NOTE

COMPARATIVE STUDIES ON THE ACTIVITIES OF LIVIDOMYCIN 5''-PHOSPHATE AND LIVIDOMYCIN ON POLYPEPTIDE SYNTHESIS DIRECTED BY POLY U IN *E. COLI* CELL-FREE EXTRACTS

MASAHITO YAMAGUCHI, FUJIO KOBAYASHI and SUSUMU MITSUHASHI*

Tokyo Research Laboratories, Kowa Co., Ltd., Higashimurayama, Tokyo, Japan
*Department of Microbiology, School of Medicine, Gunma University, Maebashi, Japan

(Received for publication April 23, 1973)

It has been reported that aminoglycosidic antibiotics inhibit protein synthesis in bacteria by interaction of the antibiotics with the ribosomes[1,2] and also cause codon misreading[3]. Lividomycin (LV)[4], a new aminoglycoside, shows antibacterial activity against various species of bacteria including *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis*[5], and inhibits poly U-directed polyphenylalanine synthesis in *Escherichia coli* cell-free system and stimulates the incorporation of leucine or serine under the same condition[6,7] In another study, it has been shown that LV was phosphorylated by an enzyme obtained from resistant bacteria, resulting in the loss of antibacterial activity[8]. This paper deals with the activity of lividomycin 5''-phosphate (p-LV)[9,10] on polynucleotide directed polypeptide synthesis in comparison with that of LV.

Materials and Methods

Antibiotics and chemicals: LV and adenosine 5'-triphosphate (ATP) were supplied from the Kowa Co., Ltd., Tokyo. P-LV, a product phosphorylated by a resistant strain (*E. coli* ML 1410 R_{m81}^{+}), was isolated by the procedure described in a previous paper[9]. Used ^{14}C-labeled amino acids were purchased from the Daiichi Pure Chemicals Co., Tokyo. Polyuridylic acid (5') (poly U), polyadenylic acid (5') (poly A) and guanosine 5'-triphosphate (GTP) were obtained from the Sigma Co., U.S.A.

Preparation of cell-free extracts and ribosomes from *E. coli*: *E. coli* ML 1410 was grown with shaking at 37°C for 4 hours in glucose broth (0.3% glucose, 0.4% meat extract, 0.5% NaCl and 1% Polypeptone). Supernatant fluid (dialyzed S-100 and preincubated S-30) and washed ribosomes were prepared from the cells at exponential growth phase (O.D. 0.7 at 650 mμ) according to the method of NIRENBERG and MATTHAEI[11] except that cells were disrupted with a sonicator.

Preparation of tRNA and ^{14}C-lysyl-tRNA: Transfer RNA was isolated by phenol extraction from *E. coli* ML 1410 cells by the method of VON EHRENSTEIN[12]. ^{14}C-Lysyl-tRNA was prepared by incubating tRNA with ^{14}C-lysine, dialyzed S-100 and ATP as reported by VON EHRENSTEIN and LIPMANN[13].

Binding of ^{14}C-lysyl-tRNA to poly A-ribosome complex: The amount of the bound ^{14}C-lysyl-tRNA was determined by the method of NIRENBERG and LEDER[14].

Assay of ^{14}C-amino acid incorporation in *E. coli* cell-free system: The reaction mixture for polypeptide synthesis was the same as that described previously[11]. The reaction was terminated by addition of 1.5 ml of 10% trichloroacetic acid (TCA). After heating the reaction mixture at 90°C for 15 minutes, the insoluble materials were filtered and washed twice with cold 5% TCA and the radioactivity was counted.

Results and Discussion

Effects on the formation of aminoacyl-tRNA and on the binding of lysyl-tRNA to the poly A-ribosome complex:

^{14}C-Aminoacyl-tRNA was formed by incubating tRNA with ^{14}C-amino acids in the presence of both the dialyzed S-100 and ATP. No inhibition was seen on the formation of phenylalanyl-, arginyl-, lysyl- or prolyl-tRNA at a concentration of 2×10^{-3}M of p-LV and LV. P-LV and LV did not show any significant effects on the activity of aminoacyl-tRNA synthetase. The binding of ^{14}C-lysyl-tRNA to the poly A-ribosome complex was investigated and the result is shown in Table 1. P-LV was found to stimulate slightly the binding of lysyl-tRNA to ribosomes in the presence of poly A. The stimulatory activity of p-LV was weaker than that of LV. These stimulations did not show any dose-response,

Table 1. Effects of p-LV and LV on the binding of lysyl-tRNA to the poly A-ribosome complex

Antibiotic (M)	cpm on filter	%
Complete	4,598	100
-poly A	986	21
p-LV 2×10^{-5}	6,013	131
2×10^{-4}	5,767	125
LV 2×10^{-6}	6,463	141
2×10^{-5}	7,612	166
2×10^{-4}	7,248	158

