CHELATION OF DIVALENT CATIONS BY LOMOFUNGIN: ROLE IN INHIBITION OF NUCLEIC ACID SYNTHESIS

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Summary: Lomofungin inhibition of yeast growth and RNA synthesis is prevented by Cu^{++} or Zn^{++} ions which chelate with the antibiotic and prevent its uptake by the cells. EDTA potentiates the inhibition. Mg^{++} ions do not protect in vivo or against the inhibition of purified bacterial RNA and DNA polymerases. Lomofungin prevents formation of the RNA polymerase. DNA initiation complex, probably by chelation with the firmly bound Zn^{++} of the enzyme.

The antibiotic lomofungin (1-carboxymethoxy-5-formyl-4, 6, 8-trihydroxyphenazine) rapidly inhibits synthesis of RNA but not of protein in yeast cells (1, 2, 3, 4). Under the conditions used, formation of messenger RNA and ribosomal RNA was preferentially affected while synthesis of transfer RNA continued (2, 3). Tests with the DNA-directed RNA polymerase of Escherichia coli indicated that lomofungin may bind to the polymerase rather than to the template (5), and the antibiotic seemed to prevent chain elongation.

We report here that lomofungin forms chelate complexes with Cu^{++} and Zn^{++} and that these ions prevent (and EDTA enhances) the inhibition of RNA synthesis. This led us to re-examine the in vitro sensitivity to lomofungin of bacterial DNA and RNA polymerases. These enzymes contain bound Zn^{++} and are activated by Mg^{++} ions (6, 7, 8).

Materials and Methods. Saccharomyces strain 1016 was grown in 0.3%

Abbreviation: EDTA, ethylenediaminetetraacetic acid

yeast extract - 0.5% peptone - 0.1 M glucose or in a defined medium (modified Vogel's medium N (9)). Exponential phase cells were harvested and suspended in fresh medium for tests of growth or RNA synthesis. Incorporation of [^{3}H]uridine was the index of RNA synthesis. The procedure has been described (2).

[^{3}H]uridine (11.6 Ci/mmole), [5,6-^{3}H]uridine-5'-triphosphate (UTP) (36.8 Ci/mmole) and [methyl-^{3}H]thymidine-5'-triphosphate (TTP) (49.7 Ci/mmole) were purchased from New England Nuclear Corp. [Thymidine-2-^{14}C]-DNA from E. coli was obtained from Amersham-Searle Corp.

Lomofungin was a gift from Dr. George Whitfield of the Upjohn Co. The procedure used in dissolving the lomofungin is very important for obtaining stable and potent stock solutions. Lomofungin (2 to 3 mg) was dissolved by repeatedly adding several drops of 0.025 N NaOH followed by vortex mixing and centrifuging the undissolved lomofungin. When the drug was completely dissolved, the pooled solutions were diluted with water to a final lomofungin concentration of 1 mg/ml; the pH should be 9.5 - 10.5. In our experience, the customary solutions in dimethylsulphoxide rapidly lose activity.

Results and Discussion. Kuo et al. (2) reported that the growth of Saccharomyces strain 1016 in the defined medium was almost completely inhibited within one hour by 5 μg of lomofungin/ml. In subsequent experiments, growth in yeast extract-peptone-glucose medium proved to be less sensitive to the antibiotic. In an attempt to explain this difference, we increased the level of trace elements in the defined medium by 200-fold and found that lomofungin was now much less effective (Figure 1); however, growth was severely reduced if addition of the trace elements occurred even

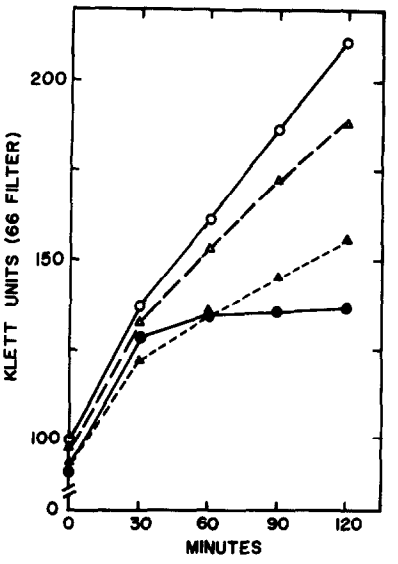

FIGURE 1 (Left). The effect of trace elements on growth inhibition by lomofungin. Lomofungin (10 μg/ml) was added at 0 min to 5 ml cultures of Saccharomyces strain 1016 (10^7 cells/ml) in defined medium at 30 C; 10 μl of trace elements solution (9) were added at the indicated times. No additions (O), lomofungin only (●), lomofungin plus trace elements at 0 min (△), lomofungin plus trace elements at 5 min (▲).

