,000 × *g* and was dissolved in 5 ml of TGMED buffer (50 mM Tris-hydrochloride, pH 7.95, 25% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM dithioerythritol, and 1 mM phenylmethylsulfonylfluoride) containing 0.05 M $(NH_4)_2SO_4$. The solution was dialyzed for 4 h against 1 liter of the same buffer at 4 C. Glycerol was then added to 50% and the extract was stored at −20 C.

Separation of the yeast RNA polymerases. The RNA polymerases were separated on a diethylaminoethyl (DEAE)-cellulose column (2 by 20 cm; Whatman DE-52) equilibrated in TGMED buffer containing 0.05 M $(NH_4)_2SO_4$. The sample (55 mg of protein [15]) was applied, and the column was washed with 20 ml of TGMED buffer containing 0.05 M $(NH_4)_2SO_4$ and then with 20 ml of TGMED buffer containing 0.1 M $(NH_4)_2SO_4$. A linear gradient was then applied (160 ml) ranging from 0.1 to 0.5 M $(NH_4)_2SO_4$ in TGMED buffer.

Enzyme assays. The assay conditions for yeast RNA polymerase were adapted from Ponta et al. (18). The standard mixture contained in 0.125 ml: 50 mM Tris-hydrochloride, pH 7.95, 1 mM EDTA, 1 mM $MgCl_2$, 0.5 mM $MnCl_2$, 0.1 mM 2-mercaptoethanol, 0.1 mM each of adenosine triphosphate (ATP), cytidine triphosphate (CTP), and guanosine triphosphate (GTP), 0.025 mM ^{3}H-uridine triphosphate (UTP; 5 μCi), 12.5 μg of denatured calf thymus DNA, and 25 μliters of sample to start the reaction. After 30 min at 37 C, the reaction was stopped with 1 ml of ice-cold 10% trichloroacetic acid containing 0.04 M sodium pyrophosphate (2). Bovine serum albumin (100 μg/tube) was added as carrier, and the precipitate was collected on Whatman glass-fiber paper (2.4 cm; GF/A). The precipitate was washed four times with 10% trichloroacetic acid containing 0.04 M sodium pyrophosphate and three times with 70% ethanol. The filters were transferred to scintillation vials containing 0.5 ml of distilled water and 10 ml of Aquasol (New England Nuclear Corp.), and radioactivity was determined with a Packard Tri-Carb liquid scintillation counter.

E. coli DNA-dependent RNA polymerase activity was measured in a final volume of 0.15 ml containing 50 mM Tris-hydrochloride, pH 7.95; 1.5 mM 2-mercaptoethanol; 1.0 mM $MgCl_2$; 1.5 mM $MnCl_2$; 0.1 mM ATP, GTP, and CTP; 0.025 mM ^{3}H-UTP (0.19 μCi); and 12.5 μg of native calf thymus DNA as template, with or without antibiotic. The reaction was started by adding the partially purified RNA polymerase (2.6 μg of protein). Incubations were carried out at 37 C for the times indicated. The reaction was stopped with 1 ml of ice-cold 10% trichloroacetic acid-40 mM sodium pyrophosphate, and the radioactivity in the trichloroacetic acid-insoluble polynucleotides was measured.

Previously described methods were used for invertase (6) and for α-glucosidase (12). Acid and alkaline phosphatases were assayed with *p*-nitrophenylphosphate as substrate as described by Torriani (22) and modified by Kuo and Lampen (13). Hexokinase was determined in a coupled enzyme assay by the conversion of glucose to glucose-6-phosphate, which was measured by the reduction of nicotinamide adenine dinucleotide phosphate in the presence of glucose-6-phosphate dehydrogenase (5).

Chemicals. Lomofungin was the gift of George B. Whitfield of The Upjohn Company, Kalamazoo, Mich. α-Amanitin was donated by David H. L. Bishop of this Institute. Rifampin, phenylmethylsulfonylfluoride, and calf thymus DNA were purchased from Calbiochem.

E. coli RNA polymerase (400 units/mg of protein) purified according to Burgess (3) was generously supplied by Francis Persico of this Institute. Peroxidase was a product of Boehringer, and all other enzymes were purchased from Sigma Chemical Co.

