LIPIARMYCIN, A NEW ANTIBIOTIC FROM *ACTINOPLANES*

III. MECHANISM OF ACTION

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In vivo, at low concentrations (≤ 1 μg/ml), the antibiotic lipiarmycin specifically inhibits RNA synthesis in *Bacillus subtilis*. At a much higher concentration (100 μg/ml), syntheses of other macromolecules such as DNA and protein also appear to be suppressed. *In vitro*, the antibiotic causes 50% inhibition of DNA-dependent RNA-polymerase from *B. subtilis* at a concentration of 0.6 μg/ml and of that from *E. coli* at 5~8 μg/ml. The activity of *Escherichia coli* DNA-polymerase I is inhibited 50% at 55~65 μg/ml. Lipiarmycin prevents ribonucleoside triphosphate polymerization only if added prior to the association between RNA-polymerase and DNA, and does not affect the elongation rate of RNA chains at concentrations up to 100 μg/ml. At that concentration, however, the antibiotic immediately blocks the polymerization of deoxyribonucleotide triphosphates catalyzed by DNA-polymerase I.

Lipiarmycin is a recently isolated, chlorine-containing, antibiotic produced by *Actinoplanes deccanensis*, nov. sp. It is active against Gram-positive bacteria but ineffective against Gram-negative bacteria, fungi and protozoa. The chemical characteristics and the biological properties of this antibiotic have been reported elsewhere.[1,2] In this paper, we wish to report the evidence which indicates that lipiarmycin acts primarily by affecting RNA synthesis, and at much higher concentrations also affects DNA synthesis.

Experiments carried out with purified bacterial RNA- and DNA-polymerases show that lipiarmycin interferes with RNA synthesis specifically at initiation of chains, whereas DNA synthesis is inhibited in the process of chain elongation.

Material and Methods

Bacterial strains:

Bacillus subtilis strain PB 556/1(thym$^-$) and *Escherichia coli* K 12 or 63 were used.

Nucleic acid polymerases:

DNA-dependent RNA-polymerase from *E. coli* was prepared according to BURGESS's method,[3] but purified only as far as the DEAE chromatography step. Purified *B. subtilis* RNA-polymerase was kindly made available to us by Dr. G. CASSANI. Unless otherwise stated, RNA-polymerase activity was assayed by the method of BURGESS.[3]

E. coli DNA-dependent DNA-polymerase I was purchased from Boehringer, Mannheim and assayed according to the method of RICHARDSON *et al.*[4]

Radiochemicals:

(Methyl-^{3}H)-dTTP, 28 Ci/mM; (2-^{3}H)-ATP, 19 Ci/mM; (5-^{3}H)-uracil, 24.2 Ci/mM; (U-^{14}C)-UTP, 48 mCi/mM; (2-^{14}C)-Thymidine, 61 mCi/mM and (U-^{14}C)-phenylalanine, 225 mCi/mM were all obtained from Radiochemical Center, Amersham.

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Radioactivity measurement:

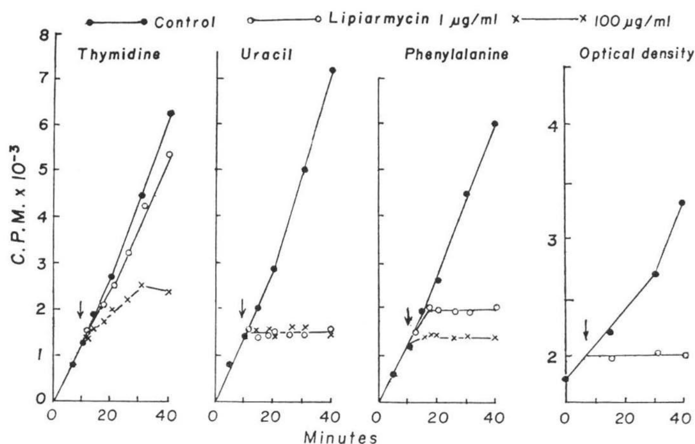
The radioactive material was collected on cellulose or glass fiber filters and the cold TCA insoluble radioactivity determined in a Philips Liquids Scintillation Analyzer with Instagel (Packard) as scintillation fluid.

