Lomofungin as an Inhibitor of Nucleic Acid Synthesis in *Saccharomyces cerevisiae*

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1. The antibiotic lomofungin was found to be a potent inhibitor of both DNA and RNA synthesis in *Saccharomyces cerevisiae*. Under selected growth conditions inhibition of DNA synthesis by the drug preceded inhibition of RNA synthesis. 2. Although in general lomofungin inhibited synthesis of ribosomal RNA and polydisperse RNA more effectively than that of low-molecular-weight RNA, under certain conditions the drug inhibited almost completely synthesis of both 4S and 5S RNA. 3. Inhibition of both RNA and DNA synthesis may be explained if RNA synthesis is required for DNA synthesis in yeast. Alternatively, lomofungin, in addition to interacting with DNA-dependent RNA polymerase, might interfere with a component(s) of the DNA-synthetic apparatus. The drug may thus prove to be of considerable value in studies of DNA synthesis in eukaryotes.

Lomofungin is a phenazine antibiotic (Tipton & Rinehart, 1970) which inhibits growth of many organisms (Johnson & Dietz, 1969; Gottlieb & Nicolas, 1969), and early studies with the drug suggested that it acted primarily as an inhibitor of RNA synthesis (Gottlieb & Nicolas, 1969). It has now been claimed that lomofungin is a potent inhibitor of DNA-dependent RNA polymerases from both *Escherichia coli* and a *Saccharomyces* strain (Cano *et al.*, 1973), and this work has indicated that the drug interacts directly with the enzyme. Recent work with *Saccharomyces cerevisiae* (Cannon *et al.*, 1973) has confirmed the inhibitory effect of lomofungin on RNA synthesis, and since the drug did not affect protein synthesis directly this inhibition allowed an estimate to be made of the decay of protein-synthetic capacity which followed addition of lomofungin *in vivo* (Cannon *et al.*, 1973).

Under certain experimental conditions some types of RNA may still be synthesized even when lomofungin is present. Thus both Kuo *et al.* (1973) and Fraser *et al.* (1973) have claimed that the drug, even at high concentrations (40–60 µg/ml), strongly inhibited synthesis of rRNA and probably mRNA, but had little or no effect on the synthesis of tRNA or 5S RNA.

In the present work we have shown that although lomofungin can inhibit synthesis of the various RNA species to different extents this effect is misleading. Thus even at low concentrations of lomofungin (5 µg/ml) synthesis of all RNA species may be almost completely inhibited. In

addition we have observed that lomofungin is a potent inhibitor of DNA synthesis. Indeed, inhibition can be detected at very early times after drug addition and under certain experimental conditions apparently precedes inhibition of RNA synthesis by the drug.

Experimental

Yeast strains and growth media

Saccharomyces cerevisiae strain Y166 (*atrp5*, *his4*, *MA1*) was grown at 30°C with shaking. YM-5 (rich medium) contained (per litre) yeast extract (Difco Laboratories, Detroit, Mich., U.S.A.; 1g), Difco peptone (2g), Difco yeast nitrogen base without amino acids (6.7g), glucose (10g), NaOH (6g) and succinic acid (10g). Complete synthetic medium contained (per litre) Difco yeast nitrogen base without amino acids (6.7g), succinic acid (10g), NaOH (6g), glucose (10g), L-histidine (40mg) and L-tryptophan (40mg). Strains were maintained on solid medium containing (per litre) Difco yeast extract (10g), Difco peptone (20g), glucose (20g) and agar (20g). Cell growth was determined by measuring E_{550} in a Bausch and Lomb Spectronic 20 colorimeter. A reading of 0.30 approximated to 10^7 cells/ml.

Preparation of spheroplasts

A culture of *S. cerevisiae* (30ml) was grown overnight to a concentration of 10^7 cells/ml. The cells

were pelleted by centrifugation for 5 min at 10000g in a Sorvall SS-34 rotor, washed once with water (30 ml) and resuspended in 1 M-sorbitol (3 ml). Glusulase (0.03 ml) was added and the suspension incubated at 23°C for 45 min with gentle shaking. The mixture was then diluted into growth medium (60 ml) containing 1 M-sorbitol and the suspension shaken gently at 30°C for 2.5 h. Spheroplasts were then ready for use in labelling experiments.

