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Alkaloid homoharringtonine inhibits polypeptide chain elongation on human ribosomes on the step of peptide bond formation

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The aim of the present study was to investigate homoharringtonine alkaloid effect on: (i) the nonenzymatic and eEF-1-dependent Phe-tRNA^{Phe} binding to poly(U)-programmed human placenta 80 S ribosomes; (ii) diphenylalanine synthesis accompanying nonenzymatic Phe-tRNA^{Phe} binding; and (iii) acetylphenylalanyl-puromycin formation. Neither nonenzymatic nor eEF-1-dependent Phe-tRNA^{Phe} binding were noticeably affected by the alkaloid, whereas diphenylalanine synthesis and puromycin reaction were strongly inhibited by homoharringtonine. It has been proposed that the site of homoharringtonine binding on 80 S ribosomes should overlap or coincide with the acceptor site of the ribosome.

Ribosome; tRNA; Homoharringtonine; Inhibition; Peptide bond

1. INTRODUCTION

Homoharringtonine and related alkaloids from *Cephalotaxus harringtonia* are known to be inhibitors of protein biosynthesis in some eukaryotic cells. It has been initially proposed that these compounds inhibit initiation in intact cells and cell lysates [1,2]. Further investigations clearly showed that such an effect was due to the prevention of the elongation cycle in contradiction to the previous proposals [3,4]. The results presented in the above-mentioned works suggested that *Cephalotaxus* alkaloids inhibited not only peptide bond formation in the fragment reaction assays but also enzymatic and non-enzymatic binding of the Phe-tRNA^{Phe} to ribosomes. In spite of being widely used in medicine as antitumour agents these compounds, however, have hardly been applied to research practice so far, since the mechanism of their action was not clear. Thus, it seems worthwhile investigating the mechanism of their inhibition of protein synthesis in eukaryotic systems, especially in human cells.

In the present paper we have studied an effect of homoharringtonine on: (i) nonenzymatic binding of individual Phe-tRNA^{Phe} simultaneously at A and P sites of poly(U)-programmed 80 S ribosomes from human placenta and diphenylalanine synthesis under these conditions; (ii) eEF1-dependent Phe-tRNA^{Phe} binding at the ribosomal A site; and (iii) acetylphenylalanyl-puromycin formation under treatment of the complex 80 S AcPhe-tRNA^{Phe} poly(U) with puromycin.

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2. MATERIALS AND METHODS

Poly(U) was purchased from Reanal (Hungary); puromycin from Fluka; [^{14}C]phenylalanine (318 Ci/mol) from UVVVR (Czechoslovakia). Enriched [^{14}C]Phe-tRNA^{Phe} (1400 pmol/A_{260} unit) and Ac-Phe-tRNA^{Phe} from *E. coli*-MRE-600 were prepared as in [5,6]. eEF-1α from the rabbit liver was kindly given to us by Dr S. Tuhachkova. Human placenta ribosomal subunits were prepared as described previously [7]. The subunits were reactivated for 10 min at 37°C in TKM buffer (13 mM $MgCl_2$, 0.6 mM EDTA, 120 mM KCl, 20 mM Tris-HCl, pH 7.5), mixed in the molar ratio of 40 S:60 S as 1:1.3. 1 A_{260} unit was assumed to be 50 pmol of 40 S and 25 pmol of 60 S subunits [8]. Poly(U)-dependent nonenzymatic binding of [^{14}C]Phe-tRNA^{Phe} (7×10^{-7} M, if not indicated otherwise) to 80 S ribosomes (1.65×10^{-7} M) was carried out in TKM buffer for 40 min at 0°C. Diphenylalanine formation was checked as in [9]. eEF-1-dependent [^{14}C]Phe-tRNA^{Phe} binding to 80 S ribosomes was performed in two steps. First, 7 pmol of 80 S ribosomes were incubated with 0.6 A_{260} unit of *E. coli* tRNA and 0.2 A_{260} unit of poly(U) for 5 min at 37°C in TKM buffer to block ribosomal P site. Second, a ternary complex was obtained by incubation of 4 μg of eEF-1α, 9 pmol of Phe-tRNA^{Phe} and 6 nmol of GTP in buffer (100 mM KCl, 3 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.5) at 0°C for 15 min. These two complexes were mixed and incubated at 0°C for 15 min. Phe-tRNA^{Phe} binding was tested using nitrocellulose filtration technique. Puromycin reaction was performed according to [10].

Homoharringtonine (Sigma) was converted to the HCl-form and dissolved in the TKM buffer. It was used in concentration 10^{-4} M in reaction mixtures; the ribosomes were preincubated with the alkaloid in the same concentration (unless otherwise specified) at 20°C, for 10 min.

3. RESULTS AND DISCUSSION

Binding capacity of poly(U)-programmed 80 S ribosomes was tested by their titration with [^{14}C]Phe-tRNA^{Phe} in the absence of translation factors (fig.1). Under the conditions of ribosomal saturation with Phe-tRNA^{Phe}, the amount of diphenylalanine formed was analysed (see table 1).