The complete reaction mixture contained the following materials in 0.5 ml of 0.1 M Tris-HCl buffer (pH 7.2): NH_4Cl 30 μmoles, $Mg(C_2H_3O_2)_2$ 7 μmoles, β-mercaptoethanol 3 μmoles, washed ribosomes 2.0 mg, poly A 50 μg and ^{14}C-lysyl-tRNA 41,000 cpm. The mixture was incubated at 25°C for 20 minutes. Bound ^{14}C-lysyl-tRNA was determined by Millipore filter method described by NIRENBERG and LEDER[12].

suggesting nonspecific reactions. A similar result was also obtained with both drugs on the binding of ^{14}C-prolyl-tRNA to the poly C-ribosome complex.

Inhibition of polyphenylalanine synthesis:

As shown in Fig. 1, p-LV at 2×10^{-6}M had no inhibitory effect on polyphenylalanine synthesis directed by poly U, but an inhibitory effect was seen at concentrations greater than 2×10^{-5}M. On the other hand, polyphenylalanine synthesis was inhibited by low concentration (2×10^{-7}M) of LV and was reduced to 10% that of control by addition of 2×10^{-4}M of LV. The results indicate that p-LV was less effective than LV in inhibiting polyphenylalanine synthesis.

Codon misreading activity on polypeptide synthesis:

The incorporation of leucine or serine into polypeptide in the *E. coli* poly U system was studied. P-LV had no significant effects on leucine-incorporation at a concentration of 2×10^{-3}M. However, the drug stimulated serine-incorporation at the same concentration and the minimum stimulatory dose of p-LV was approximately 2×10^{-5}M (Table 2). On the contrary, LV stimulated the incorporation of leucine and serine at a concentration of 2×10^{-6}M, indicating that LV possessed codon misreading activity (Table 3). The data reveal that there is an optimum concentration of LV ($2\times10^{-6}\sim2\times10^{-5}$M) for codon misreading activity in the

Fig. 1. Inhibition of poly U-directed polyphenylalanine synthesis by p-LV and LV

The reaction mixture contained the following materials in 0.5 ml of 0.02 M Tris-HCl buffer (pH 7.8): NH_4Cl 30 μmoles, $Mg(C_2H_3O_2)_2$ 7 μmoles, β-mercaptoethanol 3 μmoles, preincubated S-30 3.0 mg protein, *E. coli* tRNA 100 μg, poly U 20 μg, nineteen ^{12}C-amino acids (minus phenylalanine) 0.1 μmole (each amino acid), ATP 1 μmole, GTP 0.2 μmole, phosphocreatine 2.5 μmoles, creatine kinase 10 μg, ^{14}C-phenylalanine 0.2 μCi and antibiotic of indicated amount. The mixture was incubated at 37°C for 30 minutes and the radioactivity in the hot TCA-insoluble fraction was counted.

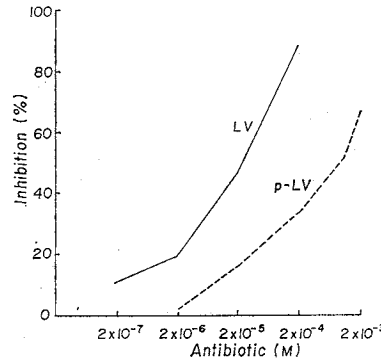

Table 2. Amino acid incorporation directed by poly U in the presence of p-LV

p-LV (M)	Phenylalanine		Leucine		Serine	
	cpm/mg protein	%	cpm/mg protein	%	cpm/mg protein	%
Complete	11,562	100	1,532	100	431	100
- poly U	156	1.3	132	9	124	30
2×10^{-6}	11,159	97	1,407	92	415	100
2×10^{-5}	9,671	84	1,516	99	473	115
2×10^{-4}	7,791	67	1,628	106	877	212
2×10^{-3}	3,712	32	1,556	102	1,531	371

The reaction mixture and method are the same as described in the legend of Fig. 1, except that other ^{14}C-amino acids were used.

Table 3. Amino acid incorporation directed by poly U in the presence of LV

LV (M)	Phenylalanine		Leucine		Serine	
	cpm/mg protein	%	cpm/mg protein	%	cpm/mg protein	%
Complete	12,531	100	1,510	100	494	100
- poly U	191	1.5	151	10	135	27
- poly U 2×10^{-5}	165	1.4	149	10	161	33
2×10^{-7}	11,196	89	2,977	197	2,538	514
2×10^{-6}	10,182	81	4,120	223	4,956	1,003
2×10^{-5}	6,664	53	3,492	231	4,939	1,000
2×10^{-4}	1,280	10	1,288	85	813	165

The reaction mixture and method are the same as described in the legend of Fig. 1, except that other ^{14}C-amino acids were used.

