MOLECULAR PHARMACOLOGY, 12, 167-176

Mode of Action of the Antitumor Compound Bruceantin, an Inhibitor of Protein Synthesis

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(Received June 16, 1975)

SUMMARY

LIAO, LON-LON, KUPCHAN, S. MORRIS & HORWITZ, SUSAN B. (1976) Mode of action of the antitumor compound bruceantin, an inhibitor of protein synthesis. *Mol. Pharmacol.*, 12, 000-000.

The plant derivative bruceantin, an antitumor compound, irreversibly inhibits protein synthesis in HeLa cells, rabbit reticulocytes, and reticulocyte lysates. Bruceantin has a secondary effect on DNA synthesis, but little effect on the synthesis of RNA. After addition of bruceantin to a reticulocyte lysate, there is a delay of 2-3 min before inhibition of protein synthesis is observed. At a concentration of 0.1 mM, bruceantin induces sequential breakdown of polyribosomes to monosomes; concomitantly, nascent peptides are released from the ribosomes as completed chains of globin. These observations suggest that the principal inhibitory effect of the drug is on initiation of protein synthesis. Higher concentrations of bruceantin (1 mM) inhibit elongation of peptide chains; under these conditions, breakdown of polyribosomes is incomplete. Bruceantin does not prevent binding of radioactive poly U, poly C, or *Escherichia coli* tRNA to reticulocyte ribosomes, nor does it alter the conformation of ribosomal subunits. The structure-activity relationships of bruceantin and its analogues, as determined by their effects on protein synthesis in HeLa cells, rabbit reticulocyte cells, and reticulocyte lysates, correlate well with their activity as antitumor agents in experimental animals. These studies demonstrate that the side chain of bruceantin is important for transport of the drug into intact cells and that partial unsaturation in ring A is required for inhibition of protein synthesis.

INTRODUCTION

Bruceantin is a cytotoxic antitumor compound isolated from *Brucea antidysenterica*, a plant used in Ethiopia in the treatment of cancer. The structures of bruceantin and its analogues are shown in Fig. 1. Certain of these compounds exhibit antitumor activity against P 388 leukemia, the Lewis lung carcinoma, and the B-16 melanocarcinoma in mice (1-3).

The present paper describes effects of bruceantin on protein synthesis in HeLa cells, rabbit reticulocytes, and reticulocyte lysates, and demonstrates that the primary effect of this drug is on the initiation of protein synthesis. Studies with bruceantin and several structural analogues indicate that the side chain of bruceantin is important for the transport of the drug

This work was supported by Division of Cancer Treatment Contract NO1-CM-43787 and United States Public Health Service Grants CA-15714 and CA-11718 from the National Cancer Institute, National Institutes of Health. A preliminary account of this work has appeared [(1974) *Fed. Proc.*, 33, 2086].

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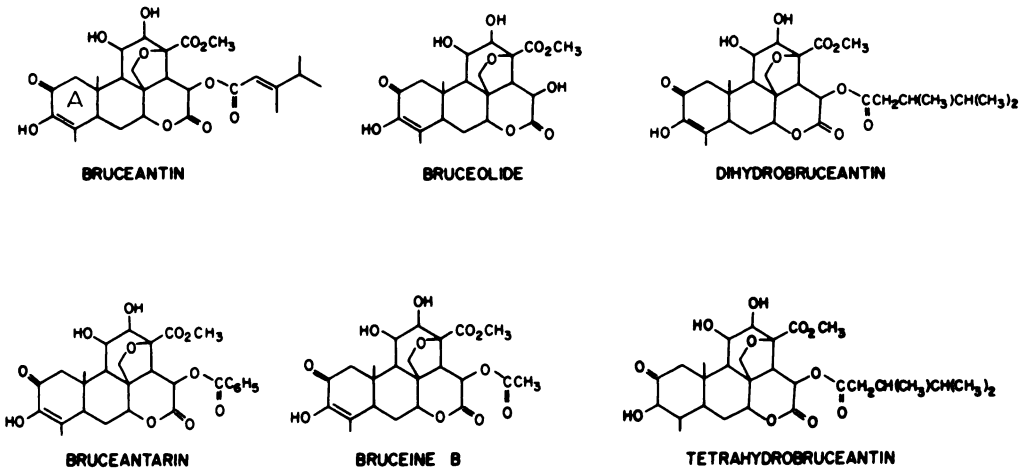

FIG. 1. *Structural formulae of bruceantin and analogues*

into intact cells and that the double bond in ring A is required for inhibition of protein synthesis. The effects of bruceantin on protein synthesis may account for the reported chemotherapeutic properties of the drug.

METHODS

Materials. A 0.01 M stock solution of bruceantin and its analogues was prepared in dimethyl sulfoxide and stored at −20°. Pyrocatechol violet was purchased from Aldrich Chemical Company; cycloheximide and emetine, from Sigma Chemical Company; [^{14}C]leucine (270 mCi/mmole), [^{14}C]thymidine (55 mCi/mmole), and [^{14}C]uridine (50 mCi/mmole), from New England Nuclear Corporation; and [^{3}H]polyuridylic acid (78.1 Ci/mole of phosphorus), [^{3}H]polycytidylic acid (67.3 Ci/mole of phosphorus), and *Escherichia coli* [^{3}H]tRNA, from Miles Laboratories.

