MOLECULAR PHARMACOLOGY, 7, 632-644

Studies on Camptothecin:

I. Effects on Nucleic Acid and Protein Synthesis

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(Received January 22, 1971)

SUMMARY

The plant alkaloid camptothecin inhibits the synthesis of DNA and RNA in HeLa cells at concentrations that do not initially influence protein synthesis. DNA, isolated from cells inhibited by camptothecin, has a sedimentation constant of approximately 40 S under alkaline conditions, in contrast to the high molecular weight DNA found in untreated cells. Inhibition of RNA synthesis in intact cells is completely reversed by removing the antibiotic from the medium, while DNA synthesis is only partially reversed by this procedure. Camptothecin does not significantly inhibit activity of the DNA-dependent RNA polymerase isolated from *Escherichia coli* or of DNA and RNA polymerases prepared from HeLa cells. The structure-activity relationships of camptothecin and its analogues, as determined by their effects on nucleic acid synthesis in HeLa cells and on the sedimentation of DNA obtained from drug-treated cells, correspond closely to their activity in suppressing the growth of tumors in experimental animals. The inhibitory effects of camptothecin on nucleic acid synthesis may account for its chemotherapeutic properties, and the reversibility of the effects on RNA synthesis suggests that camptothecin may be a useful tool for the study of macromolecular synthesis in animal cells.

INTRODUCTION

Camptothecin is a cytotoxic alkaloid found in the stem wood of *Camptotheca acuminata* (family Nyssaceae). The structures of camptothecin and its analogues, as determined by Wall *et al.* (1, 2), are shown in Fig. 1. Certain of these compounds exhibit potent antitumor activity against leukemia L1210 in mice and Walker 256 tumors in rats (4). Some pharmacological properties of camptothecin have been reported (5), preliminary clinical studies have been published (6), and Kessel[3] and others (7–10) have described various effects of camptothecin on macromolecular synthesis.

The present paper describes effects of camptothecin on nucleic acid synthesis in HeLa cells. Low concentrations of the drug inhibit the synthesis of DNA and RNA but do not initially influence protein synthesis. Camptothecin induces the conversion of high molecular weight DNA to a form sedimenting at approximately 40 S under alkaline conditions. These effects on nucleic acid metabolism may account for the re-

This investigation was supported by grants from the National Institutes of Health (CA-10666) and the American Cancer Society (T-418). This is publication 228 from the Joan and Lester Avnet Institute of Molecular Biology. A preliminary account of this work has appeared [*Pharmacologist* 12, 283 (1970)].

[1] Career Development Awardee (K4-GM-11,147) of the United States Public Health Service.

[2] Career Scientist of the Health Research Council of the City of New York.

[3] D. Kessel, (36) and personal communication.

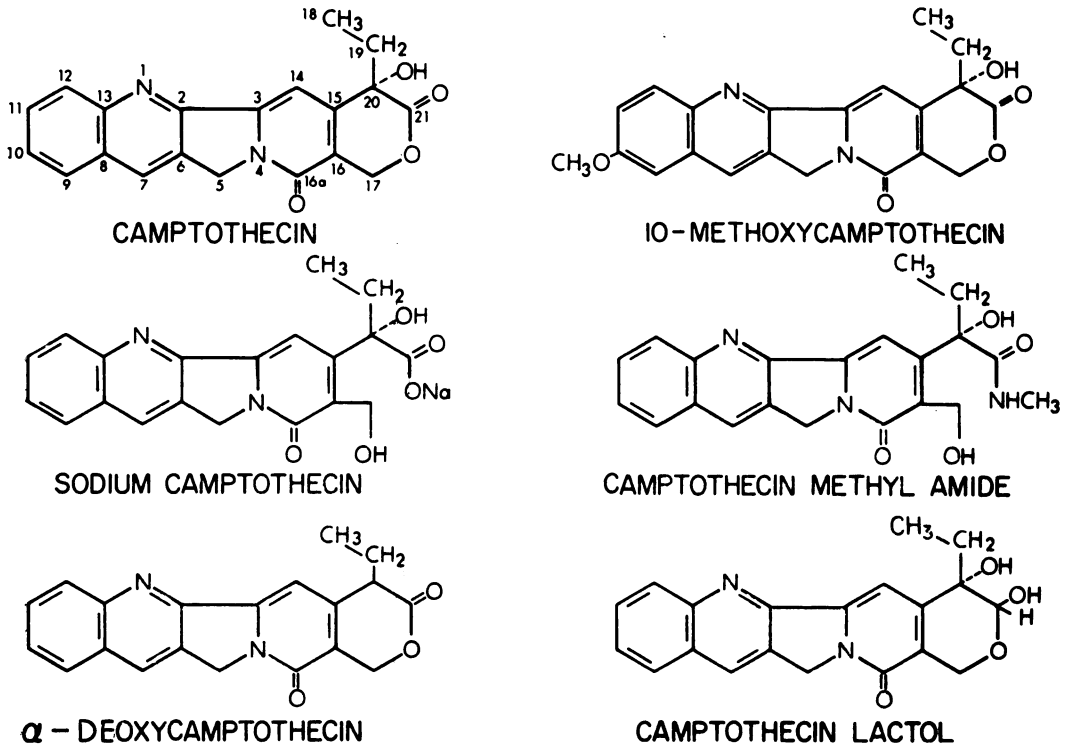

FIG. 1. *Structural formulae of camptothecin and analogues*
The numbering system used is that suggested by Shamma (3).

ported chemotherapeutic properties of the drug.

