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Inhibition of Protein Synthesis by RA-VII[1]

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The antitumor natural product RA-VII has been evaluated as an inhibitor of protein synthesis *in vitro*. Complete inhibition of protein synthesis in rabbit reticulocyte lysates is observed with 5 μM RA-VII. Mechanistic studies using purified elongation factors and ribosomes identify RA-VII as a peptidyltransferase inhibitor. Thus, similar to the related natural products bouvardin and RA-XII, RA-VII appears to function by binding to eukaryotic ribosomes. © 1995 Academic Press, Inc.

INTRODUCTION

The cyclic peptides, called RA-peptides, isolated from plants of the *Rubiaceae* family, display potent antineoplastic activity against carcinomas of the cervix, testicles, kidney, colon, breast, and lung. RA-VII (Fig. 1) has been selected for phase-I clinical evaluation (*1, 2*).

The structures of the RA peptides were proposed based on NMR experiments and total synthesis (*3–11*), and conformational analyses have provided three-dimensional models (*12–16*). The isodityrosine moiety is thought to be the active pharmacophore of these molecules, based on *in vitro* assays of model compounds (*8, 17–19*). Apparently, the remainder of the peptide acts as a conformational constraint enforcing a *cis* amide linkage between the two tyrosine residues, which is essential for cytotoxicity.

RA peptides are believed to function *in vivo* by inhibition of protein biosynthesis (*2*). Evaluation of both RA-VII and the closely related natural product bouvardin (*20*), indicates that these compounds prevent the incorporation of radiolabeled amino acids into proteins in intact cells and eukaryotic cell lysates (*1, 21, 22*). Furthermore, studies of bouvardin *in vitro* indicate that this compound inhibits both the aminoacyl-tRNA binding and the translocation steps of protein synthesis, probably by binding directly to the ribosome (*22*). We have studied the mechanism of action of RA-VII using *in vitro* biochemical assays. Our results indicate that unlike bouvardin, RA-VII has no effect upon aminoacyl-tRNA binding, but inhibits peptide bond formation. No effect of RA-VII upon translocation has been observed. We propose that RA-VII also interacts directly with 80S ribosomes.

[1] Dedicated to Jeremy R. Knowles on the occasion of his 60th birthday.

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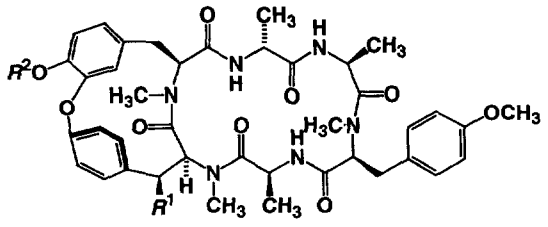

	R^1	R^2
Bouvardin	OH	H
RA-V	H	H
RA-VII	H	CH_3
RA-XII	H	1-β-D-Glc

FIG. 1. RA-peptides. The structures of three peptides from *Rubiaceae akane* and a related natural product from *Bouvardia ternifolia*.

METHODS

Materials

L-[^{35}S]Methionine (1000 Ci mmol^{-1}) and [^{14}C]-Phenylalanine (>450 mCi mmol^{-1}; ~1000 cpm pmol^{-1}) were obtained from New England Nuclear (Wilmington, DE). Rabbit reticulocyte lysates were obtained from Boehringer Mannheim (Indianapolis, IN) or from Green Hectares (Oregon, WI). Tobacco mosaic virus (TMV) mRNA and poly(U) were purchased from Boehringer Mannheim. Liquid scintillation counting was performed using Scintiverse (5 ml per sample. Fisher Scientific: Pittsburgh, PA) in a Packard 1600 TR liquid scintillation spectrometer. Sparsomycin was a gift from Mr. Don Harper of the Upjohn Chemical Co. and was prepared as a stock solution in DMSO (2.5 mM). RA-VII was provided by Professor Hedeji Itokawa and was prepared as a stock solution in DMSO (12.5 mM). Yeast eEF-1α was isolated following the procedure reported by Thiele and coworkers (*23*). Yeast eEF-2 was provided by Dr. Kalpana Chakraburtty (University of Wisconsin). Rabbit 80S ribosomes, Phe-tRNA^{Phe} and Ac-Phe-tRNA^{Phe} were prepared as described previously (*24–26*).

