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Harringtonine, an Inhibitor of Initiation of Protein Biosynthesis

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SUMMARY

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The *Cephalotaxus* alkaloid harringtonine inhibits protein biosynthesis in HeLa cells, intact rabbit reticulocytes, and reticulocyte lysates. DNA synthesis in HeLa cells is partially inhibited by a 200 nM concentration of the alkaloid, while synthesis of RNA is unaffected; the inhibitory effects on protein and DNA synthesis are partially reversible. Harringtonine induces breakdown of polyribosomes to monosomes with concomitant release of completed globin chains. When the alkaloid is added to a reticulocyte lysate, a delay of 2 min occurs before inhibition of globin synthesis is observed. Based on the preceding observations, the principal effect of harringtonine appears to be on initiation of protein synthesis. In contrast to some other inhibitors of initiation, harringtonine does not inhibit binding of polyuridylic acid or tRNA to ribosomes, nor does it inhibit chain elongation even at high concentrations of drug. These observations provide a biochemical basis for the cytotoxic and chemotherapeutic properties of harringtonine and suggest its use as a tool for the study of protein synthesis in animal cells.

INTRODUCTION

Harringtonine is a cytotoxic alkaloid isolated from *Cephalotaxus harringtonia* (1, 2). The structures of harringtonine and related *Cephalotaxus* alkaloids (Fig. 1) have been established by Powell *et al.* (3). Some of these alkaloids exhibit antitumor activity against Leukemias L1210 and P388 in mice (4, 5).[1]

The present paper describes the inhibitory effects of harringtonine on protein, RNA, and DNA synthesis in HeLa cells and on protein synthesis in rabbit reticulocyte lysates. These studies provide a biochemical basis for some of the toxic and therapeutic properties of harringtonine. As a drug that acts selectively on initiation of protein synthesis in eukaryotes, harringtonine should provide a useful tool for future studies on protein synthesis in eukaryotes.

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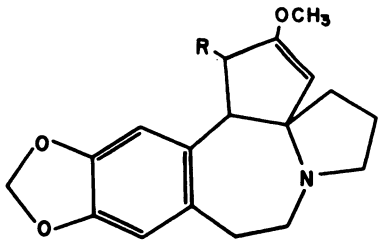
[1] Assays were performed under the auspices of the Cancer Chemotherapy National Service Center by procedures described in ref. 4.

MATERIALS AND METHODS

Materials. Harringtonine and related *Cephalotaxus* alkaloids were gifts from Dr. R. G. Powell and Dr. D. Weisleder, Northern Regional Research Laboratory, Ill. Cycloheximide and emetine were purchased from Sigma Chemical Company, and [^{14}C]leucine (200 mCi/mmole), [^{14}C]thymidine (50 mCi/mmole), [^{14}C]uridine (50 mCi/mmole), and [^{3}H]puromycin (700 mCi/mmole), from New England Nuclear Corporation. The sources of [^{14}C]tRNA and other materials used in these experiments are described elsewhere (6, 7). Su-



I CEPHALOTAXINE — R = —OH

II HARRINGTONINE — R = $CH_3C(CH_2)_2CCH_2CO_2CH_3$ (OH, CH_3, CO_2^-)

III ISOHARRINGTONINE — R = $CH_3CH(CH_2)_2C-CHCO_2CH_3$ (OH OH, CH_3, CO_2^-)

IV HOMOHARRINGTONINE — R = $CH_3C(CH_2)_3CCH_2CO_2CH_3$ (OH, CH_3, CO_2^-)

V DEOXYHARRINGTONINE — R = $CH_3CH(CH_2)_2C-CH_2CO_2CH_3$ (OH, CH_3, CO_2^-)

VI PSEUDO-DEOXYHARRINGTONINE — R = $CH_3CH(CH_2)_2CCH_2CO_2^-$ (OH, CH_3, CO_2CH_3)

FIG. 1. *Structures of harringtonine and some related alkaloids from Cephalotaxus harringtonia*

crose (enzyme grade) was obtained from Schwarz BioResearch; calf serum and tissue culture media, from Grand Island Biological Company, and Triton X-100, from Rohm and Haas.