Measurement of DNA, RNA, and protein synthesis in HeLa cells. HeLa S_3 cells were grown in suspension culture (4), and macromolecular synthesis was measured as previously described (5). [^{14}C]Leucine (0.025 μCi), [^{14}C]thymidine (0.05 μCi), or [^{14}C]uridine (0.025 μCi) was added to the cell suspension (1 ml), and the rates of synthesis of protein, DNA, and RNA were determined by measuring the respective uptake of these labeled precursors into cold trichloracetic acid-insoluble material. One milliliter of the cold salt solution described by Earle (6) (buffer A) was added at the end of the indicated period of incubation, and the cells were collected by centrifugation for 3 min at 1000 × *g*. The supernatant solution was decanted, and the cells were lysed by the addition of 1.0 ml of cold water. Then 2 ml of 10% trichloroacetic acid were added, the mixtures were chilled for 10 min, and the precipitates were collected on Millipore membrane filters. The filters were washed three times with 5% trichloroacetic acid, and the radioactivity was determined as described below.

Measurement of protein synthesis in rabbit reticulocytes. Reticulocytosis was induced in New Zealand rabbits by the administration of acetophenylhydrazide (7). Blood was obtained by cardiac puncture, and the plasma was separated from packed reticulocytes by centrifugation at 1500 × *g* for 15 min at 4°; the cells were washed twice with a solution composed of 0.13 M NaCl, 5 mM KCl, and 7.5 mM $MgCl_2$ and suspended in the same solution (8, 9). Hemoglobin synthesis in intact reticulocytes was measured as previously described (7, 10–12). Incorporation of [^{14}C]leucine into trichloroacetic aid-insoluble material was used to estimate the rate of globin synthesis in reticulocytes.

Measurement of globin synthesis by reticulocyte lysates. Lysates were prepared by adding an equal volume of cold 1 mM $MgCl_2$ to packed reticulocytes and shaking

gently for 10 min at 4°. Cell membranes and debris were removed by centrifugation for 20 min at 15,000 rpm in a Sorvall SS-34 rotor; the crude lysate was divided into small aliquots (0.7 ml) and stored in liquid nitrogen. Biosynthesis of globin was measured as previously described (8, 13, 14). The standard reaction mixture contained 10 mM Tris-HCl (pH 7.4), 74 mM KCl, 1 mM ATP, 0.2 mM GTP, 15 mM creatine phosphate, 2 mM magnesium chloride, 2 μM [^{14}C]leucine (315 Ci/mole), 6 mM 2-mercaptoethanol, a 0.1 mM mixture of 19 amino acids, 0.9 mg/ml of creatine phosphokinase, 60 μM hemin, and 0.10–0.13 ml of lysate in a final volume of 0.5 ml. Following incubation at 33°, aliquots (50 μl) were removed from the reaction mixture and added to 3 ml of 5% trichloroacetic acid. The solution was heated for 15 min at 95° and then chilled in an ice bath. Precipitates were collected on Millipore membrane filters and washed three times with cold 5% trichloroacetic acid, and the radioactivity was determined as described below

Sucrose density gradient centrifugation. Density gradient centrifugation was performed according to the procedure of Britten and Roberts (15). Samples of 1.5 ml were layered over 36 ml of 10–20% (w/v) sucrose gradients prepared in reticulocyte standard buffer (16), composed of 0.01 M Tris-HCl (pH 7.4), 0.01 M KCl, and 1.5 mM $MgCl_2$. All gradients were centrifuged at 4° for 165 min at 25,000 rpm in a Spinco SW 27 swinging bucket rotor, and the absorbance at 260 nm was determined during collection of the gradients by means of a flow cell (light path, 0.2 cm) attached to a Gilford spectrophotometer. Fractions of 1.2 ml were collected from the bottom of the tube for determination of radioactivity.

Isolation of reticulocyte ribosomes. Ammonium chloride-washed ribosomes were prepared from reticulocytes as previously described (7, 17–19).

Determination of radioactivity. ^{14}C-Labeled precipitates collected on 2.5-cm Whatman GF/C glass filters were dried and counted in a Nuclear-Chicago low-background gas flow counter. Filters containing tritium were counted in a Packard scintillation counter in vials containing 0.5 ml of Triton X-100 and 57 mg of 2,5-diphenyloxazole in 10 ml of toluene.