Analysis of radioactively labelled RNA

Cells were grown overnight in complete synthetic medium containing [¹⁴C]uracil (0.01 μ Ci/ml) to a concentration of 10^7 cells/ml (steady-state label). Spheroplasts were prepared, suspended in complete synthetic medium and samples (10 ml) exposed to lomofungin (5 μ g/ml) for various time-intervals. Samples were then incubated for 5 min at 30°C with [³H]uridine (50 μ Ci, containing 2 μ g of uridine); cycloheximide (100 μ g/ml) was added and the samples were poured over frozen 1 M-sorbitol (10 ml). Spheroplasts were collected by centrifugation for 5 min at 8000g and lysed with 0.25 ml of a solution containing 50 mM-NaCl, 100 mM-sodium acetate, pH 5.4, 10 mM-EDTA and 0.5% sodium dodecyl sulphate. The lysates were layered on 5–20% (w/v) sucrose gradients (volume 5 ml) made in the same buffer and centrifuged at 15°C for 2.5 h at 40000 rev./min in a Spinco SW50.1 rotor. Gradients were analysed in an Isco gradient analyser and 6-drop fractions were collected on 2.4 cm discs of filter paper (Whatman 3MM). From each 5 ml gradient 60 fractions were collected. The discs were washed three times with ice-cold 10% (w/v) trichloroacetic acid, once with ethanol-ether (1:1, v/v) and once with ether. Filters were air-dried and counted for radioactivity in a toluene-based scintillation fluid (Tipper, 1973).

Incorporation of [³H]adenine into nucleic acids

Cells were grown in either YM-5 medium or complete synthetic medium. When spheroplasts were used conditions for their preparation were as described above. [³H]Adenine (2.5 μ Ci/ml, plus unlabelled adenine, 1.5 μ g/ml) was added and the cells were incubated at 30°C. At selected time-intervals samples (1 ml) were placed into ice-cold 15% trichloroacetic acid (2 ml) containing adenine (0.25 mg/ml), and further samples (1 ml) were removed into 0.6 M-KOH (1 ml). Samples precipitated by trichloroacetic acid were filtered on to Whatman GF/C filter discs (2.4 cm) and washed three times with 10% trichloroacetic acid (3 ml) containing adenine (0.25 mg/ml). Discs were dried and counted for radioactivity as above to determine incorporation of [³H]adenine into total nucleic acids. Samples treated with KOH

were incubated at room temperature (25°C) for 18 h and were then neutralized with Dowex 50 resin and divided into two halves. One set of samples (0.5 ml each) was mixed with carrier bovine serum albumin (50 μ g) and precipitated with 10% trichloroacetic acid (3 ml) containing adenine (0.25 mg/ml). Samples were filtered and washed seven times with the acid solution (3 ml portions). Filters were dried and counted for radioactivity as above to determine amounts of nucleic acid resistant to alkaline hydrolysis (i.e. DNA). The second set of samples (0.5 ml each) were made 50 mM with respect to Tris-HCl, pH 7.4, and 50 mM with respect to magnesium acetate. Samples were incubated with deoxyribonuclease (Worthington Biochemical Corp., Freehold, N.J., U.S.A.; 50 μ g/ml) for 2 h at 37°C. Samples were then precipitated with trichloroacetic acid and processed as above. This procedure is necessary to check that all radioactivity remaining in total nucleic acids after KOH treatment is present in DNA.