Measurement of protein, DNA, and RNA synthesis in HeLa cells. HeLa S_3 cells were grown at 37° on Eagle's minimal essential medium (6) supplemented with 5% calf serum. For individual experiments, cell suspensions (35–45 × 10^4 cells/ml) were equilibrated with 5% CO_2 and incubated at 37° with constant stirring. Methods for the measurement of protein, DNA, and RNA synthesis in intact HeLa cells have been described in detail (6).

Measurement of globin synthesis by reticulocyte lysates. Reticulocytosis was induced in New Zealand rabbits by daily injection of acetophenylhydrazine (8). Globin synthesis in reticulocyte lysates was measured by a modification of the procedure of Maxwell and Rabinovitz (9), as previously described (8). The standard reaction mixtures contained, in a final volume of 0.1 ml, 10 mM Tris-HCl (pH 7.4), 75 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, 0.2 mM GTP, 6 mM mercaptoethanol, 2.1 mg/ml of creatine phosphate, 0.9 mg/ml of creatine phosphokinase, 2 μM [^{14}C]leucine (200 mCi/mmole), 30 μM mixtures of 19 amino acids, 60 μM hemin, and 0.05 ml of lysate. Reactions were incubated at 33° for 10 min, terminated by the addition of 5% trichloracetic acid, then heated at 90° for 15 min, and chilled in an ice-water bath. Precipitates were collected on Millipore membrane filters, and radioactivity was determined as described below.

Determination of peptidylpuromycin

formation. Puromycin was allowed to react with nascent peptides on polyribosomes, using a minor modification of the procedure of Pestka *et al.* (10). Each reaction mixture contained the following components in a final volume of 0.1 ml: 0.5 M KCl, 5 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.4), 2–4 A_{260} units of rabbit reticulocyte polyribosomes, and 5 μM [^{3}H]puromycin (700 mCi/mmole). Reactions were incubated at 25° for 1 min and terminated by addition of 3 ml of cold 5% trichloracetic acid, and precipitates were collected on Millipore filters for determination of radioactivity. In the control reactions, 1792 cpm of [^{3}H]puromycin were incorporated into peptidylpuromycin.

Measurement of radioactivity. Radioactive precipitates, collected on membrane filters, were counted either with a Nuclear-Chicago low-background gas flow counter or with a Packard scintillation counter in vials containing 5 ml of a solution composed of 35 ml of Liquifluor and 330 ml of Triton X-100 per liter of toluene.

RESULTS

Effects of harringtonine on biosynthesis of protein, DNA, and RNA in HeLa cells. Initial rates of incorporation of leucine, thymine, and uridine into trichloracetic acid-insoluble material were utilized to estimate the rates of protein, DNA, and RNA synthesis, respectively, in HeLa cells. The effect of varying concentrations of harringtonine on these parameters is shown in Fig. 2. During the first hour of incubation after addition of the drug, the average rate of protein synthesis was inhibited by 50% and 90% at concentrations of 0.05 μM and 0.2 μM, respectively. Ninety-nine per cent inhibition was achieved by increasing the concentration of drug to 1 μM. Parallel, but less complete, inhibitory effects were observed on the rate of synthesis of DNA; at maximal inhibition the rate of DNA synthesis remained at 30% of control values and RNA synthesis was inhibited by 15%.

The effect of 1 μM harringtonine on protein and DNA synthesis in nonsynchronized suspension cultures of HeLa cells was apparent within several minutes after exposure to the drug (Fig. 3). Inhibition of protein synthesis preceded that of DNA synthesis, while the effect of the drug on RNA synthesis became apparent after 45 min.