RESULTS

Effect of lomofungin on E. coli DNA-dependent RNA polymerase. Lomofungin rapidly inhibits purified *E. coli* DNA-dependent RNA polymerase (Table 1). The effect is concentration-dependent; approximately 24% inhibition occurred at 1 μg/ml over the time intervals indicated, and concentrations greater than 20 μg/ml virtually eliminated enzyme activity (data not shown). The other antibiotics tested gave results in accord with their known modes of action. Rifampin inhibits bacterial RNA polymerases (9, 23) whereas α-amanitin is mainly effective in eukaryotic systems (16, 24). Cycloheximide, which inhibits protein synthesis in eukaryotes, was without effect (14).

Rifampin inhibits RNA synthesis by binding to the polymerase protein (9, 23), whereas antibiotics such as actinomycin D and chromomycin act by complexing with the DNA template (19, 22). To determine which component of the enzyme system was affected by lomofungin, the drug was incubated with various concentrations of the polymerase or of DNA before the other component was added to start the reaction. Incubating the DNA and lomofungin together for 5 min before adding the polymerase did not affect the subsequent response to suboptimal levels of DNA (Fig. 1A). In contrast, when lomofungin and increasing levels of the enzyme preparation were incubated together and the DNA was then added, no polymerase activity was subsequently detected until its level exceeded 0.65 μg (Fig. 1B); additional amounts showed essentially unimpaired activity. Thus, lomofungin at 20 μg per ml (as in Fig. 1) appeared to inhibit completely about 0.65 μg of the polymerase preparation. The shape of the inhibition curve (Fig. 1B) suggests that there is a very sharp saturation to the inhibitory effect with a large degree of cooperativity, but that this is not certain in light of the fact that the antibiotic probably exists as relatively large

TABLE 1. *Inhibitory effect of various antibiotics on E. coli RNA polymerase*

Antibiotic	Percent inhibition at		
	2.5 min	5 min	10 min
None	0[a]	0	0
Lomofungin			
1 μg/ml	17	31	24
5 μg/ml	45	35	45
10 μg/ml	43	56	60
20 μg/ml	70	66	75
α-Amanitin, 50 μg/ml	0	0	3
Cycloheximide, 50 μg/ml	0	11	6
Rifampin, 50 μg/ml	98	99	99

[a] Actual values were 4,076, 7,175, and 14,257 counts/min for 2.5-, 5-, and 10-min incubations, respectively.

aggregates under the usual experimental conditions. In other experiments (data not shown), the degree of inhibition was not altered by increasing the concentrations of the nucleoside triphosphates. We conclude, therefore, that lomofungin prevents RNA synthesis by a direct interaction with the enzyme, not with the polynucleotide template or substrates. This conclusion is supported by the observation that lomofungin does not prevent in vitro synthesis of protein from messenger RNA (8).

To determine whether lomofungin prevents chain initiation or elongation, we examined the kinetics of its action on *E. coli* RNA polymerase (Fig. 2). The addition of lomofungin, either at the onset of the reaction or after polynucleotide synthesis had begun, produced an immediate cessation of polynucleotide formation. Rifampin did completely prevent polynucleotide formation when added at the same time as the DNA template—but, if addition was delayed for 5 min, during which time a number of nucleotide chains had been initiated, rifampin produced only a gradual decline in activity. Thus, lomofungin definitely halts chain elongation, whereas rifampin inhibits initiation, but not elongation.

Effect of lomofungin on yeast DNA-dependent RNA polymerases. The scheme employed for isolation of the enzymes was adapted from that of Ponta et al. (18), who demonstrated their isolated polymerases to be nuclear in origin and not mitochondrial. Figure 3 shows the chromatographic separation on DEAE-cellulose of the RNA polymerases obtained from *Saccharomyces* strain 1016. Fractions representing the three peaks were pooled and tested for enzymatic activity in the presence of lomofungin (Table 2).

Each of the polymerase peaks was almost totally inhibited by lomofungin at 20 μg per ml. α-Amanitin inhibited both of the polymerase peaks tested (B and C), with peak C being the more sensitive. The effect of rifampin on peak A, though unexpected, was not without precedent, since one species of polymerase isolated from coconut nuclei chromatin has been reported to be inhibited by a high concentration of this drug (17).

Specificity of lomofungin. The possible inhibition of a variety of enzymes by lomofungin was tested as a criterion of its specificity for DNA-dependent RNA polymerase (Table 3). The only enzyme inhibited was α-glucosidase, and full activity of this enzyme was regained after dialysis. Also, addition of bovine serum albumin at 100 μg/ml did not prevent inhibition of the *E. coli* RNA polymerase (results not shown). These limited observations demonstrate that lomofungin is not a general protein reactant, although it is not entirely specific for RNA polymerases.