Results

In Vivo Effects on Macromolecule Synthesis

The addition of lipiarmycin at 1 μg/ml to an exponentially growing culture of *B. subtilis* results in a rapid cessation of cellular growth, concomitant with a suppression of uracil incorporation. After a short lag period, protein synthesis is also arrested, whereas DNA synthesis remains virtually unaffected for at least thirty minutes (Fig. 1). This pattern of inhibition is typical of compounds known to inhibit RNA synthesis.

Fig. 1. Effect of lipiarmycin on growth and macromolecular syntheses in *B. subtilis*.
Radioactive precursors were added at zero time to an exponentially growing culture of *B. subtilis*, in DAVIS minimal medium supplemented with 0.2% (w/v) casein aminoacids, at 37°C. Lipiarmycin was added at the time indicated by the arrow.
Radioactive precursors were added as follows: [U-^{14}C] phenylalanine, 0.66 μCi/ml; [5-^{3}H] uracil, 0.1 μCi/ml [2-^{14}C] thymidine, 0.2 μCi/ml.
Growth was determined as optical density at 595 nm.



With a concentration of 100 μg/ml, DNA synthesis is also depressed within a few minutes after addition of antibiotic to the growing culture, while protein synthesis stops almost immediately. In order to identify the subcellular target of lipiarmycin, the antibiotic was tested on the activities of purified RNA- and DNA-polymerases.

DNA-dependent RNA-polymerase

The effect of antibiotic concentration on inhibition of *E. coli* RNA-polymerase in the presence of different templates is shown in Fig. 2. Natural templates (calf thymus and T4 DNA) were compared with a synthetic one, poly dA-T. 50% inhibition of *E. coli* RNA-polymerase primed with the natural templates is achieved at concentrations of 5 and 8 μg/ml. When the primer is poly dA-T, 50 μg/ml are required to give 50% inhibition.

Using calf thymus DNA as template, and comparing the RNA-polymerases of *E. coli*

Fig. 2. Inhibition of *E. coli* RNA-polymerase by lipiarmycin.

The reaction mixture contained 40 mM Tris-HCl pH 7.9, 0.15 M KCl 0.1 mM dithiothreitol, 10 mM $MgCl_2$, 4 mM $MnCl_2$, 0.4 mM Na phosphate buffer pH 7.4; 0.16 mM UTP, CTP, GTP and ^{3}H-ATP (12.5 mCi/mM), 150 μg/ml of calf thymus DNA, or T4-DNA, or poly dA-T, and 5 units/ml of *E. coli* RNA polymerase in a final volume of 0.1 ml. In the assay of poly dA-T primed polyribonucleotide synthesis, CTP and GTP were omitted.

Assay tubes were incubated at 36°C for 15 minutes.

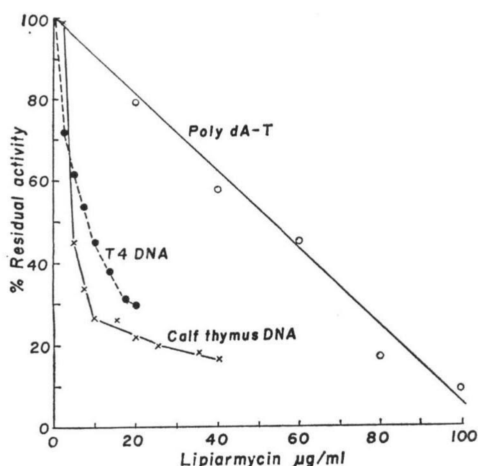
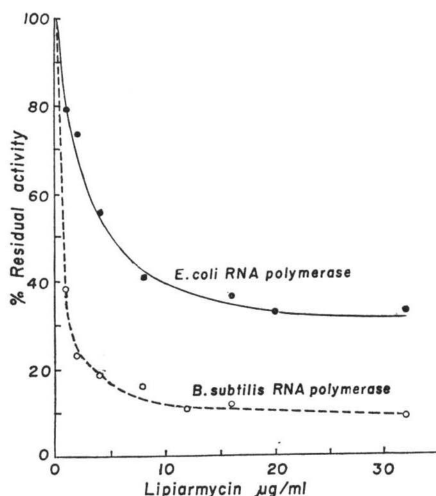