RESULTS

Effects of bruceantin on DNA, RNA, and protein synthesis in HeLa cells. The initial rates of incorporation of thymidine, uridine, and leucine into acid-insoluble material were utilized to estimate rates of DNA, RNA, and protein synthesis, respectively. The effects of bruceantin on macromolecular synthesis are illustrated in Fig. 2. During the first hour after addition of bruceantin, the average rate of protein synthesis is reduced by 50% and 90% at concentrations of 30 and 400 nM, respectively. Parallel, but less complete inhibitory effects are also observed on the rate of synthesis of DNA, which is inhibited by 50% at a concentration of 80 nM. Maximum inhibition of DNA synthesis was 60% at a concentration of 2 × 10^3 nM. The rate of RNA synthesis was only slightly inhibited by 2 × 10^3 nM bruceantin.

Irreversible inhibition of protein synthesis by bruceantin. The irreversible nature

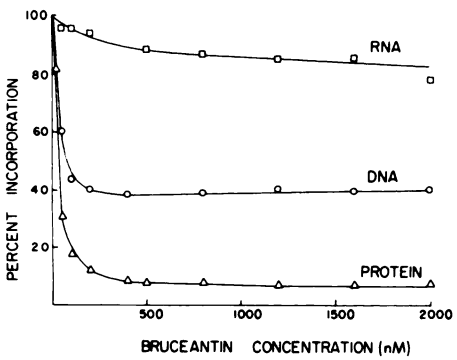

FIG. 2. *Effect of various concentrations of bruceantin on synthesis of protein, DNA, and RNA in HeLa cells*

Cells were incubated for 60 min at 37°, and incorporation of radioactivity into trichloroacetic acid-insoluble material was used to calculate the rates of synthesis of protein (Δ——Δ), DNA (○——○), and RNA (□——□), which in the control reactions were 4000, 6700, and 9800 cpm/ml, respectively. The percentage incorporation is expressed relative to these controls. Protein, DNA, and RNA synthesis was measured as described under METHODS, and bruceantin was present at the final concentrations indicated in the figure.

of inhibition of protein synthesis by bruceantin in HeLa cells is shown in Fig. 3. After exposure to the drug for 30 min, the cells were washed twice and resuspended in fresh medium, and the rate of protein synthesis was determined. Cycloheximide and emetine (20, 21), established reversible and irreversible inhibitors of protein synthesis, respectively, were used as controls for this experiment. Inhibition of protein synthesis by bruceantin in HeLa cells was irreversible after exposure to 0.1 μM bruceantin for 30 min.

Effect of bruceantin on rate of synthesis of globin. When bruceantin is incubated with intact reticulocytes for 1 hr at concentrations of 0.01, 0.1, and 1 μM, the average rate of hemoglobin synthesis is reduced by 14%, 79%, and 95%, respectively. Rabbit reticulocyte lysates were utilized as a cell-free system in which globin synthesis was inhibited by 48%, 77%, and 79% at concentrations of 1, 10, and 100 μM bruceantin, respectively. The effect of various concentrations of bruceantin on the rate of globin synthesis is shown in Fig. 4A. Even at high concentrations of bruceantin (100 μM), there is a delay of several minutes before onset of inhibition is observed; this delay does not depend on the time of addition of bruceantin to the reaction mixture (Fig. 4B). Pyrocatechol violet and emetine (22, 20), established inhibitors of initiation and elongation of protein synthesis, respectively, were used as experimental controls. Pyrocatechol violet inhibits protein synthesis after a lag period, whereas emetine stops it immediately.

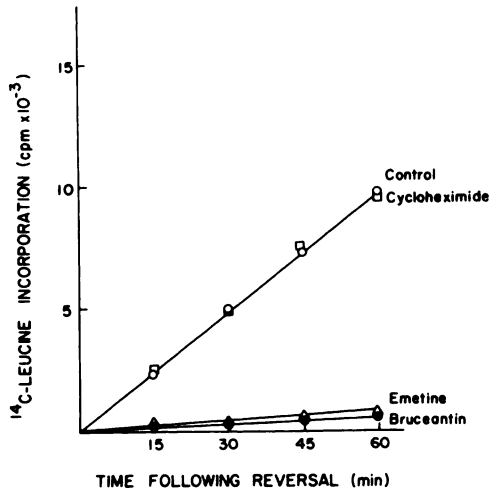

FIG. 3. *Irreversible inhibition of protein synthesis induced by bruceantin in HeLa cells*

Cells were incubated at 37° with 0.1 μM bruceantin (●——●), 10 μM cycloheximide (□——□), 10 μM emetine (△——△), or no inhibitor (○——○). After 30 min, the cultures were washed twice with warm minimal essential medium and resuspended in medium supplemented with 5% calf serum. One nanomole of [^{14}C]leucine (270 mCi/mmole) was added, and the rate of protein synthesis was measured as described under METHODS.

Effect of bruceantin on polyribosome structure and on release of nascent peptides. The effect of bruceantin on the structure of polyribosomes and on the release of nascent peptides is shown in Fig. 5. Reticulocyte lysates were incubated with inhibitors (100 μM) for 15 sec, then exposed to [^{14}C]leucine for 4 min. At this concentration, bruceantin induces breakdown of polyribosomes and releases radioactive peptides from the ribosome (Fig. 5B). This result is similar to that seen with pyrocatechol violet (Fig. 5D), an established inhibitor of protein synthesis (22). In contrast, emetine (Fig. 5C), an inhibitor of elongation, immobilizes polyribosomes, and radioactive leucine is not incorporated into peptides (20).