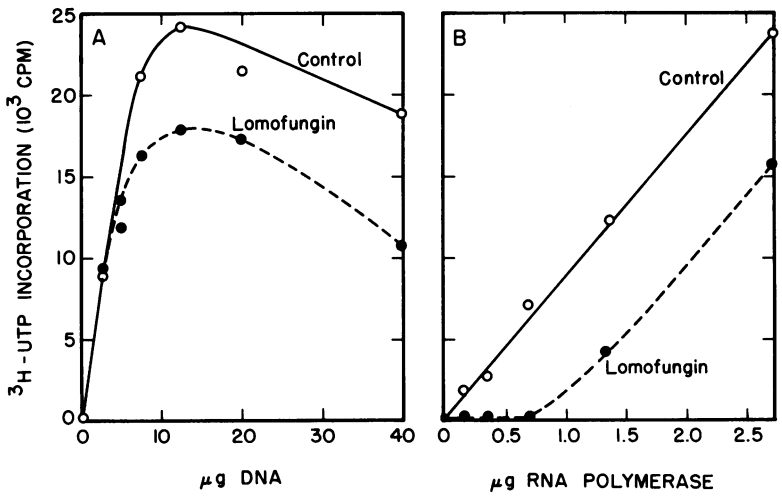

FIG. 1. *Enzyme activity as a function of concentration of DNA template and RNA polymerase, and the effect of preincubation with lomofungin. (A) The indicated amount of calf thymus DNA and the usual components of the reaction mixture were incubated for 5 min with lomofungin (20 μg/ml) or with no antibiotic, and the reaction was then started by the addition of E. coli RNA polymerase (2.6 μg of protein). (B) As A except that the indicated level of enzyme preparation was present during the 5-min incubation with lomofungin or with no antibiotic, and the reaction was started by the addition of 12.5 μg of DNA. The assay procedure is described in Materials and Methods. The reaction time was 10 min at 37 C.*

DISCUSSION

It is clear from our results that lomofungin inhibits the DNA-dependent RNA polymerases of *E. coli* and *Saccharomyces* strain 1016. The action of lomofungin on *E. coli* RNA polymerase resembles that of rifampin in that it prevents synthesis of RNA by interacting with the polymerase, but differs from that of actinomycin D which binds with the DNA template (22, 23). Both lomofungin and rifampin completely prevented polynucleotide formation when added to the polymerase system simultaneously with the DNA template; however, only lomofungin halted chain elongation when added after polynucleotide synthesis had begun.

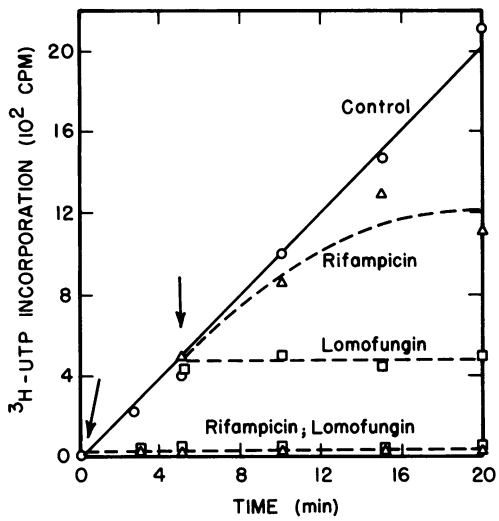

FIG. 2. *Kinetics of antibiotic inhibition of E. coli RNA polymerase. Assay conditions as described in Materials and Methods. Lomofungin and rifampin, when added (arrow), were at 20 μg/ml.*

Lomofungin was also effective against the RNA polymerases isolated and partially purified from *Saccharomyces* strain 1016. Three discrete polymerase peaks were obtained, all of which were inhibited by lomofungin (20 μg/ml). α-Amanitin inhibited peaks B and C (A not tested), and rifampin inhibited only peak A.

