METABOLIC PRODUCTS OF MICROORGANISMS 133* INHIBITION OF RIBOSOMAL PEPTIDYL TRANSFERASE BY HIKIZIMYCIN, A NUCLEOSIDE ANTIBIOTIC

KENJI UCHIDA and HEINZ WOLF

Institut für Biologie II, Lehrbereich Mikrobiologie I, Universität Tübingen, D-74 Tübingen Auf der Morgenstelle 28, Germany

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The nucleoside antibiotic hikizimycin was found to inhibit protein biosynthesis in intact cells of *Pseudomonas syringiae* and the poly U-directed polyphenylalanine biosynthesis in a cell-free system of *E. coli*. The antibiotic did not affect the transfer of phenylalanine to tRNA or the attachment of phenylalanyl-tRNA to the ribosome·poly U complex. Hikizimycin acts as a marked inhibitor in the puromycin reaction.

The nucleoside antibiotic hikizimycin ($C_{21}H_{37}N_5O_{14}$) inhibits the growth of some bacteria and some fungi. The isolation and characterization[2] as well as the partial structure[3,4] of hikizimycin have already been reported. The total structure of the antibiotic[5] is now proposed as 1-N-[2-O-(3-amino-3-deoxy-β-D-glucopyranosyl)-4-amino-4-deoxy-D-glycero-D-galacto-D-gluco-β-D-undecopyranosyl] cytosine, which is characteristically analogous to the so-called aminoacyl-4-aminohexosyl cytosine antibiotics, known as inhibitors acting on 50 S subunit of ribosome in the process of protein biosynthesis.[6]

This observation led us to evaluate the effect of hikizimycin on the protein biosynthesis in *in vivo* and *in vitro* system.

The present paper describes the effects of hikizimycin on the protein biosynthesis in intact cells of *Pseudomonas syringiae* and cell-free systems of *Escherichia coli*.

Materials and Methods

1. Materials: Ribosomes and supernatant enzymes (S-100 fraction) from *E. coli* A19,[7] a RNAase I-less mutant, were prepared as described.[8] After high-speed centrifugation, a fraction of the uppermost supernatant fluid was dialyzed with 0.01 M tris-HCl buffer (pH 7.6) containing 0.01 M magnesium acetate and 0.006 M 2-mercaptoethanol. The ribosomes were suspended in 0.01 M magnesium acetate. For the binding assay ribosomes were washed three times with 0.01 M tris-HCl buffer (pH 7.6) containing 0.01 M magnesium acetate and 0.5 M NH_4Cl. All collected enzymes and ribosomal particles were kept until use at −65°C. One A_{260} unit was taken to represent 25 pmole of 70 S ribosomes.[9]

[^{14}C]-Phenylalanyl-tRNA was prepared from *E. coli* MRE 600 tRNA (Boehringer, Mannheim, Germany) and [^{14}C]-phenylalanine as described.[10] The product was treated with acetic anhydride by the method of HAENNI and CHAPEVILLE[11] to obtain N-acetyl-[^{14}C]-phenylalanyl-tRNA. The acetylated preparation was passed through Sephadex G-25 and precipitated.

[^{14}C]-Isoleucine (338 Ci/mole), [^{14}C]-phenylalanine (225 Ci/mole), and [^{14}C]-uracil (62 Ci/mole) were purchased from the Radiochemical Centre, Amersham, England.

Hikizimycin was isolated according to methods described in a previous paper,[2] gougerotin

* Metabolic products of microorganisms, 132; publication see (1).

was purchased from Calbiochem.

2. Synthesis of cellular macromolecules: Isotopic incorporation studies were performed by addition of 2.5 μCi [^{14}C]-isoleucine or 2.5 μCi [^{14}C]-uracil to growth cuvettes containing 7 ml synthetic medium,[12] which was inoculated with enough log phase *Pseudomonas syringiae* ATCC 19310 cells to give a turbidometrically-determined concentration of 10^7 cells per ml. The growth cuvettes were then agitated at 27°C in a biophotometer (Jouan, Paris). At various times (see Fig. 1) during the growth period, a 0.2-ml sample of the cell suspension was withdrawn and then added to 1 ml of cold 7 % trichloroacetic acid. The precipitate formed was collected on a membrane filter (0.45 μ pore size), washed with 5 % trichloroacetic acid, dried, and the radioactivity measured.