Reversibility. Partial reversibility of the inhibitory effects of harringtonine is illustrated by the experiment shown in Fig. 4. The degree of reversibility depended on the concentration of harringtonine used and the procedure of washing. After exposure to 10 μM drug for 5 min, HeLa cells were washed twice with Earle's buffer and resuspended in fresh medium, and the rate of protein synthesis was measured. Under these conditions inhibition was essentially irreversible. However, if the cells were exposed to 1 μM harringtonine for no more than 5 min and then washed at least three times, inhibition of protein synthesis was

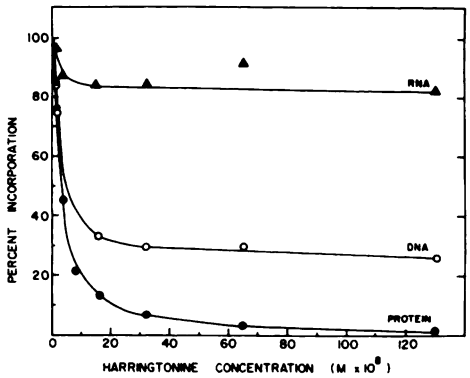


FIG. 2. *Effect of various concentrations of harringtonine on synthesis of protein, DNA, and RNA in HeLa cells*

Harringtonine was added at the indicated final concentrations to 1.5 ml of HeLa cells (4 × 10^5 cells/ml) suspended in leucine-depleted Eagle's medium containing 5% calf serum. After 1 min, 1 nmole of [^{14}C]leucine (200 mCi/mmole), 10 nmoles of [^{14}C]thymidine (50 mCi/mmole), or 5 nmoles of [^{14}C]uridine (55 mCi/mmole) were added and the cell suspension was incubated at 37° for 60 min. Reactions were terminated by addition of 3 ml of cold Earle's buffer, and the rates of protein, DNA, and RNA synthesis were determined as described under MATERIALS AND METHODS. Percentage incorporation is expressed relative to cell cultures to which no inhibitor was added. The rates of protein, DNA, and RNA synthesis in the control reactions were 21, 184, 9952, and 52,430 cpm/hr, respectively.

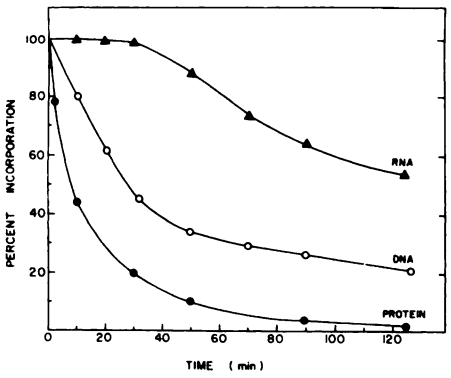


FIG. 3. *Effect of harringtonine on synthesis of protein, DNA, and RNA in HeLa cells at various times following exposure to the alkaloid*

HeLa cells (4×10^5 cells/ml), suspended in complete Eagle's medium supplemented by 5% calf serum, were divided into two portions. Harringtonine was added to one at a final concentration of 0.1 μM; then 1 nmole of [^{14}C]leucine (200 mCi/mmole), 10 nmoles of [^{14}C]thymidine (55 mCi/mmole), or 5 nmoles of [^{14}C]uridine (55 mCi/mmole) were added to both cultures. The cultures were further incubated at 37°, and at the indicated times 1 ml of sample was pipetted into 3 ml of cold Earle's buffer and the rates of protein, DNA, and RNA synthesis were determined as described under MATERIALS AND METHODS. The rates of protein, DNA, and RNA synthesis in the control reactions were 13,380, 6380, and 27,960 cpm/hr, respectively.

partially reversed after a delay of 30 min. Cycloheximide and emetine, a reversible and an irreversible inhibitor of protein synthesis, respectively, served as controls for this experiment. A concentration of each antibiotic was selected that would inhibit protein synthesis in HeLa cells by 95% for a period of at least 1 hr.

Effect of harringtonine on initiation of globin synthesis. Crude reticulocyte lysates, capable of initiating new chains of globin for periods of more than 10 min, were used to measure the rate of globin synthesis (11). The effect of varying concentrations of harringtonine on the rate of globin synthesis in reticulocyte lysates is shown in Fig. 5. There was a delay of several minutes in the onset of inhibition following addition of harringtonine to the lysate. Figure 6 shows that this delay did not depend on time of addition of the drug.