Polymerases A, B, and C of Ponta et al. (18) are usually termed I, II, and III, respectively, by other laboratories (see Adam, Schultz and Hall [1]). Since we did not find a polymerase peak eluting at a concentration of ammonium sulfate greater than 0.3 M (like polymerase C of Ponta et al. [18]) and this amanitin-insensitive enzyme is frequently not observed (5, J. Sebastian, M. M. Bhargaua, and H. O. Halvorson, Abstr. Annu. Meeting, Amer. Soc. Microbiol., p. 162, 1972), our amanitin-sensitive peak C is probably polymerase B (or II). Our peak B might then be the amanitin-insensitive polymerase A of Ponta et al. (18) and polymerase IB of

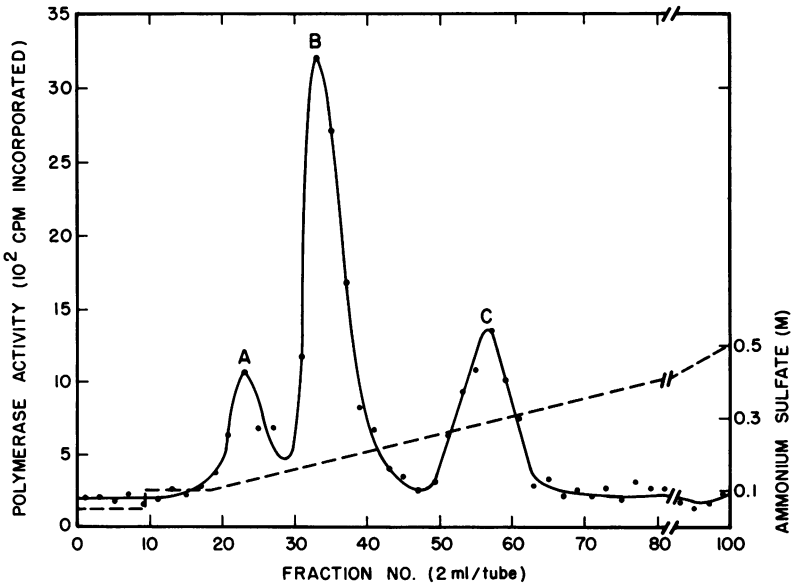

FIG. 3. *Separation of yeast RNA polymerases. Cells of strain 1016 were disrupted in a Braun homogenizer, and the extract was chromatographed on DEAE-cellulose according to Roeder and Rutter (20). Fractions (25 μliters) were assayed (18) with denatured calf thymus DNA as template and ^{3}H-UTP as the labeled trinucleotide. Both Mn^{2+} and Mg^{2+} were present.*

TABLE 2. *Effect of antibiotics on yeast RNA polymerases*

Antibiotic[a]	RNA polymerase peaks[b]		
	A	B	C
None	0[c]	0	0
α-Amanitin	—	31	80
Rifampin	99	0	0
Lomofungin	100	96	92

[a] Each antibiotic was tested at 20 μg/ml.

[b] Peak A represents fractions 22-24; B, fractions 32-34; and C, fractions 56-58 from Fig. 3.

[c] Percent inhibition. Actual values in counts per minute are 333 for A, 995 for B, and 846 for C.

TABLE 3. *Effect of lomofungin on various enzymes*

Enzyme[a]	Source	Effect[b]
External invertase	Yeast	None
Internal invertase	Yeast	None
Alkaline phosphatase	*E. coli*	None
Acid phosphatase	Wheat germ	None
Hexokinase	Yeast	None
α-Glucosidase	Yeast	Reversibly inhibited[c]
Glucose-6-phosphate dehydrogenase	Yeast	None
Glucose oxidase	*Aspergillus niger*	None
Peroxidase	Horse radish	None

[a] All enzymes were the purest available samples except for α-glucosidase which was tested as a yeast protoplast lysate.

[b] Concentrations of lomofungin were 1 and 10 μg/ml.

[c] Approximately 50% inhibition with 10 μg of lomofungin/ml.

Adam et al. (1) with some contamination by polymerase from the sensitive peak C. Our rifampin-sensitive peak A is unlike polymerase IA of Adam et al. (1), which was insensitive to rifampicin SV. This peak may well be of extranuclear origin.

Despite the uncertainties concerning the identification of the several RNA polymerase peaks obtained from *Saccharomyces* strain 1016, these initial studies indicate clearly the sensitivity of the enzymes to lomofungin. When viewed in conjunction with the high sensitivity of RNA synthesis in yeast cells and protoplasts (8, 11), these results provide evidence that lomofungin is an effective inhibitor of yeast RNA polymerase both in vivo and in vitro. The antibiotic should be useful for examining the mechanisms for regulation of enzyme synthesis by yeast.

ACKNOWLEDGMENTS

We thank Kathleen L. Meszaros for excellent technical assistance.

This work was supported by Public Health Service grant AI-04572 from the National Institute of Allergy and Infectious Diseases.

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