Fig. 3. Inhibition of DNA-dependent RNA-polymerase from *E. coli* and *B. subtilis* by lipiarmycin.

Assay tubes were incubated at 36°C for 20 minutes and contained 40 mM Tris·HCl pH 7.9, 10 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.1 mM EDTA, 1.28 mM ATP and GTP, 0.13 mM CTP and ^{14}C-UTP (3.5 mCi/mM), 100 μg/ml of calf thymus.

DNA, *E. coli* RNA polymerase (10 μg of protein) or RNA polymerase from *B. subtilis* (5 μg of protein) and lipiarmycin in a final volume of 0.1 ml. Without the antibiotic *E. coli* RNA-polymerase incorporated 1.78 mμ moles of UTP, the polymerase from *B. subtilis* 0.50 mμ moles of UTP.



and *B. subtilis*, lipiarmycin is about 8 times more effective against the *B. subtilis* enzyme, 0.6 μg/ml giving 50% inhibition (Fig. 3).

Fig. 4 shows the time courses of inhibition of *E. coli* RNA-polymerase, with calf thymus DNA primer, when inhibitor was added either at zero time or five minutes after RNA synthesis had begun. The effects of lipiarmycin are compared with those of rifampicin, which is known to be an inhibitor of chain initiation[6] and streptolydigin, which is an inhibitor of chain elongation.[7] When added at zero time, all three antibiotics cause an immediate and virtually complete inhibition of RNA synthesis. However, when added after nucleotide polymerization has begun, lipiarmycin, like rifampicin, suppressed RNA synthesis only after a lag of several minutes, whereas streptolydigin suppressed it immediately. This would suggest that lipiarmycin also is active at the initiation step of nucleotide polymerization.

This hypothesis was further tested in an experiment in which RNA-polymerase and template DNA were mixed and preincubated for 5 minutes prior to the addition of the antibiotic and the four nucleoside triphosphates. In a parallel assay, enzyme and DNA were preincubated together with lipiarmycin. As shown in Fig. 5, when lipiarmycin is present during the preincubation, nucleotide polymerization is completely blocked. There is no inhibition if it is added after preincubation of enzyme and template, suggesting that it interferes with the formation of

Fig. 4. Time course of RNA synthesis by *E. coli* RNA-polymerase in presence of various antibiotics.

The reaction mixture had the same composition as in Fig. 2 except that calf thymus DNA (150 μg/ml) was used at template.

The antibiotics were added either at zero time or after 5 minutes, as indicated by the arrow. Lipiarmycin and streptolydigin were 100 μg/ml, rifampicin was 1 μg/ml.

Incubation was at 30°C, at intervals aliquots of 0.1 ml were withdrawn and the incorporated radioactivity determined.

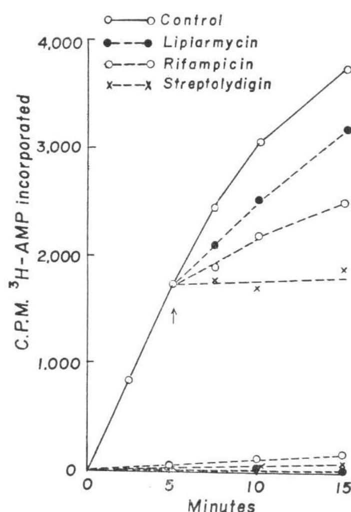
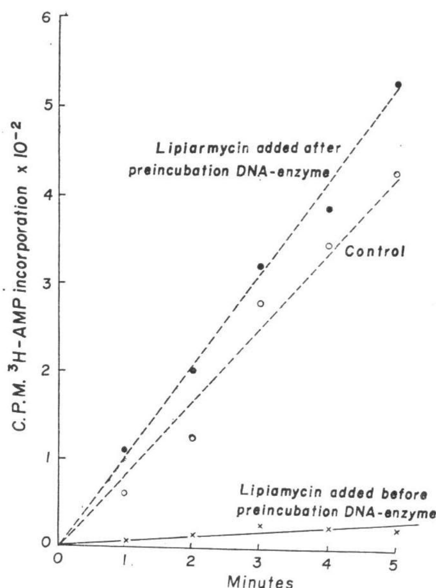