During incubation of a reticulocyte lysate, leucine is incorporated into nascent peptides bound to polyribosomes, and complete peptides are released from polyribosomes (Fig. 6A–C). Bruceantin induces breakdown of polyribosomes over a period of several minutes (Fig. 6D–F); during this time some leucine is incorporated into trichloroacetic acid-insoluble peptides that can be recovered from the top of the gradient. The nature of these peptides formed in the presence of bruceantin was determined. Lysates were incubated as described in the experiment shown in Fig. 6, and radioactive peptides released from the polyribosomes were analyzed by polyacrylamide gel electrophoresis (Fig. 7). ^{3}H-Labeled peptides formed in the presence of bruceantin were indistinguishable from α and β chains of globin, synthesized in the absence of the inhibitor. These results indicate that bruceantin does not prevent

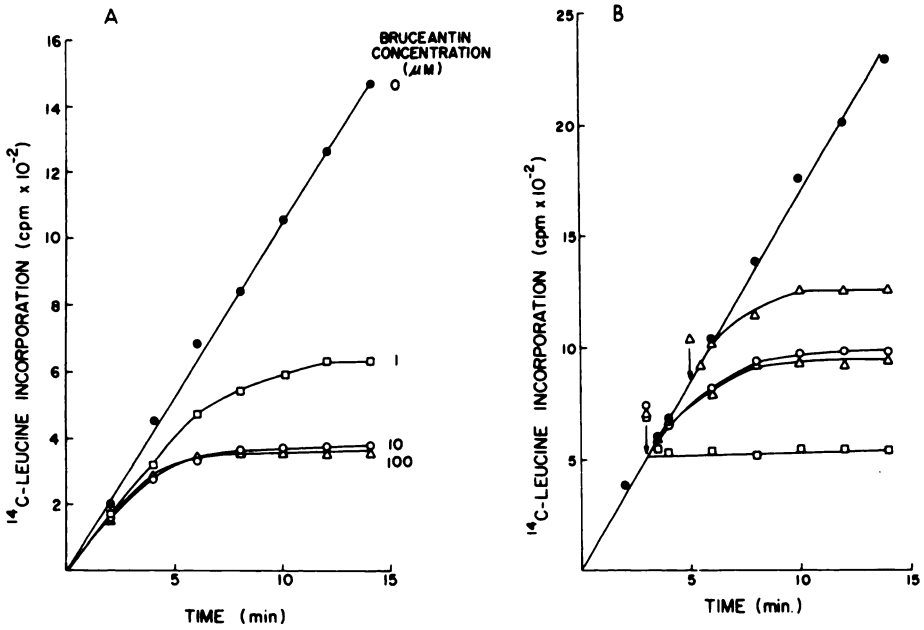

FIG. 4. *Effect of bruceantin on synthesis of globin by reticulocyte lysates*

A. The standard reaction mixture (1 ml) described under METHODS was incubated at 37° in the presence of various concentrations of bruceantin as indicated in the figure. At the times indicated, 0.1-ml aliquots were removed and 3 ml of 5% trichloroacetic acid were added. Globin synthesis was measured as described under METHODS.

B. Standard reaction mixtures (1 ml) (●——●) were prepared as described under METHODS. Bruceantin (△——△), pyrocatechol violet (○——○), or emetine (□——□), each at a final concentration of 100 μM, was added to the standard mixture at the times indicated by arrows. Globin synthesis was measured as described under METHODS, and 0.1-ml aliquots were added to 3 ml of 5% trichloroacetic acid.

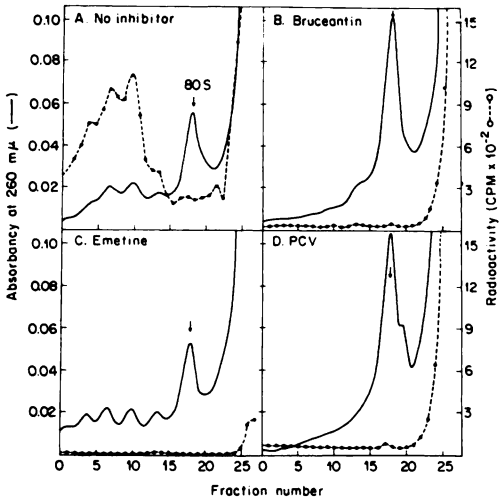

FIG. 5. *Effect of bruceantin on polyribosome structure and release of nascent peptides in reticulocyte lysates*

Standard reaction mixtures (0.5 ml), prepared as described for the synthesis of globin, were incubated with 100 μM bruceantin (B), emetine (C), or pyrocatechol violet (PCV) (D) at 32° for 15 sec, then pulsed with 4 nmoles of [^{14}C]leucine (270 mCi/mmole) for 4 min. Reactions were terminated by rapid cooling to 0°. Then 1 ml of cold reticulocyte standard buffer was added, and the mixture was layered onto 10–25% sucrose gradients. Gradients were centrifuged and fractionated as described under METHODS.

chain elongation, nor does it cause the release of incomplete polypeptides from polyribosomes.