E. coli poly U system. It was found further that polypeptide synthesis with poly U was inhibited with 2×10^{-4}M or higher concentrations of LV and that the minimum concentration required for codon misreading was about 6×10^{-8}M. Therefore, the misreading activity of p-LV is approximately 330-fold weaker than that of LV. The decrease of activity of p-LV is not understood, but it may be due to weakened interaction of the drug with ribosomes as a result of the change of charge and configuration in the LV molecule following 5″-phosphorylation.

From a study of the synthesis of 5″-deoxy LV and its amino derivative, YAMAMOTO *et al*[15] suggested that the 5″-hydroxyl group of LV was a requisite for antibacterial activity. The fact that 5″-phosphorylation of LV causes loss of antibacterial activity confirms the importance of the 5″-hydroxyl group of LV.

Acknowledgment

Authors are greatly indebted to Prof. S. YAMAGISHI, Faculty of Pharmaceutical Science, Chiba University, and Mr. H. MORI, the Tokyo Research Laboratories, Kowa Co., Ltd., for their useful advice and supports during this study.

References

1) LUZZATTO, L.; D. APIRION & D. SCHLESSINGER: Mechanism of action of streptomycin in *E. coli*. Interruption of the ribosome cycle at the initiation of protein synthesis. Proc. Nat. Acad. Sci. 60 : 873~880, 1968
2) WALLANCE, B.J. & B.D. DAVIS: Cyclic blockade of initiation sites by streptomycin-damaged ribosomes in *Escherichia coli*: an explanation for dominance of sensitivity. J. Mol. Biol. 75 : 377~390, 1973
3) DAVIES, J.; L. GORINI & B.D. DAVIS: Misreading of RNA code-words induced by aminoglycosidic antibiotics. Mol. Pharmacol. 1 : 93~106, 1965
4) ODA, T.: T. MORI, Y. KYOTANI & M. NAKAYAMA: Studies on new antibiotic lividomycins. IV. Structure of lividomycin A. J. Antibiotics 24 : 511~518, 1971
5) KOBAYASHI, F.; T. NAGOYA, Y. YOSHIMURA, K. KANEKO, S. OGATA & S. GOTO: Studies on new antibiotic lividomycins. V. *In vitro* and *in vivo* antimicrobial activity of lividomycin A. J. Antibiotics 25 : 128~136, 1972
6) MACHIYAMA, N.: Mechanism of action of lividomycin A, a new aminoglycosidic antibiotic. J. Antibiotics 24 : 706~707, 1971
7) YAMAGUCHI, M.; J. EDA, F. KOBAYASHI & S. MITSUHASHI: Mode of action of lividomycin on protein synthesis in *Escherichia coli*. Antimicr. Agents & Chemoth. in press
8) MITSUHASHI, S.; F. KOBAYASHI, M. YAYAGUCHI, K. O'HARA & M. KONO: Enzymatic inactivation of aminoglycoside antibiotics by resistant strains of bacteria; in bacterial plasmids and antibiotic resistance. pp. 337~341, Avicenum, Prague; Springer, Heidelberg, 1972
9) YAMAGUCHI, M.; T. KOSHI, F. KOBAYASHI & S. MITSUHASHI: Phosphorylation of lividomycin by *E. coli* carrying an R factor. Antimicr. Agents & Chemoth. 2 : 142~146, 1972
10) YAMAMOTO, H.; S. KONDO, K. MAEDA & H. UMEZAWA: Synthesis of lividomycin A 5″-phosphate, an enzymatically inactivated lividomycin A. J. Antibiotics 25 : 485~486, 1972
11) NIRENBERG, M.W. & J.H. MATTHAEI: The dependence of cell-free protein synthesis in *E. coli* upon naturally occurring or synthetic polynucleotides. Proc. Nat. Acad. Sci. 57 : 759~766, 1961

12) VON EHRENSTEIN, G.: Isolation of sRNA from intact *Escherichia coli* cells. in "Method in Enzymology" 12 : 588~596, Acad. Press, New York and London, 1968
13) VON EHRENSTEIN, G. & F. LIPMANN: Experiments on hemoglobin biosynthesis. Proc. Nat. Acad. Sci. 47 : 941~950, 1961
14) NIRENBERG, M. W. & P. LEDER: RNA codewords and protein synthesis. The effect of trinucleotides upon the binding of sRNA to ribosomes. Science 145 : 1399~1407, 1964
15) YAMAMOTO, H.; S. KONDO, K. MAEDA & H. UMEZAWA: Synthesis of 5″-deoxylividomycin A and its amino derivative. J. Antibiotics 25 : 487~488, 1972