METHODS

Materials. Camptothecin and its analogues were kindly provided by Dr. M. E. Wall. Sodium camptothecin was obtained from Dr. J. L. Hartwell and Dr. H. B. Wood, Jr., of the Cancer Chemotherapy National Service Center, National Cancer Institute. Unless otherwise stated, camptothecin was used in the form of its sodium salt and was dissolved in water immediately prior to use. Actinomycin D was purchased from Mann Research Laboratories; ^{3}H-actinomycin D (3.4 Ci/mmole), from Schwarz BioResearch; calf serum and media for tissue culture, from Grand Island Biological Company; calf thymus DNA and pancreatic DNase, from Worthington Biochemical Corporation; ribo- and deoxyribonucleotides and nucleosides, from P-L Biochemicals; and Triton X-100, from Rohm and Haas. Anthramycin, in the form of its methyl ether, and adenovirus 2, containing ^{14}C-thymidine-labeled DNA, were gifts from W. Leimgruber of Hoffmann-La Roche and M. Horwitz of the Albert Einstein College of Medicine, respectively. Uniformly labeled ^{14}C-leucine (252 mCi/mmole), ^{14}C-uridine (52 mCi/mmole), ^{14}C-thymidine (54 mCi/mmole), Aquasol, and Liquifluor were obtained from New England Nuclear Corporation, and ^{3}H-GTP (1.3 Ci/mmole), ^{3}H-TTP (10.4 Ci/mmole), ^{3}H-uridine (13.8 Ci/mmole), and ^{3}H-thymidine (6.7–24 Ci/mmole), from Schwarz BioResearch.

Measurement of DNA, RNA, and protein synthesis in HeLa cells. HeLa S_3 cells were grown in suspension culture in Eagle's minimal essential medium (11) supplemented with 5% calf serum. The cells were harvested by centrifugation for 3 min at 1000 × *g* and then resuspended in minimal essential medium supplemented with 5% calf serum, at a concentration of 3–4 × 10^5 cells/ml. Leucine-depleted medium (minimal essential medium with the leucine con-

centration reduced to 0.02 mM) supplemented with 5% calf serum was used for measuring protein synthesis, and minimal essential medium supplemented with calf serum, for measurements of DNA and RNA synthesis. ^{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 millilter of the cold buffered salts solution described by Earle (12) (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 $\times$ *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% trichloracetic 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% trichloracetic acid, and the radioactivity was determined as described below.

Measurement of ribosomal RNA synthesis in HeLa cells. ^{14}C-Uridine (2.5 μCi) was added to 150 ml of HeLa cells (3–4 $\times$ 10^5 cells/ml) suspended in minimal essential medium supplemented with 5% calf serum. After 4 hr the cells were harvested by centrifugation, washed by resuspension in fresh medium, then centrifuged again, and suspended in a buffer composed of 0.01 M acetate (pH 6.0), 0.1 M NaCl, and 1 mM EDTA (ANE buffer). Labeled RNA was extracted from the cells with cold phenol, using the method described by Perry and Kelley (13). After precipitation with ethanol, the RNA was dissolved in 1 ml of ANE buffer and residual phenol was removed by shaking with ether. Approximately 7 absorbancy units were suspended in 1.0 ml of ANE buffer, then layered on 36 ml of a 5–20% (w/v) sucrose gradient prepared in the same buffer. After centrifugation for 18 hr at 20,000 rpm in a Spinco SW 27 rotor at 4°, fractions of 1.0 ml were collected from the bottom of the gradient while the optical density was simultaneously recorded by means of a flow cell attached to a Gilford recording spectrophotometer. RNA was precipitated on Millipore membrane filters by the addition of trichloracetic acid and the radioactivity was determined as described below.

Measurement of leucine incorporation by lysates prepared from rabbit reticulocytes. Reticulocytosis was induced in New Zealand rabbits by the administration of acetophenylhydrazide (14). Crude lysates were prepared from these reticulocytes, and the synthesis of globin was measured by the method of Adamson *et al.* (15), using modifications described by Zucker and Schulman (16).

