LIPIARMYCIN, A NEW ANTIBIOTIC FROM ACTINOPLANES

III. MECHANISM OF ACTION

SOMMA SERGIO, G. PIRALI, R. WHITE* and F. PARENTI

Research Laboratories, Lepetit S.p.A., Milano, Italy

(Received for publication April 21, 1975)

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

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 li-parmycin 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.

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 li-parmycin, 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. 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 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 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 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...
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
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) phenanthroline9) and, apparently, the acidic dyes, Congo Red,11,12) Gallin12) 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 proflavine13) 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

We wish to thank Mr. L. GASTALDO for his skilful technical assistance and Mr. G. SARTORI for performing the in vivo uptake experiments. Dr. G. C. LANCINI is gratefully acknowledged for helpful discussions.

References