Fig. 5. Effect of preincubation of RNA-polymerase with template DNA on lipiarmycin inhibition.

The experimental conditions were the same as described in Fig. 4, except that the four nucleotide triphosphates were added after 5-minutes incubation at 36°C.

Lipiarmycin was added at the beginning of the experiment or together with the nucleotide triphosphates, at 25 μg/ml, final concentration.



Enzyme-DNA complex.

DNA-dependent DNA-polymerase

Since high concentrations of lipiarmycin inhibit *in vivo* DNA synthesis, we have investigated its action on purified DNA-polymerase I from *E. coli*. As shown in Fig. 6, inhibition is concentration-dependent. 50 % inhibition was seen at 55 μg/ml with poly dA-T as template, and at 65 μg/ml with calf thymus DNA as template. Thus, DNA-polymerase is 10 times less sensitive to the drug than is RNA-polymerase, when a natural template is used, but equally sensitive when the polymerization is primed with poly dA-T.

The time course of the effect on DNA-polymerase differs from that observed with DNA-polymerase. The drug causes immediate inhibition when added either at zero time or 5 minutes after the beginning of the polymerization reaction (Fig. 7).

Lack of Effect on Protein Synthesis

At concentrations up to 100 μg/ml, no effect of the antibiotic was observed in a cell-free peptide synthetizing system, programmed with either poly-U or endogenous m-RNA. In this assay system, uncharged t-RNA is used. Therefore, it can be deduced that lipiarmycin does not interfere with aminoacylation of t-RNA. The *in vivo* effect on protein synthesis, *i.e.*, the

Fig. 6. Inhibition of *E. coli* DNA-polymerase I by lipiarmycin.

The reaction mixture contained 67 mM potassium phosphate buffer, pH 7.4, 6.7 mM $MgCl_2$, 1 mM β mercaptoethanol, 0.1 mM each dATP, dGTP, dCTP and ^{3}H.dTTP (20 mCi/mM), 150 μg/ml of either calf thymus DNA or poly dAT and 5 units/ml of *E. coli* DNA-polymerase I. When poly dAT primed polydeoxyribonucleotide synthesis was assayed, dGTP and dCTP were omitted.

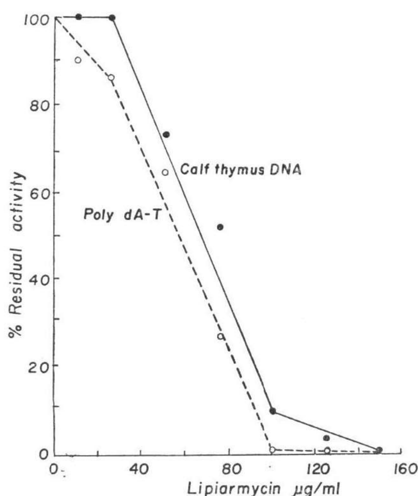
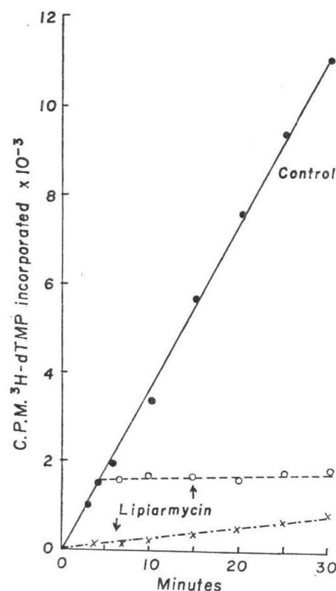


Fig. 7. Time course of DNA synthesis by *E. coli* DNA-polymerase I in the presence of lipiarmycin.