3. Protein biosynthesis: Experiments for polyphenylalanine biosynthesis were carried out by a method similar to that of TRAUB *et al.*[13] Each incubation mixture (50 μl) contained: 0.01 M tris-HCl pH 7.8, 0.03 M NH_4Cl, 0.01 M magnesium acetate, 0.006 M 2-mercaptoethanol, 0.001 M ATP, 0.03 mM GTP, 0.05 mM 19 amino acids (omitting phenylalanine), 0.005 M phosphoenolpyruvic acid, 1 μg pyruvate kinase, 0.02 μCi [^{14}C]-phenylalanine, 10 μg poly U, 5 μl enzyme fraction (S-100), and 0.2 A_{260} unit of 70 S ribosomes. The reaction mixture was incubated for 45 minutes at 30°C. The incorporation of radioactivity into hot 5 % trichloroacetic acid-precipitable material was measured by assaying a sample (40 μl) of the reaction mixture by the paper disk method.[14]

4. Determination of aminoacyl-tRNA binding to ribosomal particles: The binding of [^{14}C]-phenylalanyl-tRNA to ribosomes was assayed as described.[15] Fifty μl binding buffer (0.1 M tris-acetate pH 7.2, 0.01 or 0.02 M magnesium acetate, 0.05 M KCl) contained 2 A_{260} units of washed ribosomes, 20 μg poly U, and 0.01 μCi [^{14}C]-phenylalanyl-tRNA. The reaction mixture was incubated for 10 minutes at 24°C, then diluted by addition of 3 ml of ice-cold binding buffer, and filtered through a Millipore filter (HA 0.45 μ pore size). The filter was dried, and the radioactivity measured.

5. Puromycin reaction: The formation of N-acetyl-[^{14}C]-phenylalanyl-puromycin was assayed by the method of LEDER and BURSZTYN.[16] Each reaction mixture (50 μl) contained: 0.1 M tris-acetate (pH 7.2), 0.05 M KCl, 0.01 M magnesium acetate, 20 μg poly U, N-acetyl-[^{14}C]-phenylalanyl-tRNA (4,400 cpm), and 2.4 A_{260} units of ribosomes. The reaction mixture was incubated at 30°C for 40 minutes. After 10 minutes of the incubation time, 0.05 μmoles puromycin contained in 5 μl water was added.

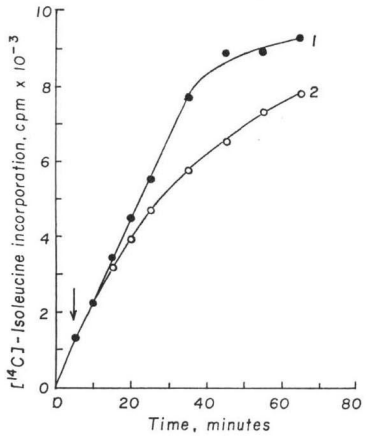
Results

The effect of hikizimycin on the incorporation of [^{14}C]-isoleucine and [^{14}C]-uracil by intact cells of *Pseudomonas syringiae* was determined. The incorporation of [^{14}C]-thymidine could not be measured, because uptake was insufficient. Hikizimycin reduced isoleucine incorporation within less than 15 minutes after addition, whereas the rate of uracil incorporation remained essentially unchanged during the incubation period after addition of the antibiotic (Fig. 1). We inferred from these data that the primary action of hikizimycin consists in the inhibition of the protein biosynthesis. This conclusion was further confirmed by the following *in vitro* experiments.

As shown in Fig. 2, hikizimycin inhibited the poly U-directed polyphenylalanine biosynthesis in the *in vitro* system of *E. coli* completely at the concentration of 10^{-4} M. Fifty percent inhibition was observed at a ratio of 9 moles of hikizimycin/mole of ribosome. Gougerotin, which was tested in the same system for comparison, inhibited the peptide biosynthesis to the extent of approximately 13 % at the concentration of 10^{-4} M. However, 50 %

Fig. 1. Effect of hikizimycin on the incorporation of [^{14}C]-isoleucine and [^{14}C]-uracil by intact cells of *Pseudomonas syringiae* (time of addition of the antibiotic is indicated by the arrow. 1: control. 2: hikizimycin 0.8 mg/ml.

(a)



(b)

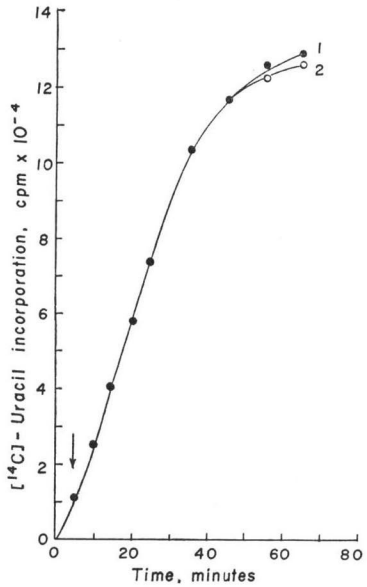


Fig. 2. Effect of concentrations of hikizimycin (1) and gougerotin (2) on the polyphenylalanine biosynthesis in the cell-free system of *E. coli*.