Measurement of uridine and thymidine nucleotides in acid-soluble pools. HeLa cells were suspended in 1.0 ml of minimal essential medium at a concentration of 4–5 $\times$ 10^5 cells/ml; 25 μCi of ^{3}H-thymidine (24 Ci/mmole) or ^{3}H-uridine (13.8 Ci/mmole) were added, and the suspension was incubated for 10 min at 37° in the presence or absence of camptothecin. Cells were collected by low-speed centrifugation, washed with buffer A, lysed in 0.3 ml of 5% trichloracetic acid, and allowed to stand in an ice bath for 10 min. Following centrifugation at 10,000 rpm for 20 min, precipitates were removed and 100 μl of the supernatant solution were evaporated to dryness at approximately 15° under reduced pressure. After redissolving in 10 μl of water, the appropriate nucleosides and their mono-, di-, and triphosphates (0.1–0.5 μmole each) were added as carriers and separated by thin-layer chromatography on cellulose as described below.

Assay of Escherichia coli RNA polymerase activity. E. coli RNA polymerase, previously designated ammonium sulfate fraction III (17), was a gift from Dr. Umadas Maitra. The activity of this enzyme was determined in a standard reaction mixture containing Tris-HCl, pH 7.5, 20 μmoles; $MnCl_2$, 1 μmole; 2-mercaptoethanol, 1 μmole; ATP, UTP, and CTP, 60 nmoles each; ^{3}H-GTP, 40 nmoles (25 μCi/μmole); native calf thymus DNA, 10 μg; and 0.5 unit of enzyme in a final volume of 0.25 ml. A reaction mixture lacking DNA was included to determine background incorporation of radio-

activity. Reactions were terminated by the addition of 1.5 ml of cold 5% trichloracetic acid; the mixture was chilled for 5 min and 20 μmoles of sodium pyrophosphate were added. Precipitates were collected on Millipore membrane filters and rinsed three times with 5% trichloracetic acid and three times with 1% trichloracetic acid, and the radioactivity was determined as described below.

Assay of RNA polymerase activity in HeLa cell nuclei. Nuclei were prepared as previously described (14), washed by the method of Friedman (18), and suspended in a solution composed of 0.01 M potassium phosphate (pH 7.7), 2 mM $MgCl_2$, and 15 mg/ml of bovine serum albumin. Enzyme activity was determined in reaction mixtures containing Tris-HCl, pH 8.0, 10 μmoles; KCl, 7.5 μmoles; $MgCl_2$, 0.5 μmole; NaF, 3 μmoles; cysteine, 1 μmole; ATP, 0.1 μmole; UTP, 0.04 μmole; CTP, 0.04 μmole; ^{3}H-GTP (1.3 Ci/mmole), 0.5 nmole; and 0.03 ml of nuclear suspension (40×10^6 nuclei/ml) in a volume of 0.1 ml. The reaction was terminated by the addition of 5% trichloracetic acid, and the radioactive precipitates were processed as described in the preceding paragraph.

Assay of DNA polymerase activity in HeLa cell nuclei. A suspension of nuclei was prepared as described in the preceding section. Calf thymus DNA was treated for 6 min at 37° with pancreatic DNase and heated in a boiling water bath for 10 min, as described by Friedman (18), to obtain optimal priming activity. The activity of DNA polymerase was measured in reaction mixtures containing Tris-HCl, pH 8.0, 3.5 μmoles; $MgCl_2$, 1.2 μmoles; dATP, dCTP, and dGTP, 0.12 μmole each; ^{3}H-TTP (75 μCi/μmole), 40 nmoles; primer DNA, 25 μg; and 0.03 ml of the nuclear suspension (30×10^6 nuclei/ml) in a final volume of 0.1 ml. The reaction was terminated with 5% trichloracetic acid and the radioactive precipitates were processed as described above.

Sucrose density gradient analysis of DNA obtained from HeLa cells. Alkaline sucrose gradients were prepared by a slight modification of the method described by Hodge and Scharff (19). Fourteen milliliters of a 5–20% linear sucrose gradient, containing 1.0 M NaCl, 0.19 M NaOH, and 0.01 M EDTA, were layered over a 0.5-ml cushion of cesium chloride (density, 1.8 g/ml). The gradients were overlaid with 0.5 ml of a solution composed of 1.0 M NaCl, 0.19 M NaOH, 0.01 M EDTA, and 0.5% sodium deoxycholate. HeLa cells used in these experiments were collected by centrifugation, resuspended in 0.3 ml of 0.15 M NaCl, layered on the gradient, and immediately centrifuged in a Spinco SW 27 rotor at 15,000 rpm for 15 hr at 4°. Fractions of 0.6–0.7 ml were collected by means of a finger pump, beginning at a position 2.5 cm above the bottom of the tube. The solution remaining in the tube was disrupted by sonic oscillation for 20 sec in a Branson model W 185 Sonifier set at position 6. All fractions, including the sonicated material, were adjusted to a final concentration of 12.5% trichloracetic acid, and, after standing for 15 min at 4°, precipitates were collected on Millipore membrane filters. Filters were washed three times with 5% trichloracetic acid, and the radioactivity was determined as described below.