5 min after the lomofungin. After preliminary tests the salts in the mixture were all added at 0.4 mM. $CuSO_4$ gave the best results, with the Cu^{++} being the active ion. Cu^{++}, Fe^{++} and Zn^{++} offered good protection; Mn^{++}, Mg^{++}, Ca^{++}, K^+, Na^+ and NH_4^+ were ineffective.

Previous studies with similar phenazines have shown that the carboxyl and hydroxyl groups function in chelate formation and form a stable complex with Cu^{++}, Ni^{++} or Zn^{++} (10). Lomofungin also chelates these metals as demonstrated by a shift in its absorption maxima (pH 5.8) from 340 and 410 nm to 360 and 430 nm in the presence of 0.4 mM $CuSO_4$ (Pavletich, unpublished results).

Protection of RNA synthesis. In the defined medium at pH 5.8, RNA synthesis was almost completely protected when 0.4 mM $CuSO_4$ was added

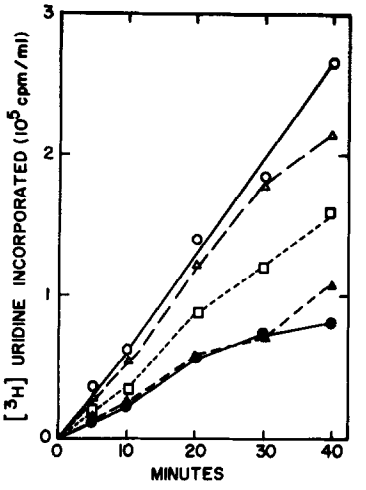

FIGURE 2 (Right). Prevention by $CuSO_4$ of the inhibition of RNA synthesis by lomofungin. Lomofungin (10 μg/ml) was added to 5 ml cultures (10^7 cells/ml) at -5 min and 40 μCi [^{3}H]uridine at 0 min. Samples were removed at the designated times and incorporation of uridine assayed as described in Materials and Methods. Additions: 0.4 mM $CuSO_4$ at -5 min (no lomofungin) (O), no $CuSO_4$ (●), 0.4 mM $CuSO_4$ at -5 min (△), 0.2 mM $CuSO_4$ at -5 min (□) 0.4 mM $CuSO_4$ at 0 min (▲).

along with the lomofungin (Fig. 2). If the addition of $CuSO_4$ was delayed 5 min, the protective effect was largely lost. EDTA (1 mM) markedly increased the activity of lomofungin in inhibiting RNA synthesis in intact cells (a similar sensitization was observed by Fraser and Creanor with Schizosaccharomyces pombe, personal communication), whereas 0.1 mM $ZnSO_4$ essentially nullified the inhibition (Fig. 3). As with growth, RNA synthesis was more sensitive in the defined medium than in the yeast extract-peptone-glucose medium (48% and 20% inhibition, respectively, by 10 μg lomofungin/ml in another experiment).

Since the pH optima of most RNA polymerases are at pH 7 or above, we examined the ability of divalent cations to protect RNA synthesis by cells suspended in 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

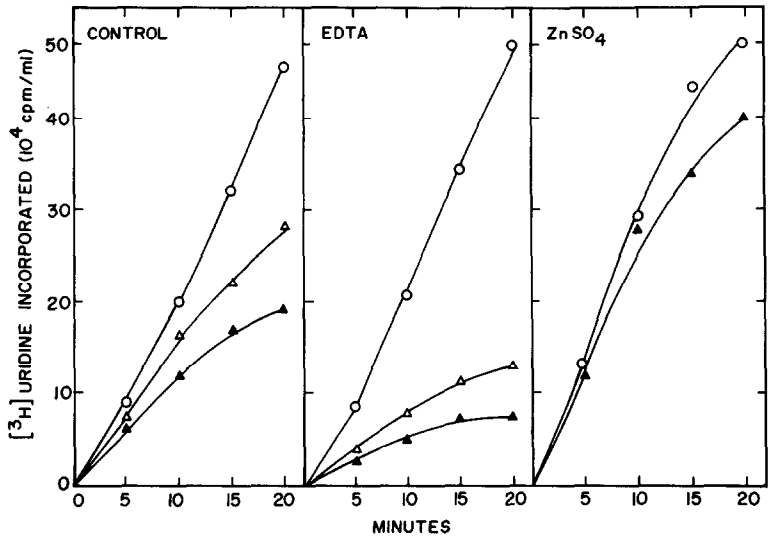

FIGURE 3. Effect of EDTA and $ZnSO_4$ on the inhibition of RNA synthesis by lomofungin. Strain 1016 was grown overnight in the defined medium with 0.2 M glucose and suspended (4×10^7/ml) in fresh medium with 100 mM glucose. At 0 min, lomofungin and, where indicated, EDTA (1 mM) or $ZnSO_4$ (0.1 mM) and 8 μCi/ml of [^{3}H]uridine (20 Ci/mmole) were added. No lomofungin (O), 5 μg lomofungin/ml (Δ), 10 μg lomofungin/ml (▲).