The reaction mixture had the same composition as in Fig. 6; the template was calf thymus DNA. Lipiarmycin was added either at zero time or after 5 minutes, at a 110 μg/ml concentration, as indicated by the arrow.



rapid arrest of amino acid incorporation observed at high concentrations of antibiotic, might therefore be due to some general damage to the cell, rather than to a specific inhibition.

Attempts to show Interaction of Lipiarmycin with DNA

Lipiarmycin at a concentration of 100 μg/ml does not affect the rate of DNA hydrolysis catalyzed by pancreatic deoxyribonuclease.

At 50 μg/ml, lipiarmycin does not alter the melting point or the renaturation profile of *B. subtilis* DNA. Finally, the absorption spectrum of the drug was not modified on incubation with DNA.

Discussion

The data presented indicate that the antibiotic lipiarmycin specifically inhibits nucleic acid synthesis both *in vivo* and *in vitro*. In both circumstances, however, RNA synthesis is more sensitive to the antibiotic than is DNA synthesis. Lipiarmycin has been reported recently to inhibit also RNA-polymerase from calf thymus nuclei although at a concentration 10 times higher than that needed to inhibit *E. coli* RNA-polymerase.[7]

Lipiarmycin appears to impair the initiation of RNA chain synthesis in a highly specific manner. The antibiotic prevents RNA synthesis *in vitro* completely only when it is added before the reaction initiates. Addition of the drug after the reaction has begun, results in a lag period before synthesis stops, since RNA chain completion continues for several minutes. Similar kinetics of inhibition has been obtained by TALPAERT *et al.*[7] In the same way,

preincubation of RNA-polymerase with the DNA template protects the polymerization reaction from lipiarmycin inhibition for at least five minutes, during which time the rate of RNA chain elongation remains unaffected. The inhibition observed at the later time presumably reflects the effect on initiation of new RNA chains. Even at the high concentration of lipiarmycin which also inhibits DNA synthesis, the inhibitory effect on RNA synthesis is on initiation.

The effect of lipiarmycin on DNA synthesis shows characteristics markedly different from its effect on RNA synthesis. First, the DNA-polymerase reaction is about 10 times less sensitive to the drug per unit of enzyme. Second, the dose-effect relationship is the same with both natural and synthetic templates, whereas RNA-polymerase shows a quantitatively different inhibition response depending on whether natural DNA or poly dA-T is used. Last, in DNA polymerization, chain elongation and not initiation is blocked by the antibiotic.

Furthermore, inhibition of DNA-polymerase I by lipiarmycin does not imply that the inhibition of DNA synthesis seen *in vivo* at high drug concentration is a consequence of such activity. DNA-polymerase I, in fact, is not essential for DNA synthesis *in vivo*.

These data do not allow us to decide definitively whether lipiarmycin affects the template or the enzyme function in the synthesis of nucleic acids. The specificity of its inhibitory effect on initiation in RNA synthesis would suggest a possible binding to the RNA-polymerase, as has been found to be the case with other initiation-specific inhibitors, such as the rifamycins,[6,7] the related streptovaricins,[8] phenanthroline[10] and, apparently, the acidic dyes, Congo Red,[11,12] Gallin[12] and aurintricarboxylic acid.[13] On the other hand, a preferential inhibition of RNA chain initiation has also been reported for drugs interacting with DNA, such as proflavine[15] and distamycin,[14] so that alternative can not be excluded on this basis alone. However, attempts to show interaction of the antibiotic with DNA were unsuccessful, which would seem to favor the hypothesis that the inhibitor acts on the enzyme.

Acknowledgements

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