Protein Synthesis Assays

In vitro translation was performed using commercially prepared cell-free translation systems as described in the manufacturer's instructions (Boehringer Mannheim), except that reaction mixtures (25 μl) contained inhibitor solution (1 μl) and TMV mRNA (4 μl of 40 μg ml^{-1} stock). Protein synthesis was initiated by the addition of TMV mRNA and the reaction mixtures were incubated at 37°C for 60

min unless otherwise specified. The amount of protein synthesized was determined by precipitation of an aliquot of the reaction mixture onto a glass fiber filter (Whatman GF/C) using 10% TCA containing 2% casamino acids. The filter was dried at 60°C for 10 min and the precipitated protein was measured by scintillation counting.

Peptidyltransferase

Peptidyltransferase activity was assayed using the puromycin reaction as described by Busiello and coworkers (27). Sucrose-cushioned 80S ribosomes (26 pmol), GTP (1 mM, pH 7.0), poly(U) (20 μg), and Ac-[^{14}C]-Phe-tRNA^{Phe} (26 pmol) in buffer A (100 μl) containing 50 mM Tris–HCl (pH 7.5), 80 mM KCl, 10 mM $MgCl_2$, and 2.5 mM DTT were incubated at 37°C for 20 min. To measure the amount of Ac-[^{14}C]-Phe-tRNA^{Phe} bound to the ribosomes, a 10-μl aliquot of this mixture was removed, diluted with buffer A (300 μl), and filtered through a Millipore type HA nitrocellulose filter. To the remaining mixture (90 μl) was added 10× concentrated Hepes buffer (14 μl) [Hepes–KOH (200 mM, pH 7.5), KCl (1.0 M), DTT (10 mM), and $MgCl_2$ (100 mM)], GTP (15 μl of a 10 mM solution, pH 7.0), eEF-2 (0.5 μl of a 0.79 mg ml^{-1} stock), and H_2O (13 μl). The assay mixture was incubated at 37°C for 20 min. A sample of inhibitor stock solution or carrier (15 μl) was added and incubation was continued at 37°C for 15 min. Puromycin was added (15 μl of a 10 mM solution, pH 7.0) and following further incubation at 37°C for 20 min, NaOAc (0.3 M, 300 μl) was added and the Ac-Phe-puromycin was extracted (vortexed then centrifuged) into ethyl acetate (800 μl). The amount of radioactivity in the organic extract was measured by scintillation counting of a 600-μl aliquot. In a typical experiment, approximately 20% of the Ac-Phe-tRNA^{Phe} binds to ribosomes, and 25% of the bound Ac-Phe-tRNA^{Phe} is reactive with puromycin, indicating that it is located in the ribosomal P-site.

Peptide bond formation between Ac-Phe-tRNA^{Phe} and Phe-tRNA^{Phe} was assayed in 100-μl reaction mixtures containing sucrose-cushioned ribosomes (14 μl, 50 pmol), poly(U) (20 μg), GTP solution (10 μl of a 10 mM stock, pH 7.0), yeast eEF-1α (2 μg of a 200 μg ml^{-1} stock), eEF-2 (0.5 μl of a 0.79 mg ml^{-1} stock), and Ac-[^{14}C]-Phe-tRNA^{Phe} (26 pmol) in buffer [Tris–HCl (50 mM, pH 7.5), KCl (80 mM), $MgCl_2$ (10 mM) DTT (2.5 mM)]. The mixtures were incubated at 37°C for 20 min. A 10-μl aliquot of each mixture was used to measure the amount of Ac-[^{14}C]-Phe-tRNA^{Phe} bound to ribosomes by dilution into buffer (0.4 ml) and filtration through a nitrocellulose filter, which was rinsed, dried, and counted as before. The amount of P-site bound Ac-[^{14}C]-Phe-tRNA^{Phe} was determined for an identical sample using the puromycin reaction. Mixtures were quenched with NaOAc (400 μl, 0.1 M, pH 5.5) and extracted with ethyl acetate (800 μl) by vortexing. The amount of Ac-[^{14}C]-Phe-puromycin formed was measured by scintillation counting of 600 μl of the organic layer. Following binding of Ac-[^{14}C]-Phe-tRNA^{Phe} to the ribosomal P-site, inhibitor solution (10 μl) was added and the mixtures were incubated at 37°C for 15 min. To each sample was added [^{14}C]-Phe-tRNA^{Phe} (20 pmol) yeast eEF-1α (10 μl of a 200 μg ml^{-1} stock), buffer (10 μl, 10× previous concentrations), KCl (14 μl of a 280 mM solution), enough inhibitor solution to maintain the same concentration, and H_2O, increasing the volume of each assay to 200 μl. These