(HEPES) buffer, pH 7.0. $ZnSO_4$ was highly effective at 0.1 mM; Mg^{++} salts (1 mM) were ineffective and Ca^{++} gave little, if any, protection. EDTA (1 mM) and, to some extent, the transition metal-chelating agent o-phenanthroline (0.1 mM) sensitized RNA synthesis to lomofungin, but the Ca^{++}-chelating agent, ethylene glycol-bis (beta-aminoethyl ether) N,N'-tetraacetic acid had no effect at 1 mM.

Binding of lomofungin. Strain 1016 cells bind lomofungin at pH 5.8 (Table 1) and at pH 6.8. The process was complete in < 1 min. $CuSO_4$ (0.4 mM) largely prevented the binding of the antibiotic and enhanced the release of previously bound material (Table 1).

The protection of growth and RNA synthesis by Cu^{++} and Zn^{++} salts (Fig. 1 - 3) was probably the result of their forming a chelate with

Table 1. The Effect of $CuSO_4$ on the Binding and Release of Lomofungin

$CuSO_4$		Lomofungin bound ($\mu g/2 \times 10^7$ cells) 5 min	15 min
(A)	-	7.0	7.7
	0.4 mM	1.8	1.5
		Lomofungin released ($\mu g/2 \times 10^7$ cells) 5 min	15 min
(B)	-	1.5	1.5
	0.4 mM	3.5	3.7

A. Lomofungin (10 μg/ml) was added to Saccharomyces 1016 cells suspended at 2×10^7/ml in defined medium. $CuSO_4$, when present, immediately preceded the lomofungin. Samples were centrifuged at various times and unbound lomofungin in the supernatant fluids estimated by A_{550} in comparison with a standard curve of lomofungin in the same medium without cells.

B. Cells treated with lomofungin for 15 min, at which time 70% of the lomofungin (7 μg/ml) had been bound, were incubated in fresh medium with or without $CuSO_4$ to assess release of lomofungin.

lomofungin that is not taken up by the yeast cells (Table 1); however, the chelating property of lomofungin is probably important to its mechanism of action since a number of chelating agents are known to inhibit RNA and DNA polymerases (7, 8, 11). Lomofungin might inhibit by chelating the dissociable divalent cations (Mg^{++} or Mn^{++}) that are essential for polymerase activity, as suggested by Fraser and Creanor (12) for the action of 8-hydroxyquinoline and for lomofungin as well. This possibility can be discarded, however, on the basis of the data presented in Figure 4. The inhibition of the purified DNA and RNA polymerases by lomofungin was not prevented at high Mg^{++} levels. Also the concentration of Mg^{++} in the defined medium (0.81 mM) is much higher than the concentrations of lomofungin required for inhibition (10 μg/ml is 0.03 mM). Finally, Mg^{++}