Thin-layer chromatography. Thin layers of cellulose, mounted on plastic backings, were obtained from Eastman Organic Chemicals. Nucleosides and nucleotides were separated on cellulose, using a solvent system (20) composed of 1-butanol, acetone, acetic acid, 5% NH_4OH and water (45:15:10:10:20). Compounds were visualized with ultraviolet light, the chromatograms were cut into 0.5-cm pieces, and the radioactivity was determined as described below.

Determination of radioactivity. Radioactive precipitates containing tritium were collected on Millipore filters and treated with 0.6 ml of NH_4OH for 15 min, dissolved in 10 ml of the scintillation mixture described by Bray (21), and counted with an efficiency of 14% in a Packard Tri-Carb liquid scintillation counter. Precipitates obtained from alkaline sucrose gradients were collected on Millipore filters, dissolved in 10 ml of Aquasol, and counted with an efficiency of 25%. Precipitates containing ^{14}C were also collected on Millipore filters, glued to planchets, dried, and counted with an efficiency of 21% in a Nuclear-Chicago low-background coun-

ter. Sections of thin-layer chromatograms were placed in 10 ml of a solution composed of Liquifluor, 330 ml; Triton X-100, 2 liters; and toluene, 4 liters.

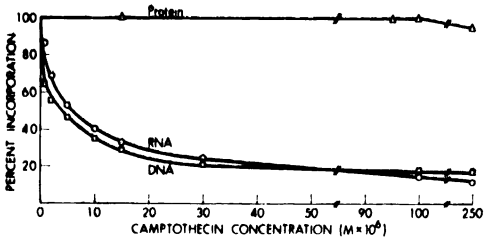

FIG. 2. *Effect of various concentrations of camptothecin on synthesis of protein, RNA, and DNA in HeLa cells*

Cells were incubated for 60 min at 37°, and the incorporation of radioactivity into trichloracetic acid-insoluble material was used to calculate the rates of synthesis of protein (△——△), RNA (○——○), and DNA (□——□), which, in the control reactions, were 1500, 2492, and 8670 cpm/ml, respectively. The percentage incorporation shown is expressed relative to these controls. Protein, RNA, and DNA syntheses were measured as described under METHODS, and camptothecin was present at the final concentrations indicated in the figure.

RESULTS

Effects of camptothecin 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 the rates of DNA, RNA, and protein synthesis, respectively. The effects of camptothecin on these parameters during the first hour after addition of the drug to the medium are illustrated in Fig. 2. The rate of protein synthesis was unaffected by 100 μM camptothecin, while synthesis of both DNA and RNA was inhibited by approximately 50% at a concentration of 5 μM.

The effects of camptothecin on the rate of DNA and RNA synthesis in HeLa cells were observed immediately after addition of the drug to the medium (Fig. 3A). After 15 min, synthesis of both DNA and RNA was inhibited by more than 70%. In contrast to these results, the effects of actinomycin on DNA synthesis were not observed until at least 1 hr after inhibition of RNA synthesis had occurred (Fig. 3B). Similar effects of actinomycin have been reported by other investigators (22).

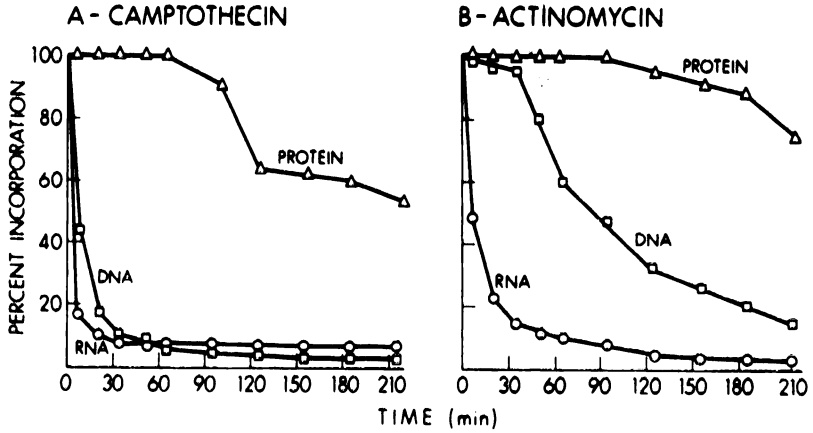

FIG. 3. *Effect of camptothecin (A) and actinomycin (B) on synthesis of protein, RNA, and DNA in HeLa cells at various times following exposure to inhibitor*

Hela cells were incubated at 37° in medium containing 50 μM camptothecin or 0.66 μM actinomycin, or in the absence of inhibitors. At the intervals indicated, 1-ml aliquots were removed from each of the three cultures, the appropriate isotope was added, and the rates of synthesis of protein (△——△), RNA (○——○), and DNA (□——□) were determined, as described under METHODS, during the succeeding 10-min intervals. Incorporation of radioactivity into trichloracetic acid-insoluble material during each time period is represented as the midpoint of the interval. The percentage incorporation shown is expressed relative to controls, in which incorporation of ^{14}C-leucine (△——△), ^{14}C-uridine (○——○), and ^{14}C-thymidine (□——□) was 280, 360, and 312 cpm/ml, respectively, during the initial 10 min of the experiment, and 400, 445, and 350 cpm/ml, respectively, at the end of the experiment.