Effect of harringtonine on structure of polyribosomes and release of nascent peptides. Harringtonine induced complete breakdown of polyribosomes to monosomes over a period of 3 min; concomitantly, completed radioactive peptides were released from the polysomes and polyribosomes dissociated into monosomes (Fig. 7).

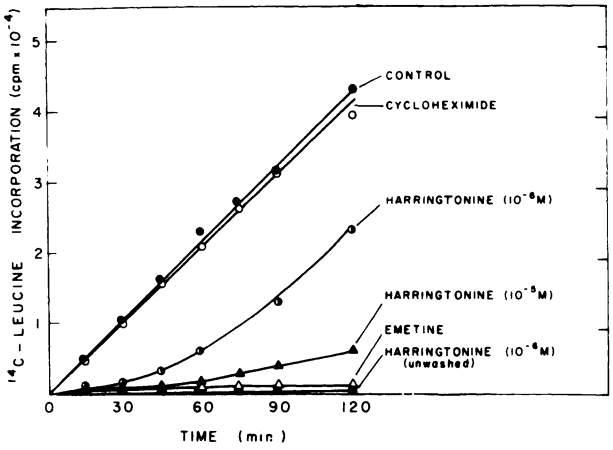


FIG. 4. *Reversal of inhibition of protein synthesis induced by harringtonine and other antibiotics*

HeLa cells (5×10^4 cells/ml), suspended in complete Eagle's medium containing 5% calf serum, were incubated for 5 min in the presence of harringtonine, cycloheximide (10 μM), or emetine (1 μM). The control reaction contained no antibiotics. All cultures, including the control, were centrifuged, and the cells were washed three times with Earle's buffer at 37° and the resuspended in fresh leucine-depleted medium. [^{14}C]-leucine (1 nmole) (200 mCi/mmole) was added, and the rate of protein synthesis was measured as described under MATERIALS AND METHODS.

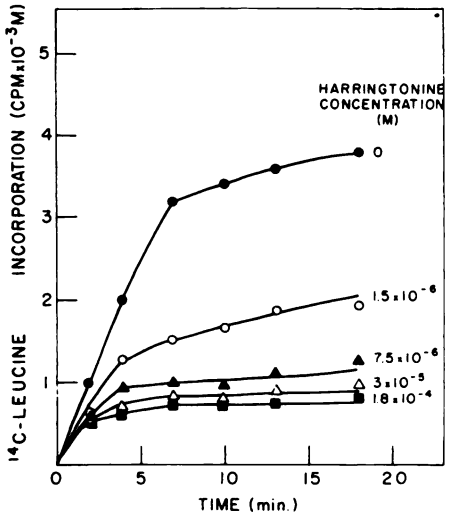


FIG. 5. *Effect of varying concentrations of harringtonine on synthesis of globin by reticulocyte lysates*

The standard reaction mixture (0.5 ml) described under MATERIALS AND METHODS was incubated at 33° in the presence of various concentrations of harringtonine as shown. At the indicated times 0.05-ml aliquots were removed and added to 5% trichloracetic acid. Globin synthesis was measured as described under MATERIALS AND METHODS.

During this time there was an increase in the number of polyribosomes in the uninhibited control reaction as well as a decrease in the number of 80 S monosomes and an increase in radioactive nascent peptides bound to polyribosomes (Fig. 7A and B). When harringtonine was present in the lysate prior to the addition of [^{14}C]leucine, some radioactive peptide was released to the top of the sucrose gradient (Fig. 7D).

Completion and release of globin chains in the presence of harringtonine. To determine whether radioactive peptide released from polyribosomes in the presence of harringtonine (0.2 mM) represented completed chains of globin or prematurely released incomplete polypeptides, a lysate was incubated at 33° for 3 min in the presence or absence of the drug as described for Fig. 7B and C. The soluble reaction product released from the ribosomes was then analyzed by polyacrylamide gel electrophoresis. ^{3}H-peptides formed in the presence of harringtonine were indistinguishable from those formed in the absence of the drug; radioactivity was found in completed α- and β-globin chains but not in smaller peptides (Fig. 8). Authentic [^{14}C]globin, composed of α- and β-globin, was used as a standard marker for this experiment.