The effects of bruceantin on ribosomal subunits can be seen more clearly when centrifugation of the sucrose gradients is continued for longer periods of time (Fig. 8). At a concentration of 100 μM, bruceantin has no effect on sedimentation of 80 S ribosomes or 60 S and 40 S ribosomal subunits. In contrast, the 40 S ribosomal subunit sediments more slowly in the presence of pyrocatechol violet.

Effects of various concentrations of bruceantin and bruceolide on polyribosomes.

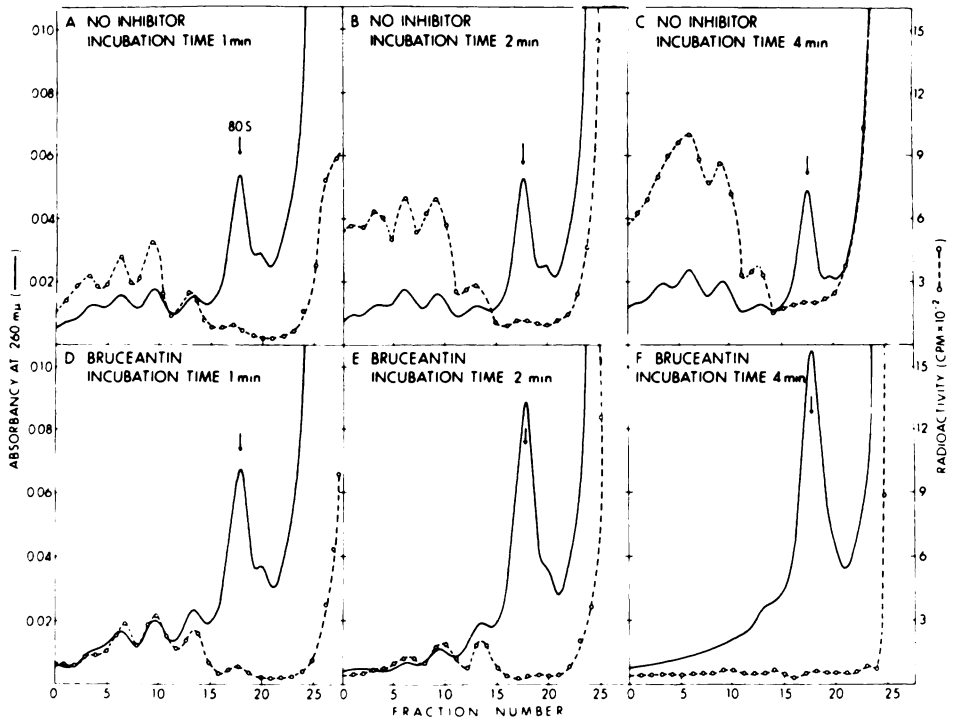

FIG. 6. *Effect of bruceantin on incorporation of amino acids into polyribosome-bound peptides*

Six standard reaction mixtures (0.5 ml), each containing 4 nmoles of [^{14}C]leucine (270 mCi/mmole), were prepared as described for the synthesis of globin. Samples were incubated at 33° for various periods of time, as indicated in the figure. Bruceantin was added to reactions D, E, and F. Sucrose density gradient centrifugation was performed as described under METHODS.

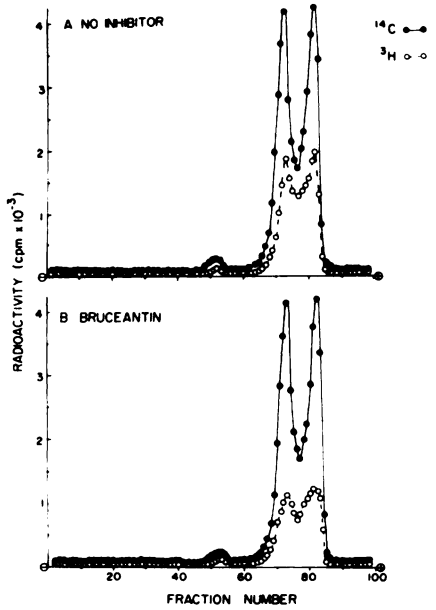

FIG. 7. *Comparison by acrylamide gel electrophoresis of nascent peptides formed in the presence and absence of bruceantin*

Two reaction mixtures (1 ml) were incubated as described in the legend to Fig. 6C and F. [^{3}H]Leucine (1 nmole, 20 Ci/mmole) was used in place of [^{14}C]leucine. Reactions were terminated by adding an equal volume of cold reticulocyte standard buffer and filtering through Millipore membrane filters to remove ribosomes. Filtrates containing ^{3}H-labeled peptides were dialyzed for 24 hr against three changes of distilled water. Then 200 μg of ^{3}H-labeled peptides were mixed with 70 μg of authentic [^{14}C]globin in a solution composed of 10 mM phosphate buffer (pH 7.2), 0.15 M mercaptoethanol, and 0.2% sodium dodecyl sulfate. Samples were heated at 100° for 1 min, cooled to room temperature, then subjected to electrophoresis for 16 hr at 4 V/cm on 20-cm 10% acrylamide gels prepared in 100 mM phosphate buffer, pH 7.2, containing 0.1% sodium dodecyl sulfate. Migration is toward the anode on the right. Gels were crushed automatically and suspended in 10 ml of Aquasol, and the radioactivity was determined. A. Peptide synthesized in the absence of bruceantin. B. Peptide synthesized in the presence of bruceantin. O- - -O, ^{3}H-labeled peptides; ●——●, [^{14}C]globin.