samples were incubated at 37°C for a further 20 min and a 20-μl aliquot was precipitated onto nitrocellulose as described earlier to estimate the amount of ribosome-bound ^{14}C-label. The remainder of the reaction mixture was diluted with 2 vol of KOH (0.3 M) and incubated at 50°C for 15 min. The hydrolyzed samples were adjusted to pH 7.0 with acetic acid (20 μl) and supplemented with unlabeled Phe, Ac-Phe and Ac-Phe-Phe (prepared from commercially available phenylalanine). The Phe, Ac-Phe, and Ac-Phe-Phe were separated by HPLC and the labeled material was quantified by scintillation counting. HPLC was performed on a Waters system 625 LC equipped with a 3.9 × 150 mm Delta Pak C-18 column and a Waters 994 photodiode array detector, eluting with a convex gradient of 0–65% acetonitrile in 10 mM K_2HPO_4, pH 2.1, at 0°C; elution times: Phe, 9.5 min; Ac-Phe, 13.2 min; Ac-Phe-Phe, 14.8 min (*28*). Greater than 75% of the label was recovered in each experiment.

Aminoacyl-tRNA Binding

Elongation factor-1α-dependent aminoacyl-tRNA binding experiments were performed following the procedures outlined by Merrick (*24*). Reaction mixtures (50 μl) containing Hepes-buffer [Hepes–KOH, (20 mM, pH 7.5), $MgCl_2$ (10 mM), KCl (100 mM), and DTT (1.0 mM)], poly(U) (20 μg), 80S ribosomes (50 pmol), [^{14}C]-Phe-tRNA^{Phe} (15 pmol), yeast eEF-1α (10 μl of a 200 μg ml^{-1} stock), and GTP (5 μl of a 10 mM solution, pH 7.0) were incubated at 37°C for 30 min, in the presence of different concentrations of inhibitor. To determine the amount of ribosome-bound [^{14}C]-Phe-tRNA^{Phe}, an aliquot (20 μl) of the assay mixture was removed, diluted with 0.3 ml Hepes-buffer (0.3 ml), and filtered through a Millipore type HA nitrocellulose filter. The filter was rinsed with Hepes-buffer (5 × 2 ml), dried at 60°C for 10 min, and counted.

To assay the binding of Ac-[^{14}C]-Phe-tRNA^{Phe}, 80S ribosomes (26 pmol) were mixed with Ac-[^{14}C]-Phe-tRNA^{Phe} (13 pmol), poly(U) (20 μg), GTP (1 mM), eEF-1α (10 μl of a 200 μg ml^{-1} stock), and inhibitor in a buffer containing Tris–HCl (50 mM, pH 7.5), KCl (80 mM), $MgCl_2$ (10 mM), and DTT (2.5 mM). Following incubation at 37°C for 30 min, a 40 μl aliquot of the assay mixture was removed, diluted into Tris–HCl buffer (0.4 ml) and filtered through a type HA nitrocellulose filter. The filter was rinsed with buffer (2 × 5 ml), dried at 60°C for 10 min, and counted to measure the amount of ribosome-bound Ac-[^{14}C]-Phe-tRNA^{Phe}.