Chemicals

[5',6-³H]Uridine (42.4 Ci/mmol), [2-¹⁴C]uracil (40 mCi/mmol) and [G-³H]adenine (8.26 Ci/mmol) were obtained from New England Nuclear Corp., Boston, Mass., U.S.A. Lomofungin and cycloheximide were both kindly provided by Dr. G. B. Whitfield Jr. of the Upjohn Company, Kalamazoo, Mich., U.S.A. Various concentrations of lomofungin were used in the different experiments. In all cases lomofungin was dissolved freshly in dimethylsulphoxide, and in all experiments dimethylsulphoxide was added to either whole cells or spheroplasts at a concentration of 2 μ l/ml. Controls contained dimethylsulphoxide with no dissolved drug. The chemical had no effect on uptake of [³H]uridine or [³H]adenine into either whole cells or spheroplasts. Glusulase was obtained from Endo Laboratories Inc., Garden City, N.Y., U.S.A.

Results and Discussion

Although lomofungin, at a concentration of 5 μ g/ml, rapidly inhibited the incorporation of uridine into RNA in either whole cells or spheroplasts of *S. cerevisiae* (Cannon *et al.*, 1973) this inhibition was not complete during the 30 min period after drug addition. It was therefore decided to determine if the synthesis of all RNA species was inhibited equally in the limited synthesis which followed lomofungin addition.

The stable RNA components (mainly 27S, 15S and 4S) were labelled by growing cells of *S. cerevisiae* overnight in the presence of [¹⁴C]uracil. Cells were converted into spheroplasts, and after incubation in complete synthetic medium to restore

activity (Udem *et al.*, 1971) samples were exposed to lomofungin for various time-intervals and then incubated with [3 H]uridine for 5min. Under these conditions the 3 H label entered all the stable RNA species, although relatively little rRNA precursor was apparent. After the labelling period, spheroplasts were inhibited with cycloheximide to block protein synthesis, chilled rapidly and then lysed and analysed directly on sucrose gradients containing sodium dodecyl sulphate. In control incubations (Fig. 1a) the uridine label coincides closely with the stable RNA markers both in position on the gradient and in the relative amounts present in the different RNA species. As expected, ribosomal RNA (27S and 15S) was in considerable excess over low-molecular-weight RNA (mainly 4S). Incubation with lomofungin (5 μ g/ml) for 5min before labelling with [3 H]uridine resulted in a 37% decrease in total uridine incorporation (Fig. 1b). For comparison of RNA profiles the 3 H radioactivities for all graphs shown in Fig. 1 have been plotted on different scales so that the height of the main peak is approximately the same throughout. In the control (Fig. 1a) the predominant peak is, of course, 27S RNA. In Fig. 1(b), however, there is a large increase, relative to the other 3 H-labelled RNA components resolved on the gradient, in the amount of 3 H-labelled low-molecular-weight RNA. Further, there is also a slight increase, relative to 27S RNA, in the amount of 3 H-labelled RNA in the 15S RNA area. This area undoubtedly contains some polydisperse RNA, presumed to be mRNA (Udem & Warner, 1972). For Fig. 1(c) spheroplasts were incubated with lomofungin for 7.5min before exposure to [3 H]uridine. The gradient profile for this sample shows a continuing rise in labelling of low-molecular-weight RNA relative to other RNA components, and label in 27S RNA continues to decline relative to that in the 15S area. The inhibition of uridine incorporation by lomofungin in Fig. 1(c) was approx. 59%. After 10min incubation with lomofungin (Fig. 1d) total uridine incorporation was inhibited by 70% relative to the control (Fig. 1a). The low-molecular-weight RNA was now the predominant 3 H-labelled peak and there was a sharp decrease in 3 H label in both 15S and 27S regions, more particularly in the latter. Thus, at times up to 10min, lomofungin caused differential inhibition of synthesis of different size classes of RNA. In the last experiment of this series spheroplasts were incubated with lomofungin for 30min and then labelled with [3 H]uridine for 5min (Fig. 1e). Inhibition of incorporation was almost complete (95%) and the 3 H-labelling pattern was similar to that shown by control incubations (Fig. 1a). Thus after 30min in the presence of lomofungin there was no selective inhibition or enrichment of the synthesis of any RNA species: all were synthesized (albeit in small

amounts) and their synthesis was affected by lomofungin to a similar degree.