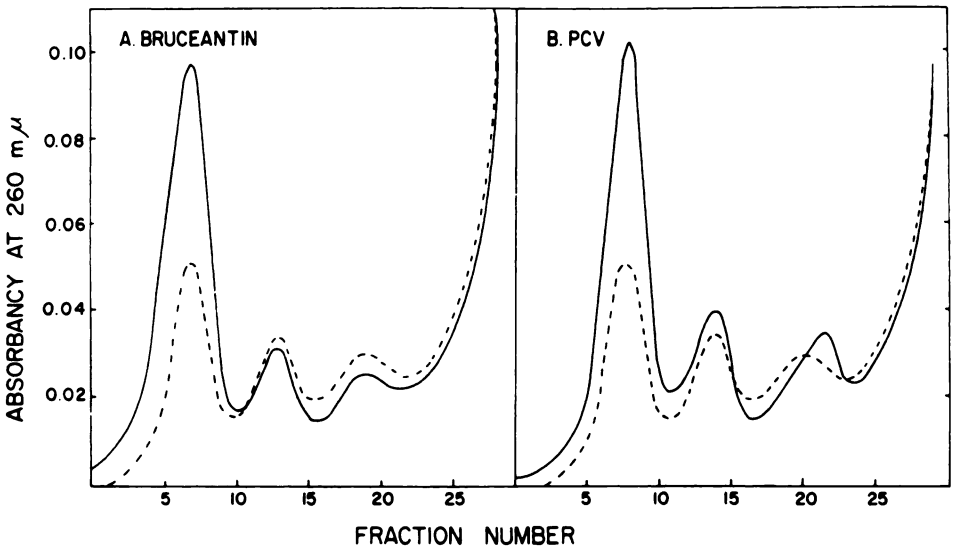

FIG. 8. *Effect of bruceantin or pyrocatechol violet (PCV) on ribosomes and ribosomal subunits in reticulocyte lysates*

Standard reaction mixtures (0.5 ml), containing 100 μM bruceantin (A) or pyrocatechol violet (B), were prepared as described for the synthesis of globin, incubated for 4 min at 32°, and subjected to sucrose density gradient analysis as described under METHODS. The gradients were centrifuged for 16.5 hr at 17,000 rpm. - - -, no additions; ——, inhibitor added.

The effects of various concentrations of bruceantin and bruceolide on the structure of polyribosomes are shown in Figs. 9 and 10, respectively. Reticulocyte lysates were incubated with various concentrations of drugs for 4 min at 32°. Bruceantin dissociates polyribosomes into single ribosomes at concentrations of 100 and 10 μM (Fig. 9B and C). At higher (1 mM) and lower (1 μM) concentrations, the dissociation of polyribosomes is incomplete (Fig. 9A and D). Similar results are observed with bruceolide (Fig. 10), although incubation of bruceolide at 100 μM with reticulocyte lysates does not result in complete dissociation of polyribosomes.

Effect of bruceantin on binding of [³H]poly U, [³H]poly C, and [³H]tRNA to ribosomes. Bruceantin, at a concentration of 100 μM, does not prevent binding of [³H]poly U, [³H]poly C, or uncharged [³H]tRNA from *E. coli* to ammonium chloride-washed reticulocyte ribosomes, as determined by retention of the polynucleotide-ribosome complex on Millipore membrane filters (Table 1). Pyrocatechol violet, used as a control in these experiments, inhibits the attachment of [³H]poly U, [³H]poly C, and [³H]tRNA by 98%, 89%,

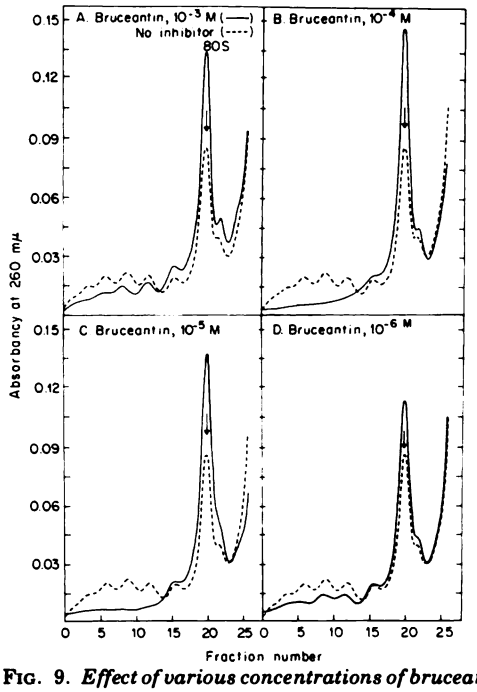

FIG. 9. *Effect of various concentrations of bruceantin on polyribosomes*

Standard reaction mixtures (0.5 ml), prepared as described for the synthesis of globin, were incubated with 1000, 100, 10, and 1 μM bruceantin for 4 min at 32°. Reactions were terminated and mixtures centrifuged as described under METHODS.