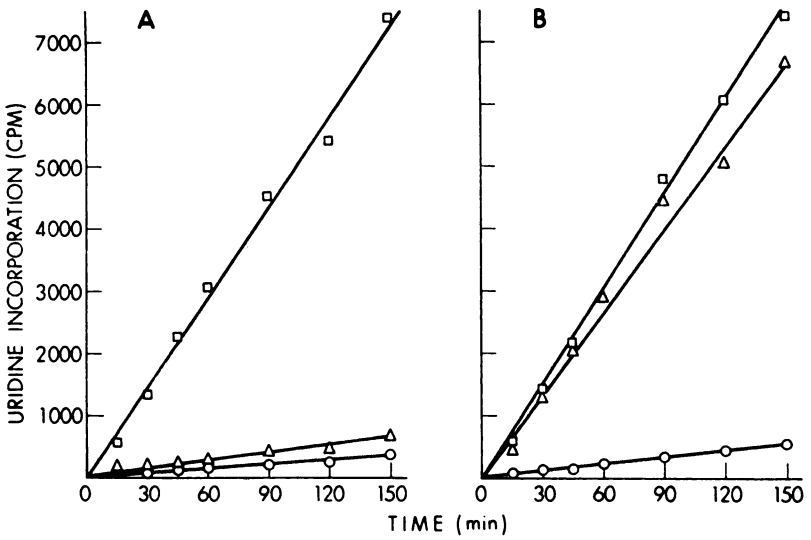

FIG. 4. *Reversal of inhibition of RNA synthesis in HeLa cells*

Hela cells were incubated at 37° with 100 μM camptothecin (△——△) or 20 μM anthramycin (○——○), or in the absence of inhibitors (□——□). After 30 min, a 10-ml sample was removed from each of the three cultures, washed twice with warm minimal essential medium, and resuspended in medium supplemented with calf serum. ^{14}C-Uridine (0.025 μCi) was added to 10 ml of the original culture (A) or the washed cells (B); 1-ml aliquots were removed at the indicated times, and the amount of RNA synthesized was measured as described under METHODS.

A decrease in the rate of protein synthesis in camptothecin-treated cells was noted 1 hr or more after inhibition of RNA synthesis had occurred. This decrease was similar to that observed in actinomycin-treated control cultures, and progressed over several hours.

Reversal of inhibition of RNA synthesis by camptothecin. Inhibition of RNA synthesis by camptothecin in HeLa cells could be reversed by collecting camptothecin-treated cells by centrifugation, washing them twice, and resuspending them in minimal essential medium. After exposure to 0.1 mM camptothecin for periods as long as 60 min, RNA synthesis was strongly inhibited (Fig. 4A). Following the washing procedure, the rate of RNA synthesis in these cells was at least 90% of that observed in uninhibited cultures subjected to similar manipulations (Fig. 4B). Anthramycin (23), an antibiotic that irreversibly inhibits RNA synthesis in HeLa cells,[4] served as a control for this experiment.

Partial reversal of inhibition of DNA synthesis. Inhibition of DNA synthesis by camptothecin in nonsynchronous cultures of HeLa cells was partially reversed after exposure to 0.1 mM camptothecin (Fig. 5B). After exposure to the antibiotic for 5 or 60 min prior to washing, the rate of DNA synthesis following the washing procedure was inhibited 47% or 63%, respectively, as compared to uninhibited cultures. In contrast, the effect of anthramycin on DNA synthesis was not reversed by similar washing procedures.

Effect of camptothecin on ribosomal RNA synthesis. HeLa cells, incubated for 4 hr in the presence of 50 μM camptothecin, synthesized 20% of the RNA observed in control cultures. When analyzed by sucrose density gradient centrifugation, very little newly synthesized RNA was found to sediment in the 28 S or 18 S regions of the gradient, while 84% of that formed was observed to sediment in the 4–5 S region (Fig. 6).

During a 4-hr incubation period, 60% of the RNA synthesized in the control culture sedimented in the 28 S region of the gradient, 18% in the 18 S region, and 21% in the 4–5 S region. The effects of 0.066 μM actino-

[4] S. B. Horwitz, C. Chang, and A. P. Grollman, unpublished observations.