In some respects our results resemble those of Fraser *et al.* (1973), although there are notable differences between the two sets of data. Fraser *et al.* (1973) studied the effect of lomofungin on RNA synthesis in the fission yeast *Schizosaccharomyces pombe*. They concluded that lomofungin selectively inhibited synthesis of rRNA and polydisperse RNA within a few minutes of addition, both RNA types being equally sensitive to various lomofungin concentrations. However, even with high concentrations of lomofungin (60 μ g/ml) synthesis of 5S RNA was completely unaffected 30–35min after drug addition and tRNA (4S) was also synthesized at normal rates 15–30min after drug addition. After 30min exposure to lomofungin there was a 34% inhibition of tRNA synthesis. Fraser *et al.* (1973) also claimed that lomofungin inhibited the processing of rRNA precursor to mature rRNA.

In agreement with Fraser *et al.* (1973) we have observed that synthesis of total low-molecular-weight RNA may be relatively insensitive to lomofungin. However, in our work this only applied to early times after drug addition, so that even though we used a tenfold lower concentration of drug than did Fraser *et al.* (1973), exposure to lomofungin for 30min resulted in inhibition of the synthesis of low-molecular-weight RNA (both 4S and 5S) as well as inhibition of rRNA synthesis. Further, there was no indication from our results that processing of rRNA precursor to mature rRNA was affected by lomofungin. Thus in all cases, labelling of spheroplasts after exposure to the drug allowed some incorporation of radioactivity into all species of RNA and accumulation of rRNA precursor was not apparent.

It has been reported (Cano *et al.*, 1973) that lomofungin, added *in vitro*, inhibits not only three DNA-dependent RNA polymerases from a *Saccharomyces* strain but also a corresponding enzyme isolated from *E. coli*. The drug did not interact with DNA and the authors inferred that lomofungin interacted directly with the polymerase. We decided to study the effect of the drug on DNA synthesis, since we had obtained preliminary results suggesting that DNA synthesis in both whole cells and spheroplasts of *S. cerevisiae* was sensitive to low concentrations of lomofungin (Cannon *et al.*, 1973). Further, Gottlieb & Nicolas (1969) had previously claimed that the drug affected the synthesis of both RNA and DNA, but that RNA formation was inhibited at lower concentrations of lomofungin than were required for inhibition of DNA synthesis.

Cells were grown in YM-5 medium to a density of approx. 10^7 cells/ml. [3 H]Adenine was added and samples were removed at various times into both KOH and trichloroacetic acid. Different

concentrations of lomofungin were added to samples 9min after the start of the incubation. This allowed time for added [^3H]adenine to equilibrate with the intracellular pool of nucleic acid precursors and ensured linear incorporation of adenine at the time of drug addition. All samples were processed, and radioactivity in both RNA and DNA was measured as described in the Experimental section. The results are illustrated in Fig. 2(a), which shows that DNA synthesis is inhibited almost immediately on addition of lomofungin. After 9min in the presence of drug, 5, 7 and 9 μg of lomofungin/ml caused respectively 44, 51 and 63% inhibition. After 45min incubation with lomofungin the corresponding values for inhibition of DNA synthesis were 60, 76 and 84%. In contrast (Fig. 2b) synthesis of RNA was inhibited less dramatically, particularly with the lower concentrations of lomofungin. Thus 9min after drug addition there was no apparent inhibition of RNA synthesis at any drug concentration studied and even after 18min incubation with lomofungin 5 and 7 μg of drug/ml produced no inhibition of RNA synthesis, whereas 9 μg /ml inhibited the process by only 35%. After 45min incubation with lomofungin the corresponding values for inhibition of RNA synthesis were 12, 46 and 68%. The experiment was repeated by using yeast spheroplasts instead of whole cells growing in YM-5 medium and the results obtained were essentially the same as those already shown in Figs. 2(a) and 2(b). We conclude that synthesis of DNA in *S. cerevisiae* is inhibited by lomofungin to a greater extent and at earlier times after drug addition than is RNA synthesis. This result is in direct contrast with the earlier experiments of Gottlieb & Nicolas (1969). These authors studied DNA synthesis by measuring incorporation of [$\text{methyl-}^3\text{H}$]thymidine into growing cells of *S. cerevisiae*. Although we attempted to repeat their experiments under the conditions reported we consistently found that our strain of yeast failed to incorporate thymidine, the cells apparently being impermeable to this compound.