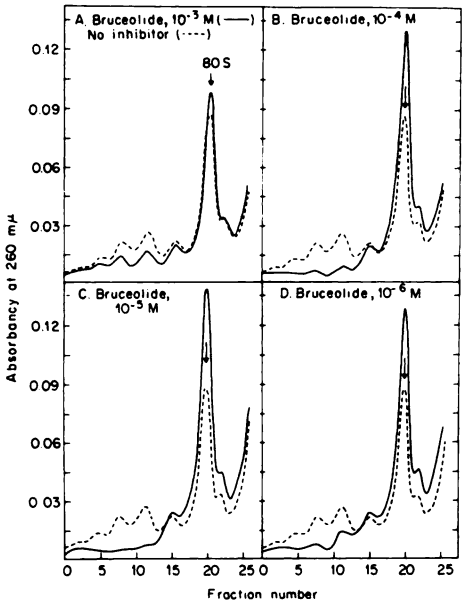

FIG. 10. *Effect of various concentrations of bruceolide on polyribosomes*

Standard reaction mixtures (0.5 ml), prepared as described for the synthesis of globin, were incubated with 1000, 100, 10, and 1 μM bruceolide for 4 min at 32°. Reactions were terminated and mixtures centrifuged as described under METHODS.

and 96%, respectively, as previously reported (22).

Structure-activity relationships. The effect of certain structural analogues of bruceantin (Fig. 1) was determined on protein synthesis in HeLa cells, whole reticulocytes, and reticulocyte lysates. Bruceantin, dihydrobruceantin, bruceantarin, and bruceine B are more potent inhibitors of protein synthesis than bruceolide and tetrahydrobruceantin in HeLa cells (Fig. 11) and reticulocytes (Table 2). Bruceantin and dihydrobruceantin exhibit essentially identical activity in these two cells. With the exception of tetrahydrobruceantin, all the compounds are quite similar in their ability to inhibit protein synthesis in reticulocyte lysates (Table 2).

DISCUSSION

The predominant effect of bruceantin in HeLa cells, rabbit reticulocytes, and reticulocyte lysates is on protein synthesis. The partial inhibition of DNA synthesis in HeLa cells probably results from a primary effect on protein synthesis, since the latter process is required for DNA synthesis in animal cells (23–26).

At concentrations of 100 and 10 μM, bruceantin has a selective effect on the initiation of protein synthesis in reticulocyte lysates. This is based on (a) a delay of several minutes before inhibition of globin synthesis is observed, (b) breakdown of polysomes to monosomes, and (c) completion of nascent globin peptides in the presence of bruceantin. The preceding are characteristics observed with aurintricarboxylic acid (9), pyrocatechol violet (22), sodium fluoride (27–29), pactamycin (30–33), and harringtonine (34), established inhibitors of initiation of protein synthesis. At higher concentrations (1 mM), bruceantin freezes polyribosomes and prevents elongation of peptide chains. Bruceantin does not inhibit the binding of poly U, poly C, or *E. coli* tRNA to ribosomes.

Bruceantin can be distinguished from other reported inhibitors of protein initiation. Aurintricarboxylic acid (9) and pyro-

TABLE 1

Effect of bruceantin on binding of [³H]poly U, [³H]poly C, and E. coli [³H]tRNA to reticulocyte ribosomes

The reaction mixture contained, in a volume of 0.25 ml, 8 mM $MgCl_2$, 60 mM KCl, 25 mM Tris-HCl (pH 7.5), 50 μg of ammonium chloride-washed ribosomes, and 0.01 μCi of [³H]poly U, [³H]poly C, or [³H]tRNA. Reaction mixtures were incubated at 37° for 10 min, filtered through a Millipore membrane filter, and washed seven times with a buffer containing the same ionic composition as that used in the reaction mixture. Filters used in this experiment were first treated by immersion in 0.5 N NaOH at room temperature for 30 min, then rinsed with distilled water and stored in 0.1 M Tris-HCl, pH 7.4. Filters thus treated retain ribosomes but not poly U, poly C, or *E. coli* tRNA. In the absence of inhibitors, 8100 cpm of [³H]poly U, 750 cpm of [³H]poly C, or 2200 cpm of [³H]tRNA were bound to the filter. This binding was dependent on the presence of ribosomes.