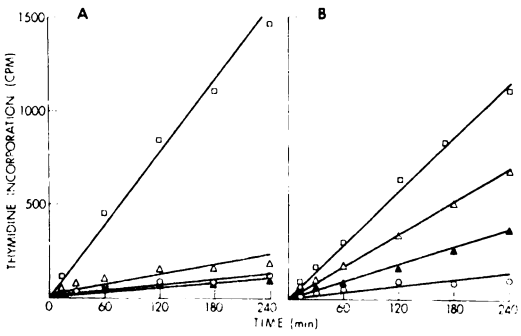

FIG. 5. *Reversal of inhibition of DNA synthesis in HeLa cells*

Hela cells were incubated at 37° in the presence of 100 μM camptothecin for 5 min (△——△) or 60 min (▲——▲), with 20 μM anthramycin for 60 min (○——○), or in the absence of inhibitors (□——□). Following this incubation, 10 ml of cells were removed from each of the cultures, washed twice with warm minimal essential medium, and resuspended in medium supplemented with calf serum. ^{14}C-Thymidine (0.05 μCi) was added to 10 ml of the original culture (A) or the washed cells (B); 1-ml aliquots were removed at the indicated times, and the amount of DNA synthesized was measured as described under METHODS.

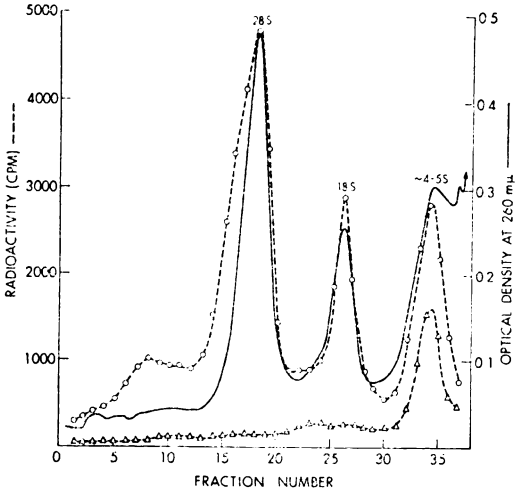

FIG. 6. *Effect of camptothecin on ribosomal RNA synthesis*

Cultures of HeLa cells were exposed to ^{14}C-uridine for 4 hr in the presence (△- - -△) or absence (○- - -○) of 50 μM camptothecin. RNA was extracted from whole cells and analyzed by sucrose gradient sedimentation, as described under METHODS. The optical density is shown only for the control, as it was essentially the same in both cultures.

mycin on RNA synthesis were compared under similar experimental conditions; very little newly synthesized RNA sedimented in the 28 S and 18 S regions, while 81% was observed in the 4–5 S region.

Effect of camptothecin on protein synthesis in reticulocyte lysates. Camptothecin did not alter incorporation of leucine into globin by lysates prepared from rabbit reticulocytes (Table 1). Emetine, an alkaloid that is known to inhibit globin synthesis (24), served as a control for this experiment.

Effect of camptothecin on accumulation of uridine and thymidine nucleotides in acid-soluble pools. At a concentration of 0.1 mM, camptothecin partially inhibited intracellular accumulation of uridine and the subsequent conversion of this nucleoside to its nucleotides (Table 2). However, no effect was observed at 10 μM, a concentration which inhibited RNA synthesis by more than 50% (Fig. 2). The uptake and conversion of thymidine were unaffected, even at 0.1 mM concentrations of camptothecin.

Effect of camptothecin on activity of DNA and RNA polymerases. As shown in Table 3, camptothecin had little effect on the activity of crude DNA polymerase prepared from HeLa cell nuclei, even at concentrations of 1 mM. Actinomycin, which served as a control, inhibited the activity of this enzyme by more than 50% at a concentration of 80 μM.

The effect of camptothecin on RNA polym-

TABLE 1

Effect of camptothecin on synthesis of globin in reticulocyte lysates

Globin synthesis was determined in lysates prepared from rabbit reticulocytes by measuring ^{14}C-leucine incorporation into hot trichloracetic acid-insoluble material, as described under METHODS. Reaction mixtures were incubated for 8 min at 33°.

Addition	Concentration	Incorporation	Inhibition
	μM	*cpm*	%
None		1650	
Camptothecin	1000	1590	4
	100	1620	2
	1	1550	6
Emetine	100	22	99

TABLE 2

Effect of camptothecin on uridine and thymidine nucleotides in acid-soluble pools in HeLa cells

Nucleotide pools were labeled by adding ^{3}H-thymidine or ^{3}H-uridine to cultures of HeLa cells, and the intracellular concentrations of acid-soluble nucleotides and nucleosides were determined as described under METHODS. The solvent system used for thin-layer chromatography did not completely resolve either nucleotide diphosphate from its respective triphosphate.

Nucleoside or nucleotide(s)	Incorporation		
	Control	Camptothecin, 100 μM	Camptothecin, 10 μM
	cpm	*cpm*	*cpm*
Uridine	992	737	1,100
UMP	27,200	13,800	30,100
UDP + UTP	358,000	228,000	421,000
Thymidine	1,100	1,900	
TMP	612	618	
TDP + TTP	18,700	17,800	

TABLE 3

Effect of camptothecin on activity of DNA polymerase in nuclei prepared from HeLa cells

Enzyme activity was determined as described under METHODS. The assay was initiated by the addition of DNA, after which the mixture was incubated at 37° for 40 min.