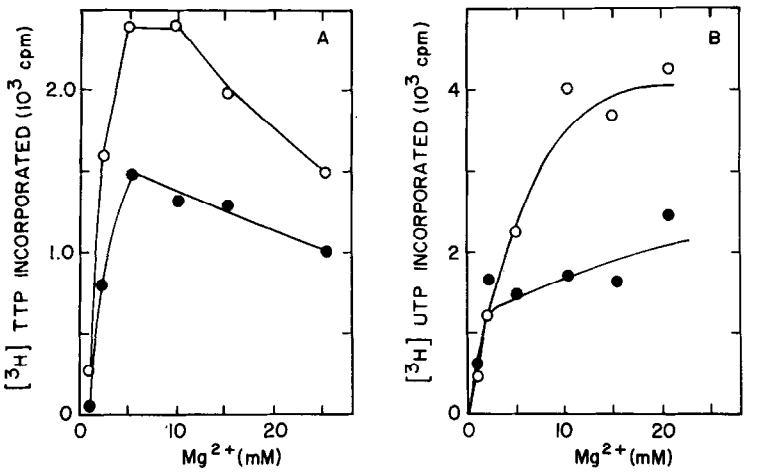

FIGURE 4. Effect of Mg^{++} concentration on polymerase activity in the absence (O) and presence (●) of lomofungin. (A) Incorporation of [^{3}H]TTP by Micrococcus luteus DNA polymerase (Sigma Chemical Co.); procedure based on an assay by Bollum (13). The reaction mixture (0.25 ml) contained 0.025 mM [^{3}H]TPP (1 μCi), 7 μg enzyme, 6.2 μg heat-denatured calf thymus DNA and varying concentrations of $MgCl_2$. The mixture minus the DNA was equilibrated for 5 min at 37 C; the lomofungin (40 μg/ml) was added at -5 min and the DNA at 0 min. The reaction was terminated after 5 min by 1 ml 10% CCl_3COOH - 40 mM sodium pyrophosphate. The precipitate was collected on Whatman glass fibre filters which were washed with 5% CCl_3COOH - 40 mM sodium pyrophosphate and suspended in 0.25 ml water and 5 ml Aquasol for determination of incorporated radioactivity. (B) Activity of E. coli RNA polymerase (Sigma Chemical Co.) was measured in 0.15 ml containing 50 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, 1.5 mM-2-mercaptoethanol, 0.1 mM ATP, GTP and CTP, 0.025 mM [^{3}H]UTP (1 μCi), 6.2 μg native calf thymus DNA, 2.6 μg enzyme and varying concentrations of $MgCl_2$. Lomofungin (20 μg/ml) was added at - 5 min, DNA at 0 min, and 10% CCl_3COOH at 5 min. The precipitates were treated as for DNA polymerase. No lomofungin (O), lomofungin added (●).

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or Ca^{++} (0.1 mM) did not protect RNA synthesis by yeast cells against lomofungin either at pH 5.8 or at pH 7.

DNA and RNA polymerases have been shown to contain tightly bound Zn^{++} (and possibly other divalent cations (14))which participates in the formation of the polymerase-DNA initiation complex and coordinates the growing point of the DNA (7). Formation of the initiation complex was

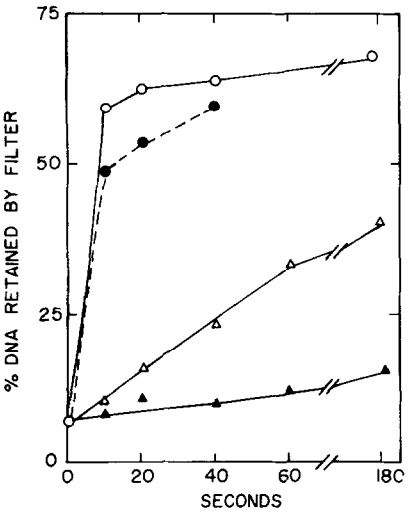

FIGURE 5. Inhibition by lomofungin of formation of the RNA polymerase· DNA initiation complex. Lomofungin was preincubated for 5 min with enzyme (0.2 μg) or with [thymine-2-^{14}C] DNA from E. coli (0.1 μg) in 20 μl and then diluted to 90 μl with binding buffer containing 2 mg bovine serum albumin/ml. The reaction was terminated at the indicated times by 0.9 ml of buffer containing 10 μg E. coli DNA/ml and the mixture filtered immediately on Millipore filters (0.45 μm). When DNA containing 8000 cpm was used, 500 to 600 cpm were bound to the filter in the absence of enzyme. No lomofungin (O), 10 μg/ml lomofungin preincubated with ^{14}C-DNA (8000 cpm) (●), 5 μg/ml (Δ) and 10 μg/ml (▲) lomofungin preincubated with E. coli RNA polymerase.

measured by the technique of Hinkle and Chamberlin (15) in which the enzyme· DNA complex is detected by its retention on membrane filters. Lomofungin inhibited formation of the complex (Fig. 5), but its effectiveness was a function of the order of addition of the reactants. Incubation of the antibiotic with the polymerase before addition of the DNA prevented complex formation almost completely. In contrast, preincubation of lomofungin with the DNA and subsequent addition of the enzyme yielded relatively little inhibition.

We propose that lomofungin (like o-phenanthroline (6, 8)) inhibits nucleic acid synthesis primarily by forming a chelate with the bound Zn^{++} of the DNA

and RNA polymerases and thus interfering with initiation. This explanation is supported by the fact that the inhibition can be reversed competitively by DNA or by the appropriate nucleotide triphosphates (K. Pavletich, unpublished data).

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