Effect of harringtonine on formation of peptidylpuromycin. Nascent peptide chains bound to the donor site on the ribosome react accordingly with puromycin. Chains localized in the acceptor site require translocation before they can react. Reticulocyte polyribosomes were used to investigate the effect of harringtonine on transpeptidation and translocation in the absence of exogenous aminoacyl-tRNA.

Incorporation of tritiated puromycin into trichloracetic acid-precipitable material was taken in the assay as a measure of peptidylpuromycin formation as described under MATERIALS AND METHODS. Harringtonine (0.5 mM) reduced the initial rate of peptidylpuromycin formation by over 3%. Anisomycin (0.1 mM), an established inhibitor of peptide bond formation, blocked the rate of synthesis of peptidylpuromycin

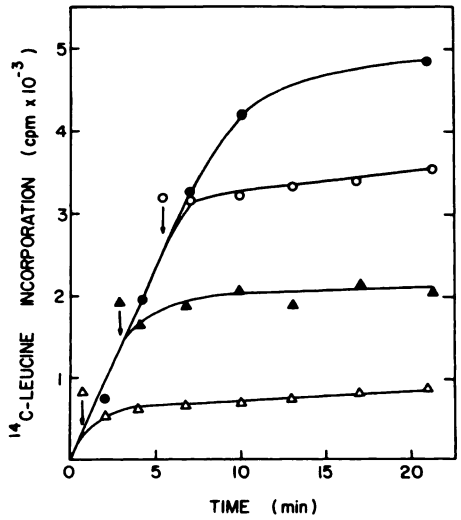


FIG. 6. *Effect of adding harringtonine at different times during synthesis of globin*

Standard reaction mixtures (0.5 ml) (see MATERIALS AND METHODS) were incubated at 33°. Harringtonine (100 μM) was added at 0 (Δ), 3 (▲) and 5 min (O); 0.05-ml aliquots were removed at intervals and pipetted into 5% trichloracetic acid, and globin synthesis was measured as described under MATERIALS AND METHODS.