Addition	Concentration	Incorporation	Inhibition
	μM	*cpm*	%
None		8820	
Camptothecin	1000	7980	10
	100	8310	6
Actinomycin	160	1990	78
	80	4330	51

erases prepared from *E. coli* and HeLa cell nuclei was also examined (Table 4). The activity of RNA polymerase purified from *E. coli* was totally dependent on the presence of template DNA. The activity of this enzyme was unaffected by 100 μM camptothecin, but was inhibited 80% by actinomycin at a concentration of 2 μM. Incubation of camptothecin with the DNA primer or with RNA polymerase for 10 min at 37° prior to beginning the assay did not increase the observed degree of inhibition. In the former case, the reaction was initiated by adding RNA polymerase to the reaction; in the latter, by adding template.

RNA polymerase prepared from HeLa cell nuclei was inhibited 29% by the highest concentration of camptothecin used (1 mM), but only 9% by a 10 μM concentration. This enzyme was sensitive to inhibition by actinomycin.

Effect of camptothecin on sedimentation of HeLa cell DNA. The results shown in Table 5 indicate that acid-insoluble radioactivity remained unchanged when HeLa cells containing ^{3}H-thymidine-labeled DNA were incubated for 3.5 hr in the presence of 50 μM camptothecin. Nevertheless, this DNA was apparently converted from a high to a lower molecular weight form, as detected by sedimentation of cells through alkaline sucrose density gradients (Fig. 7A). The lower molecular weight material sedimented at approximately 40 S, while DNA isolated

TABLE 4

Effect of camptothecin on activity of RNA polymerases prepared from E. coli and HeLa cells

Enzyme activity was determined as described under METHODS. In assays of the *E. coli* enzyme, all components of the reaction mixture, except the enzyme, were incubated for 10 min at 37° in the presence of the indicated concentrations of camptothecin and actinomycin. Enzyme was added to initiate the reaction, and the incubation was continued for 20 min. In assays of the enzyme prepared from HeLa cell nuclei, camptothecin or actinomycin was added to the reaction mixtures at the indicated concentrations; the assay was initiated by the addition of the nuclei, after which the mixture was incubated for 5 min at 37°.

Addition	Concentration	Incorporation	Inhibition
	μM	*cpm*	%
E. coli enzyme			
No addition		2120	
Camptothecin	220	2550	0
Actinomycin	2	424	80
HeLa cell nuclei enzyme			
No addition		936	
Camptothecin	1000	663	29
	100	841	10
	10	849	9
Actinomycin	8	248	74

TABLE 5

Measurement of acid-insoluble fraction prepared from HeLa cells exposed to camptothecin

HeLa cells containing ^{3}H-thymidine-labeled DNA were prepared by exposing 50 ml of a cell suspension to 2.5 μCi of ^{3}H-thymidine for 17 hr. The cells were centrifuged, washed, and resuspended in 50 ml of fresh medium. Camptothecin was added to 25 ml of the culture at a final concentration of 50 μM; the remainder served as an uninhibited control. Aliquots of 1.0 ml were removed from both cultures at the indicated times, the acid-insoluble fraction was recovered by precipitation with cold trichloracetic acid, and the radioactivity was determined as described under METHODS.

Time	Acid-insoluble radioactivity		
	Control	Camptothecin-treated	Inhibition
min	*cpm*	*cpm*	%
0	4950	4990	0
30	4670	4450	5
60	4790	4720	1
90	4390	4240	4
150	4610	5070	0
220	4740	4840	0

from untreated HeLa cells was recovered from the bottom of the gradient.

Conversion of DNA to the lower molecular weight form occurred within 10 min if cells were exposed to 20 μM camptothecin at 37°; no further changes were observed if the time of incubation was extended to 1 hr. Similar results occurred when the temperature was maintained at 0° for 10 min.

Significant quantities of lower molecular weight DNA were formed in cells treated with lower concentrations (2 μM) of camptothecin. Even at 0.2 μM, differences between treated and untreated cells were observed (Fig. 7A). Actinomycin also affected sedimentation of DNA after incubation of HeLa cells with high concentrations of this antibiotic (Fig. 7B). However, a distinct new peak did not appear in the 40 S region of the gradient. No alteration in the sedimentation of DNA was observed in cells treated with 0.2 μM actinomycin, even though this concentration of inhibitor prevents ribosomal RNA synthesis (Fig. 6). The slight breakdown of DNA observed in the control cells used in this particular experiment (Fig. 7B) was not a consistent observation.

Structure-activity relationships. Structural analogues of camptothecin (Fig. 1) were tested to determine their inhibitory effects on RNA synthesis in HeLa cells and their ability to convert DNA to a lower molecular weight species. For these experiments, camptothecin, its sodium salt, camptothecin methyl amide, deoxycamptothecin, methoxycamptothecin, and the lactol (dihydrohemiacetal) analogue of camptothecin were dissolved in dimethyl sulfoxide at a concentration of 1 mg/ml. The final concentration of dimethyl sulfoxide present in these assays had no apparent effect on the rate of macromolecular synthesis.