The experiments described in Figs. 2(a) and 2(b) were then repeated, except that incubation with

either whole cells or spheroplasts was in complete synthetic medium, and the effect of lomofungin on DNA and RNA synthesis was studied. These growth conditions were directly comparable with those used to obtain the data of Fig. 1 and the results are shown in Figs. 2(c) and 2(d). Synthesis of both RNA and DNA was severely inhibited by lomofungin, added at concentrations of 1.5, 3 and 5 μg /ml, at very early times after exposure to the drug. The kinetics of inhibition were such that it was impossible to determine whether inhibition of DNA synthesis preceded inhibition of RNA synthesis, as had been observed with cells growing in rich medium (cf. Figs. 2a and 2b). For some reason both cells and spheroplasts of *S. cerevisiae* were particularly sensitive to lomofungin when growing in minimal medium (cf. Fig. 1). This effect may be related to the different growth rates observed with yeast in the two media. In minimal medium growth was slower than in rich medium and the rates of both RNA and DNA synthesis were correspondingly lower in minimal medium. The nature and sizes of intracellular pools may well change in different growth media and other variables could presumably affect the inhibition. However, we are confident that the results of Fig. 2(a) and 2(b) are a valid indication that lomofungin can affect DNA synthesis before inhibition of RNA synthesis becomes apparent.

Eukaryotic nuclear DNA-dependent RNA polymerases can be separated as a routine into three or more fractions (I, II and III) by DEAE-cellulose or DEAE-Sephadex chromatography (Roeder & Rutter, 1969; Tipper, 1973). Similarly three discrete polymerases from *S. cerevisiae* have been described (Broggt & Planter, 1972; Cano *et al.*, 1973), and Cano *et al.* (1973) have claimed that lomofungin is a potent inhibitor of all three enzymes in isolation. Their results indicate that the drug acts rather non-specifically, and similar conclusions have been implied in personal communications from several laboratories. It is intriguing therefore that our data, as does the work of Fraser *et al.* (1973), indicate a differential inhibition by lomofungin of the synthesis of certain RNA components, particularly rRNA. In our view, however, this differential

Fig. 1. Labelling of RNA components in the presence of lomofungin

A culture of *S. cerevisiae* was grown overnight in complete synthetic medium containing [^{14}C]uracil and spheroplasts were prepared as described in the Experimental section. Samples were exposed to lomofungin for various time-intervals and labelled for 5min with [^3H]uridine. Spheroplasts were collected and analysed as described in the Experimental section. (a) Spheroplast samples (10ml) incubated for 7.5min with dimethyl sulphoxide (20 μl). In (b), (c), (d) and (e) spheroplast suspensions (10ml) were incubated with lomofungin (50 μg dissolved in 20 μl of dimethyl sulphoxide) for 5, 7.5, 10 and 30min respectively before labelling. Sedimentation in the sucrose gradients is from left to right. ----, Stable RNA components with incorporated [^{14}C]uracil. The actual experimental points are excluded to avoid overcrowding and the count profile is dotted in. ●, RNA with incorporated [^3H]uridine.