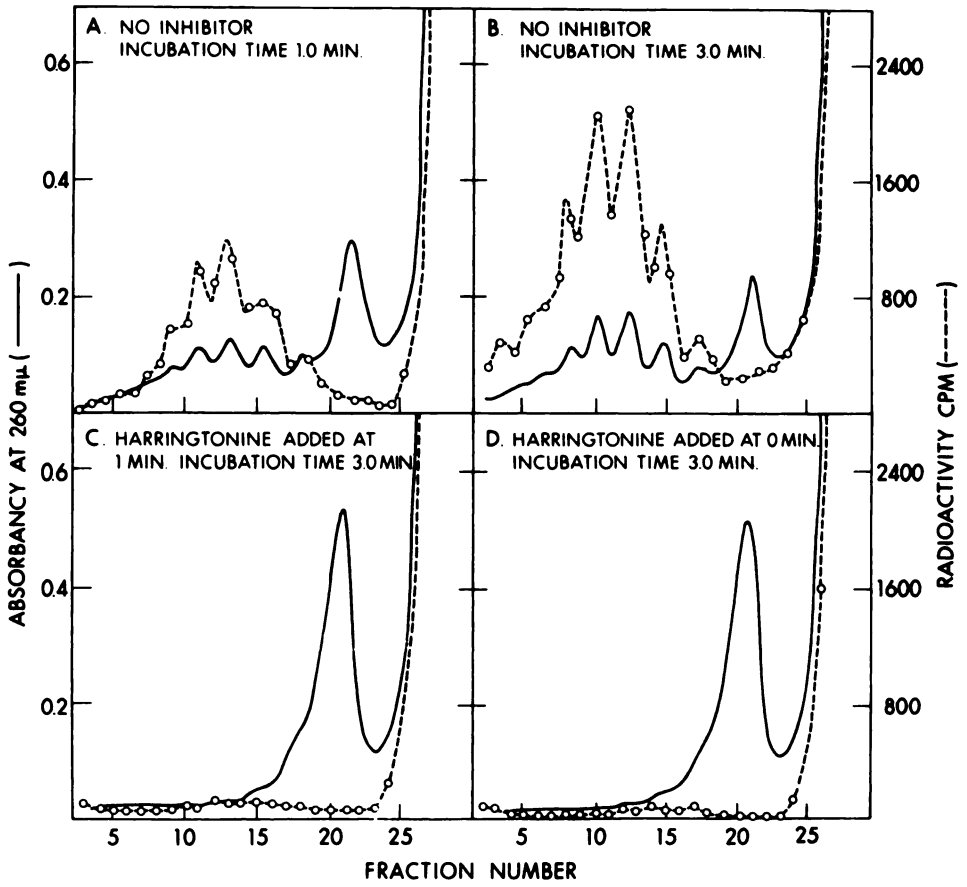


FIG. 7. *Effect of harringtonine on structure of polyribosomes and release of nascent peptides from polyribosomes*

Four standard mixtures (0.25 ml), each containing 5 nmoles of [^{14}C]leucine (200 mCi/mmole), were incubated at 33° for 1 min and then treated as follows. A. The reaction was terminated immediately. B. The reaction mixture was incubated at 33° for 2 min, then terminated. C. Harringtonine was added to the reaction mixture at a final concentration of 0.1 mM, incubated at 33° for 2 min, then terminated. D. Harringtonine was added before [^{14}C]leucine, and the reaction was incubated at 33° for 3 min, then terminated. All reactions were terminated by addition of 0.8 ml of cold reticulocyte standard buffer and layered on 10–25% sucrose gradients in the same buffer. Gradients were centrifuged as described under MATERIALS AND METHODS.

by 93%. Cycloheximide (0.1 mM), which prevents translocation under certain conditions, inhibited the reaction by 44%. Aurintricarboxylic acid (0.1 mM), an inhibitor of initiation, reduced peptidylpuromycin formation by 6%. These results suggest that harringtonine does not affect transpeptidation.

Effect of harringtonine on binding of [^{3}H]polyuridylic acid and uncharged tRNA to ribosomes. The effect of harringtonine on binding of synthetic polynucleotides to reticulocyte ribosomes was compared with that of aurintricarboxylic acid (Table 1). Harringtonine, 0.3 mM, did not affect binding of [^{3}H]polyuridylic acid, while this reaction was inhibited 94% by 0.1 mM aurintricarboxylic acid.

The effect of harringtonine on binding of uncharged *Escherichia coli* tRNA to ammonium chloride-washed ribosomes was essentially unaffected by 0.3 mM harringtonine (Table 2). Aurintricarboxylic acid (0.3 mM) inhibited this reaction by 51%.

Structural specificity. The inhibitory activities of certain analogues and isomers of

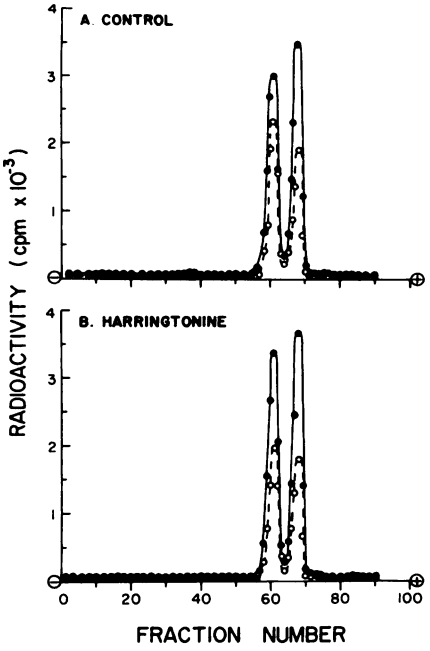


FIG. 8. *Acrylamide gel electrophoresis of reaction products formed in the presence and absence of harringtonine*