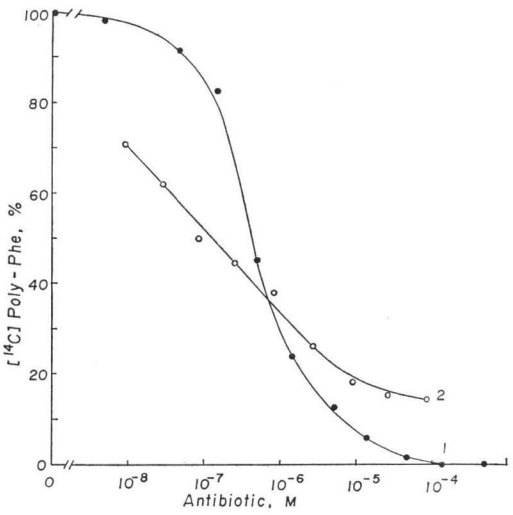
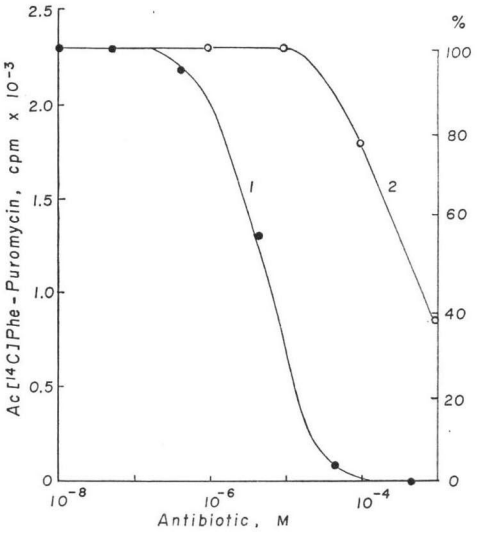


Fig. 3. Effect of concentrations of hikizimycin (1) and gougerotin (2) on the formation of N-acetyl-[^{14}C]-phenylalanyl-puromycin in the cell-free system of *E. coli*.



inhibition was obtained at a ratio of 2 moles of gougerotin/mole of ribosome. In addition, the effect of hikizimycin on partial reactions in order to locate the sensitive intermediate steps of the peptide chain elongation was studied.

As seen in Table 1, the formation of [^{14}C]-phenylalanyl-tRNA was not affected by the drug. Nonenzymatic binding of [^{14}C]-phenylalanyl-tRNA to ribosomes directed by poly U is known to take place predominantly at the *a*-site in 15~20 mM [Mg^{++}] and at the *p*-site in 5~10 mM [Mg^{++}][17,18]. The effect of hikizimycin on this reaction was studied by using the

Table 1. Effect of hikizimycin on the formation of [^{14}C]-phenylalanyl-tRNA (*E. coli*).

Hikizimycin added (M)	[^{14}C]-Phenylalanyl-tRNA synthesized	
	(cpm)	(%)
Control	2455	100
4.8×10^{-8}	2510	102
4.8×10^{-7}	2560	104
4.8×10^{-6}	2295	98
4.8×10^{-5}	2460	100
4.8×10^{-4}	2495	102

The assay was performed using the incubation mixture for polyphenylalanine biosynthesis without ribosomal particles. The incorporation of radioactivity into cold 5% trichloroacetic acid-precipitable material was measured.

filter technique of NIRENBERG and LEDER.[15] Hikizimycin did not show any inhibitory influence in our experiments with 10 and 20 mM [Mg^{++}] (see Table 2).

The synthesis of N-acetyl-[^{14}C]-phenylalanyl-puromycin is generally considered as a model system of peptidyl transfer reaction.[19] Hikizimycin was found to inhibit this reaction completely at a concentration of 10^{-4} M. Gougerotin, when tested in the same system, is less effective as an inhibitor.

The results here reported lead to the following conclusion: hikizimycin interferes with the peptide biosynthesis in the cell-free

Table 2. Effect of hikizimycin on the binding of [^{14}C]-phenylalanyl-tRNA to ribosomes (*E. coli*).

Hikizimycin added (M)	[^{14}C]-Phenylalanyl-tRNA bound to ribosomes at			
	10 mM [Mg^{++}]		20 mM [Mg^{++}]	
	(cpm)	(%)	(cpm)	(%)
Control	2640	100	3610	100
4.8×10^{-8}	2565	97	3640	101
4.8×10^{-7}	2690	102	3525	98
4.8×10^{-6}	2590	98	3625	100
4.8×10^{-5}	2860	108	3650	101
4.8×10^{-4}	2595	98	3570	99

system from *E. coli* by affecting the peptidyl transfer reaction. The inhibitory pattern points out the similarity of hikizimycin to the aminoacyl-4-aminohexosyl-cytosine group of antibiotics: gougerotin, blasticidin S, amicetin, bamicetin, and plicacetin.[6]

Discussion

Although some authors[20,21,22] have proposed structure-activity relationships for the aminoacyl-4-aminohexasyl cytosine antibiotics, very little is actually known about them. It is therefore interesting to note that hikizimycin, despite its identical mode of action and close structural analogy to these five antibiotics, still possesses distinctive structural features, which may give information to allow us to examine the structural requirements for activity.

These structural characteristics of hikizimycin compared to the other aminoacyl-4-aminohexosyl cytosine antibiotics may be summarized as follows: i) lack of amino acid components, ii) presence of N-free 4-aminoundecose (named hikosamine) instead of N-substituted 4-aminohexose, and iii) presence of a second aminosugar, 3-aminohexose (kanosamine).

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