Translocation

Inhibition of translocation was determined following the general approach described by Busiello and coworkers (*27*). Elongation factor dependent Ac-[^{14}C]-Phe-tRNA^{Phe} binding to 26 pmol ribosomes was performed as described above. The amount of P-site bound Ac-[^{14}C]-Phe-tRNA^{Phe} was determined for an identical sample using the puromycin reaction. Approximately 25% of the bound Ac-[^{14}C]-Phe-tRNA^{Phe} was puromycin active, suggesting that most of the bound Ac-[^{14}C]-Phe-tRNA^{Phe} is in the ribosomal A-site. Inhibitor (10 μl of a 10 mM solution in DMSO), or fusidic acid (10 μl of a 30 mM solution in H_2O) was added to the assay mixture, which then was incubated at 37°C for 10 min. Next, eEF-2 (0.5 μl of a

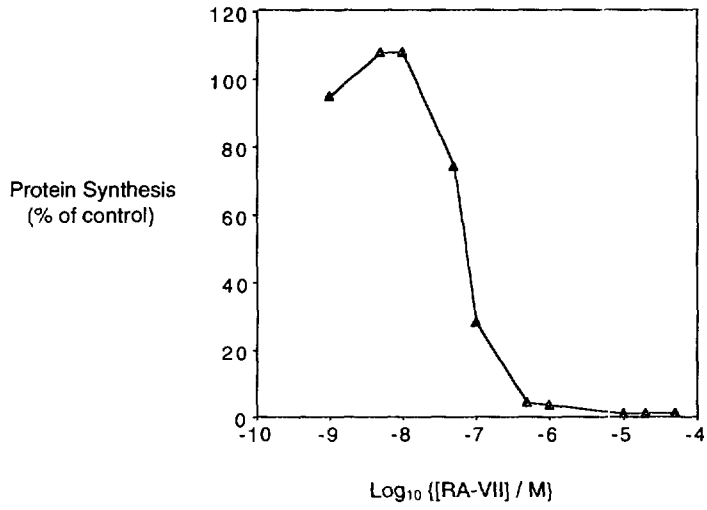

FIG. 2. Concentration-dependence plot for inhibition of *in vitro* protein synthesis by RA-VII. The extent of protein synthesis is determined by measuring the amount of [^{35}S]methionine incorporated into tobacco mosaic virus proteins in a rabbit reticulocyte lysate. Results are expressed as a percentage of protein synthesis in the absence of inhibitor and are the average of two determinations (error ~ ±10% of each value based on repetitive determinations).

0.79 mg ml^{-1} solution), 10× concentrated Hepes-buffer (15 μl), puromycin (15 μl of a 10 mM solution in H_2O, pH 7.0), and H_2O (15 μl) were added, followed by addition of GTP (5 μl of a 10 mM solution in H_2O). This mixture was incubated at 37°C for a further 30 min and then the amount of P-site bound Ac-[^{14}C]-Phe-tRNA^{Phe} was determined using the puromycin reaction as described above. Translocation of Phe-tRNA^{Phe} was assayed similarly, except that each experiment used 21 pmol of Phe-tRNA^{Phe} and 16 pmol ribosomes.

RESULTS

RA-VII completely inhibits *in vitro* protein synthesis in rabbit reticulocyte lysates at a concentration of 5 μM, with an IC_{50} ~ 80 nM (Fig. 2). It inhibits the growth of L1210 cells with IC_{50} = 2 ng ml (~2.5 nM; (*29*)). RA-VII (50 μM) inhibits poly(U)-dependent poly(phenylalanine) synthesis by approximately 50%, but no increase in inhibition was observed upon raising the concentration of RA-VII to 1 mM (data not shown). When RA-VII is added to an active *in vitro* translation system, protein synthesis stops immediately as expected for an inhibitor of polypeptide elongation (Fig. 3). Thus, RA-VII appears to inhibit protein synthesis at the elongation stage as might be predicted based on its close structural similarity to bouvardin. Therefore, the effect of RA-VII upon the individual steps in polypeptide elongation was examined, in comparison with the known elongation inhibitor sparsomycin (*30*).