Fig. 2. Incorporation of adenine into DNA and RNA and the effect of lomofungin

Experimental conditions are described in the Experimental section. Cells were incubated with [^3H]adenine for 9 min before addition of selected concentrations of lomofungin (indicated by arrow). Samples were processed and amounts of ^3H label incorporated into both DNA and RNA were measured as described in the Experimental section. (a) DNA synthesis in cells growing in rich medium; (b) RNA synthesis in cells growing in rich medium; (c) DNA synthesis in cells growing in complete synthetic medium; (d) RNA synthesis in cells growing in complete synthetic medium. For (a) and (b) symbols are ●, control; ○, lomofungin (5 μg/ml); □, lomofungin (7 μg/ml); △, lomofungin (9 μg/ml). For (c) and (d) symbols are ●, control; ■, lomofungin (1.5 μg/ml); ▲, lomofungin (3 μg/ml); ○, lomofungin (5 μg/ml).

inhibition is not complete, and with time lomofungin strongly inhibits all RNA polymerases, which are functioning in the yeast cell at the time of drug addition. In this respect the data of Cano *et al.* (1973) seem validated, but our results raise two important points. If the polymerase responsible for synthesis of 4S RNA is one of the three activities identified (Cano *et al.*, 1973) then this polymerase when present in the intact cell is apparently less sensitive, or at least less available, to lomofungin than the polymerase(s) which synthesize rRNA. Alternatively, the three activities identified may all be equally sensitive to inhibition by the drug, as shown by Cano *et al.* (1973), but none of these activities may be responsible for synthesis of 4S RNA. Implied in this consideration is the possible presence in yeast cells of a further polymerase(s),

as yet unidentified, with relatively low sensitivity to lomofungin. Adman *et al.* (1972) have in fact described a fourth peak of RNA polymerase activity from yeast, although it has been claimed (B. Hall, personal communication) that this enzyme is also sensitive to lomofungin. Clearly, further work is required before definitive statements can be made in this area.

Finally, the effect of lomofungin as an inhibitor of DNA synthesis must be considered. Since Cano *et al.* (1973) have shown that lomofungin does not interact with DNA when the drug inhibits RNA polymerase it seems unlikely that such an interaction would occur when lomofungin inhibits DNA synthesis. This interpretation is supported by preliminary studies on possible hyperchromicity changes, which could result from interaction between

the drug and DNA. In the presence of lomofungin the spectral properties of DNA at room temperature were not altered and the drug had no effect on the so-called 'melting' temperature of DNA (R. Wartell, personal communication). In bacteria initiation of DNA replication requires a transcriptional event, indicating a role for RNA in the process (Lark, 1972). DNA chains are synthesized in short pieces, which are later joined together. Each fragment of DNA starts off with a length of RNA, which is removed, by an unknown mechanism, before synthesis of the fragment is completed. Polynucleotide ligase has been implicated in the joining of the completed fragments and DNA polymerase I may also be involved. The latter enzyme could perhaps also function in removal of RNA from the DNA chains and in extension of the Okazaki fragments until these are adjacent (Hirose *et al.*, 1973; Okazaki *et al.*, 1971). A direct involvement of DNA polymerase I has also been indicated in replication of the colicinogenic factors *colE₁* and *colE₂*, which cannot be maintained in polymerase I-deficient strains (Kingsbury & Helinski, 1970). Possibly, lomofungin could interact with one of these enzymes concerned with DNA replication, either a polymerase or the ligase. Since the concentration of drug required to inhibit DNA synthesis is extremely low the process affected is highly sensitive to lomofungin. Although we have presented evidence that total DNA synthesis can be affected by lomofungin before the drug inhibits total RNA synthesis, it is clear that the two inhibitory effects are closely related. We have discussed above how RNA is involved in control of DNA replication in bacteria and it may be that synthesis of an RNA component(s) is also required for DNA synthesis in yeast. Lomofungin possibly inhibits synthesis of such an RNA component before it exerts its effect on RNA synthesis as a whole. Such a differential and immediate inhibition could be reflected by rapid cessation of DNA synthesis on lomofungin addition. We wish to stress, however, the speculative nature of such an inhibitory mechanism since from our data we cannot exclude a direct action of lomofungin on DNA polymerase. Clearly, however, further studies with lomofungin

could well help to elucidate the reactions and components involved in DNA synthesis in eukaryotes and the drug may well prove useful in unravelling the complex interrelations apparent between RNA and DNA synthesis.

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