Inhibitor added (200 μM)	[³H] Poly U bound	[³H] Poly C bound	[³H] tRNA bound
	% inhibition		
Bruceantin	2	6	0
Pyrocatechol violet	98	89	96

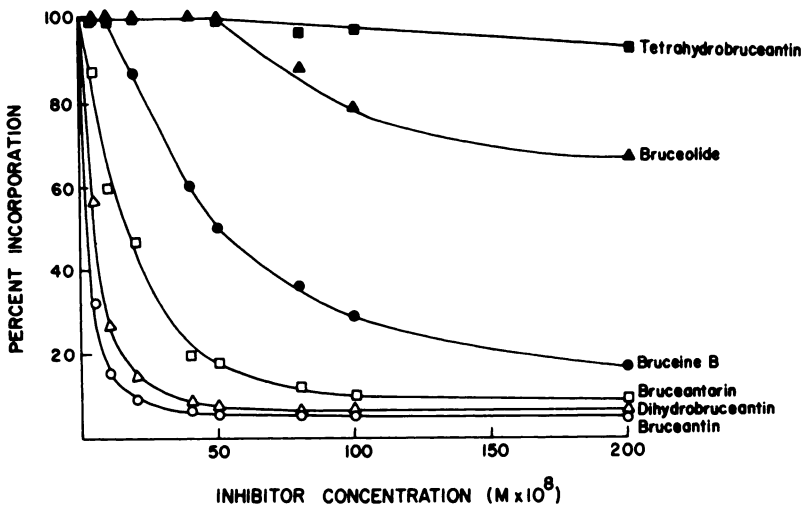

FIG. 11. *Effect of analogues of bruceantin on protein synthesis in HeLa cells*

The experiment was performed as described in the legend to Fig. 2. Incorporation of [^{14}C]leucine into trichloroacetic acid-insoluble material was used to calculate the rate of protein synthesis, which, in the control reaction, was 7200 cpm/ml. The percentage incorporation shown is expressed relative to this control. Bruceantin (○——○), dihydrobruceantin (△——△), bruceantarin (□——□), bruceine B (●——●), bruceolide (▲——▲), and tetrahydrobruceantin (■——■) were present at the final concentrations indicated.

TABLE 2

Effects of analogues of bruceantin on protein synthesis in HeLa cells, reticulocytes, and reticulocyte lysates

Protein synthesis in HeLa cells, reticulocytes, and reticulocyte lysates was measured as described under METHODS.

Inhibitor added	Concentration of inhibitor required for 50% inhibition		
	HeLa cells	Reticulocytes	Lysates
	μM	μM	μM
Bruceantin	0.03	0.04	1.6
Dihydrobruceantin	0.04	0.05	1.6
Bruceantarin	0.16	0.43	6.0
Bruceine B	0.50	0.50	2.1
Bruceolide	4.0	3.8	3.4
Tetrahydrobruceantin	8.0	3.6	180

catechol violet (22) prevent attachment of synthetic messenger RNA and tRNA to reticulocyte ribosomes, and pactamycin (35) inhibits binding of uncharged tRNA to ribosomes. Bruceantin is most similar in its mechanism of action to harringtonine (34), another antitumor drug that also penetrates animal cell membranes, has no effect on binding of tRNA or synthetic messenger RNA to ribosomes, and inhibits initiation of protein synthesis. At high concentrations, harringtonine, unlike bruceantin, does not inhibit elongation of peptide chains.

The structure-activity analysis of bruceantin and its analogues was conducted in intact cells and cell-free lysates. The activity of dihydrobruceantin is similar to that of bruceantin in all systems, indicating that saturation of the double bond in the side chain of bruceantin does not alter the biological properties of the molecule. Substitution of the side chain of bruceantin by a benzoyl or acetyl group, as in bruceantarin or bruceine B, decreases inhibitory activity in HeLa cells and in reticulocytes. Removal of the side chain of bruceantin, as in bruceolide, results in a significant loss of activity in whole cells. All these compounds, which differ only in their side chains, have similar effects in reticulocyte lysates. Such data suggest that the side chains of bruceantin and its analogues are involved in transport across cell membranes. The longer, more hydrophobic side chains present in bruceantin and dihydrobruceantin seem to be more effective in enhancing transport than the side chains in bruceantarin and bruceine

B. Bruceolide, with no side chain, is poorly transported into cells. The principal effect of bruceolide in lysates is on initiation of protein synthesis, similar to the mechanism of action of bruceantin.

Tetrahydrobruceantin, in which the double bond in the A ring of bruceantin is saturated, has reduced activity in both intact cells and lysates, although this compound has the same side chain as dihydrobruceantin. This suggests that ring A may be involved in the interaction responsible for the inhibition of protein synthesis. Our structure-activity analysis corresponds closely to the ability of these compounds to suppress the growth of tumors in experimental animals (1). Neither bruceolide nor tetrahydrobruceantin demonstrates significant antitumor activity in experimental animals.

ACKNOWLEDGMENT

The authors are grateful to Dr. Arthur P. Grollman for many helpful discussions during the course of this work.

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