Two reaction mixtures were incubated exactly as described for Fig. 7B and D, except that [^{3}H]leucine (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 filtered through Millipore membranes to remove ribosomes. Filtrates containing ^{3}H-peptides were dialyzed for 24 hr against three changes of distilled water. Then 120 μg of ^{3}H-peptides were mixed with 70 μg of authentic [^{14}C]globin in 10 mM phosphate buffer containing 0.15 M mercaptoethanol and 0.2% sodium dodecyl sulfate. Samples were heated at 100° for 1 min, then cooled to room temperature. Electrophoresis was conducted for 16 hr at 4 V/cm on 20 cm of acrylamide prepared in 0.01 M phosphate buffer containing 0.1% sodium dodecyl sulfate. Migration is from the anode. ●——●, ^{3}H-peptide; O- - -O, authentic [^{14}C]globin. Gels were crushed automatically and suspended in 10 ml of Aquasol, and radioactivity was determined. *M* phosphate buffer containing 0.1% sodium dodecyl sulfate. Migration is from the anode. ●—●, ^{3}H-peptide; O---O, authentic [^{14}C]globin. Gels were crushed automatically and suspended in 10 ml of Aquasol, and radioactivity was determined.

harringtonine, as tested in HeLa cells, intact reticulocytes, and reticulocyte lysates, are shown in Table 3. Neither cephalotaxine (I) nor the side chain of harringtonine (see Fig. 1), when tested alone, was active. Harringtonine (II), isoharringtonine (III), homoharringtonine (IV), and deoxyharringtonine (V) were equally active in-

TABLE 1

Effect of harringtonine and aurintricarboxylic acid on binding of [^{3}H]polyuridylic acid to reticulocyte ribosomes

Each reaction mixture contained, in a volume of 0.25 ml, 8 mM $MgCl_2$, 60 mM KCl, 25 mM Tric-HCl (pH 7.5), 33 μg of ammonium chloride-washed ribosomes, and 1.85 A_{260} units of [^{3}H]poly U (80,000 cpm). Inhibitors were added after the ribosomes and prior to the addition of [^{3}H]poly U. Reactions were incubated at 36° for 10 min, filtered through a Millipore membrane filter, and washed seven times with a buffer of the same ionic composition as that used in the reaction mixture. Filters used in this experiment were first treated by immersion in 0.5 M NaOH at 23° 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.

Inhibitor added	Concentration	Poly U bound	Inhibition
	mM	cpm	%
None		11,480	
Harringtonine	0.1	12,080	0
	0.3	14,490	0
	1.0	13,360	0
Aurintricarboxylic acid	0.1	69	94
	1.0	203	98

TABLE 2

Effect of harringtonine and aurintricarboxylic acid on binding of [^{14}C]tRNA to reticulocyte ribosomes

The reaction mixture contained, in a volume of 0.125 ml, 50 mM Tris-HCl (pH 7.4), 8 mM $MgCl_2$, 60 mM KCl, 1.37 A_{260} units of *E. coli* [^{14}C]tRNA (28,000 cpm), 100 μg of ammonium chloride-washed ribosomes, and the indicated concentrations of inhibitors. The reaction mixture was incubated at 0° for 20 min. Ribosomes were collected on Millipore filters, and radioactivity was determined as described under MATERIALS AND METHODS.

Inhibitor added	Concentration	tRNA bound	Inhibition
	mM	cpm	%
None		1899	
Harringtonine	0.1	1972	0
	0.3	2068	0
Aurintricarboxylic acid	0.03	967	51
	1.0	85	96

TABLE 3

Effect of harringtonine and its isomers on protein synthesis

Results are expressed as the concentration of harringtonine and its isomers required for 50% inhibition of protein and synthesis. The details of experimental procedures are described under MATERIALS AND METHODS.

Alkaloid	HeLa cells	Reticulocytes	Lysate
	μM	μM	μM
I. Cephalotaxine	100	100	440
II. Harringtonine	0.05	0.15	2
III. Isoharringtonine	0.09	0.19	7.5
IV. Homoharringtonine	0.04	0.10	3
V. Deoxyharringtonine	0.07	0.12	6.6
VI. Pseudodeoxyharringtonine	24	57	1100

hibitors of protein biosynthesis in HeLa cells, intact reticulocytes, and reticulocyte lysates. Pseudodeoxyharringtonine (VI) was less active.