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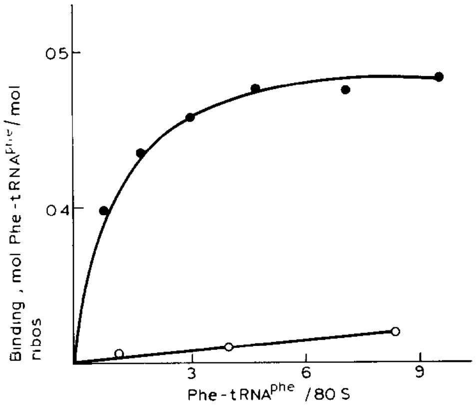

Fig.1. Titration of human placenta 80 S ribosomes with [^{14}C]Phe-tRNA^{Phe}. (●) With poly(U); (○) without poly(U).

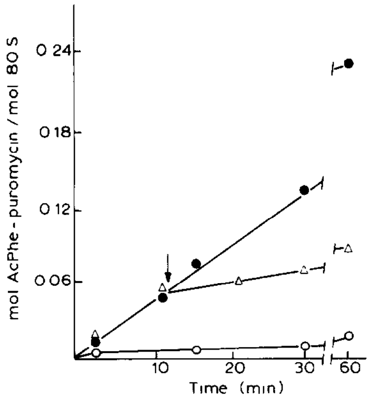

Fig.2. Kinetics of acetyl [^{14}C]phenylalanyl-puromycin formation (●), without homoharringtonine; (○) 80 S ribosomes were preincubated with the alkaloid; (△) homoharringtonine was added to the reaction mixture 10 min after puromycin addition (indicated with arrow). Binding of Ac[^{14}C]Phe-tRNA^{Phe} (1.6×10^{-7} M) to the 80 S ribosomes (1.6×10^{-7} M) and poly(U) (5 A_{260} units/ml) was carried out by incubation of the components in the TM buffer at 20°C for 20 min. The binding level of AcPhe-tRNA^{Phe} was about 0.25 mol/mol ribosomes. Puromycin treatment of the complex was performed at 37°C for 10 min.

It is seen that the maximum level of poly(U)-directed Phe-tRNA binding is about 0.7 mol/mol of 80 S ribosomes (fig.1). From the data presented in table 1 it is evident that most parts of the Phe residues bound to the ribosomes participated in $(Phe)_2$ formation. So, the majority of active ribosomes bound Phe-tRNA^{Phe} simultaneously at A and P sites and about 35% of the ribosomes were active in this binding. At a concentration of 10^{-4} M homoharringtonine does not significantly affect nonenzymatic Phe-tRNA^{Phe} binding to 80 S ribosomes, but almost completely inhibits transpeptidation (diphenylalanine formation, see table 1). The alkaloid slightly affects not only nonenzymatic but also eEF-1α dependent Phe-tRNA^{Phe} binding (see table 1).

We have also studied inhibitory action of homoharringtonine on acetylphenylalanyl-puromycin formation when complex 80 S poly(U) Ac[^{14}C]Phe-tRNA^{Phe} was treated with puromycin. The complex was obtained at relatively low AcPhe-tRNA^{Phe} concentration; earlier in the same system on *E. coli* ribosomes it was shown that under such conditions, binding was preferable at the puromycin-reactive P site [11]. As can be seen from fig.2, AcPhe-puromycin formation is strongly inhibited in the case where homoharringtonine has been added to the complex 80 S poly(U) AcPhe-tRNA^{Phe} upon the addition of puromycin and is almost completely inhibited in the case where 80 S ribosomes have been preincubated with the alkaloid before the complex formation.

Table 1

Homoharringtonine effect on: (i) nonenzymatic [^{14}C]Phe-tRNA binding to poly(U)-programmed 80 S ribosomes from human placenta and diphenylalanine synthesis; and (ii) eEF-1-dependent [^{14}C]Phe-tRNA binding to the ribosomal P site

Ribosomes	Nonenzymatic binding		eEF-1-dependent binding	
	Binding level (mol Phe-tRNA/mol ribosomes)	Phe synthesis (mol Phe/mol ribosomes)	With eEF-1	Without eEF-1
Untreated	0.70	0.30	0.30	0.05
Preincubated	0.56	0.02	0.24	0.05

So, homoharringtonine selectively inhibits peptide bond formation and almost does not hinder codon-dependent tRNA^{Phe} binding both nonenzymatically and eEF-1-dependently at ribosomal A and P sites. Since, the alkaloid inhibits AcPhe-puromycin formation independently of time of addition, one may suggest that the site of homoharringtonine binding should overlap or coincide with the acceptor site of the peptidyl transferase centre of ribosomes from human placenta.

The obtained data differ from the results reported earlier on ribosomes from rabbit reticulocytes [3]. According to [3], *Cephalotaxus* alkaloids prevent not only transpeptidation but also tRNA binding to ribosomes (both nonenzymatic and enzymatic). Probably, the mechanism of the alkaloids' inhibitory action depends on the source of ribosomes. But it is more reasonable to suggest that the observed differences should be due to different ways of alkaloid dissolution: in [3] it was dissolved in the form of a base in dimethyl sulfoxide and this solution was added to ribosomes, while in the present paper, homoharringtonine was converted into the HCl form in aqueous solution.

There is no set of antibiotics selectively inhibiting definite steps of the eukaryotic elongation cycle in contrast to prokaryotic one. Therefore homoharringtonine may be useful as a selective inhibitor of transpeptidation in the studies of different states of tRNA on ribosomes in different model systems imitating various steps of the elongation cycle.

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