The effects of homoharringtonine were examined in more detail. This analogue induces breakdown of polyribosomes to monosomes with concomitant release of complete globin chains, and when it is added to a reticulocyte lysate a delay of 2 min occurs before inhibition of globin synthesis is observed. Thus, the principal effects of homoharringtonine on initiation of protein synthesis are similar to those of harringtonine.

DISCUSSION

Harringtonine inhibits biosynthesis of protein and DNA in HeLa cells. The observed partial inhibition of DNA synthesis probably results from a primary effect on protein synthesis, since the latter process is required for concurrent synthesis of DNA in animal cells (12–15). Anisomycin (6), puromycin (12), emetine (16), tylocrebrine (17), cycloheximide (18), pactamycin (19), and other agents (20, 21) that block protein synthesis in animal cells simultaneously inhibit synthesis of DNA.

Aurintricarboxylic acid (7, 8), sodium fluoride (22, 23), and pactamycin (24–27), established inhibitors of initiation of protein synthesis, induce breakdown of polyribosomes to monosomes with concomitant release of completed chains of globin. Inhibition of amino acid incorporation occurs several minutes after addition of those drugs to cell-free lysates. In contrast, inhibitors of peptide chain elongation, such as emetine (16), anisomycin (6), cycloheximide (18), and tylocrebrine (17), block protein synthesis without observable delay, prevent the breakdown of polyribosomes to monosomes, and inhibit the release of nascent peptides. In the case of harringtonine, a delay of several minutes occurs before inhibition of globin synthesis is observed in reticulocyte lysates. The drug induces complete breakdown of polyribosomes to monosomes, and released peptides have been identified as complete globin chains.

Inhibitors of initiation and elongation can also be distinguished by measuring their effects on the rate of peptidylpuromycin formation. Rabbit reticulocyte polyribosomes bear nascent peptide chains. Some of these are bound to the "donor" site and react with puromycin; others, located in the "acceptor"site, react only after translocation. Thus anisomycin, an inhibitor of transpeptidation, blocks the reaction of puromycin with nascent peptides, and cycloheximide, which prevents translocation (28), inhibits this reaction, but only by 50%. The action of cycloheximide is complex, and this drug has also been shown to inhibit peptide bond formation (29) and chain initiation (30). Drugs such as aurintricarboxylic acid, which inhibits initiation without affecting chain elongation, do not prevent synthesis of peptidylpuromycin (29); harringtonine behaves in this manner.

Based on the preceding observations, harringtonine would appear to be an inhibitor of initiation. The drug can be distinguished from certain other reported inhibitors of initiation (20). Aurintricarboxylic acid (8) prevents attachment of mRNA and uncharged tRNA to reticulocyte ribosomes, thus affecting an early step in initiation. Pactamycin inhibits binding of uncharged tRNA to ribosomes, prevents dipeptide formation, and blocks formation of the initiation complex under certain conditions (31). Harringtonine has no effect on binding of tRNA or mRNA to

ribosomes and may exert its action on a later step in the initiation process.

The structure-activity analysis of harringtonine and its isomers in our systems *in vitro* reveals that several configurations of the side chain are associated with full inhibitory activity. In experimental animals homoharringtonine has been found to be slightly more active as an antitumor agent than harringtonine. The principal effects of this isomer also appears to be directed against initiation of protein synthesis.

Neither the side chains of harringtonine, when tested alone, nor the alkaloid lacking the side chain (cephalotaxine) are active. Certain modifications in the side chain, such as those found in pseudodeoxyharringtonine, abolish activity, while other related isomers (isoharringtonine and deoxyharringtonine) are fully active. Lack of activity does not appear to be related to transport, since the same relative inhibitory activities are observed when these compounds are tested in cell-free lysates. This information may be useful in the effort to prepare active analogues of harringtonine from the more abundant cephalotaxine for use in cancer chemotherapy (32).

Harringtonine is the only small molecule thus far reported that penetrates animal cell membranes and selectively inhibits initiation of protein synthesis without affecting chain elongation. Thus this drug may prove especially useful in studying mechanisms of protein biosynthesis in mammalian cells.

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