Camptothecin, its sodium salt, the amide, and deoxy and methoxy analogues inhibited RNA synthesis in HeLa cells by 50% or more at concentrations of 5 μM (Fig. 8). These compounds also converted approximately 90% of cellular DNA to a lower molecular weight species at a concentration of 20 μM (Table 6). The lactol analogue was the least active of the group, but still inhibited the rate of RNA synthesis by 50% at a concentration of 30 μM and induced some breakdown of DNA.

DISCUSSION

Some of the effects of camptothecin on macromolecular synthesis in HeLa cells are observed immediately after the cells have been exposed to the drug. These include inhibition of ribosomal RNA synthesis, conversion of cellular DNA from a high to a lower molecular weight form, and inhibition of DNA synthesis. Inhibition of RNA synthesis, measured as incorporation of uridine into acid-insoluble material, is completely reversed by washing the cells in fresh medium. This observation suggests that camptothecin might prove useful for studies in which RNA synthesis is related to protein synthesis or to the assembly of macromolecules. In contrast to the effects on RNA synthesis, inhibition of DNA synthesis was only partially reversed by similar washing procedures.

The rate of amino acid incorporation be-

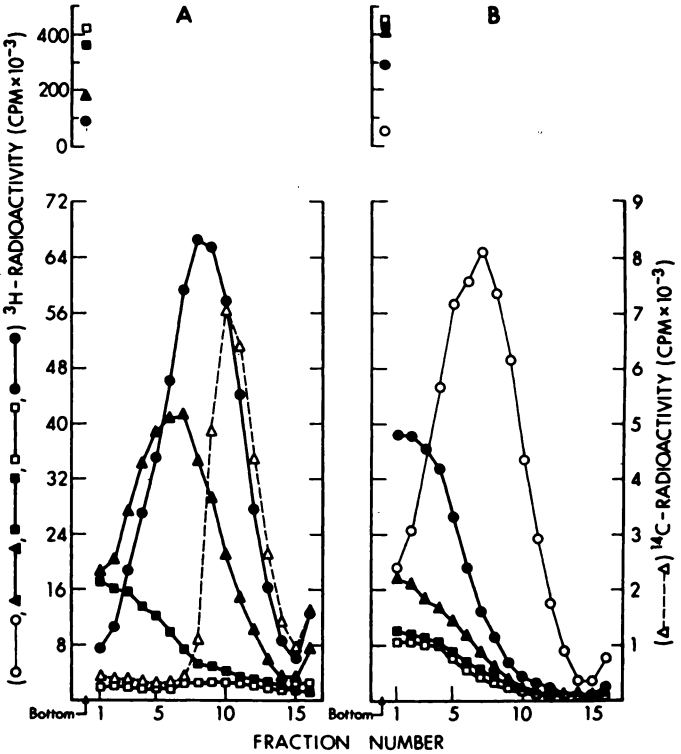

FIG. 7. *Sedimentation of DNA obtained from camptothecin- and actinomycin-treated HeLa cells*

HeLa cells containing tritium-labeled DNA were prepared by incubating 20 ml of a cell suspension (3×10^5 cells/ml) at 37° in the presence of ^{3}H-thymidine (2.5 μCi/ml, 5.7 Ci/mmole). After 1 hr, the suspension was diluted to 100 ml with cold minimal essential medium containing 20 μM ^{12}C-thymidine, washed once with 50 ml of the same solution, and resuspended in 14 ml of minimal essential medium supplemented with 5% calf serum. After incubation for 30 min at 37°, the cells were divided into aliquots of 2.0 ml, and camptothecin (experiment A) or actinomycin (experiment B) was added at the final concentrations indicated below. After 10 min of further incubation, cellular DNA was analyzed by alkaline sucrose density gradient centrifugation as described in METHODS. "Bottom" refers to the lower 2.5 cm of the gradient tube. Purified adenovirus 2, containing ^{14}C-thymidine-labeled DNA, sediments at 34 S (25) and was used as a standard marker.

A. ●——●, 20 μM camptothecin; ▲——▲, 2 μM camptothecin; ■——■, 0.2 μM camptothecin; □——□, no additions; △- - -△, ^{14}C-adenovirus 2 DNA marker.

B. ●——●, 20 μM actinomycin; ▲——▲, 2 μM actinomycin; ■——■, 0.2 μM actinomycin; □——□, no additions; ○——○, 20 μM camptothecin.

gan to decrease approximately 1 hr after exposure of cells to camptothecin. Although this could reflect a direct effect of camptothecin on protein synthesis, most compounds that primarily inhibit protein synthesis in animal cells also inhibit globin synthesis in rabbit reticulocyte lysates (26). Since camptothecin did not affect the latter process, the delayed inhibition of protein synthesis observed in camptothecin-treated HeLa cells is most likely secondary to the demonstrated effects of the alkaloid on the synthesis of nucleic acids.

Camptothecin may act by inhibiting one or several steps in the biosynthesis of RNA and DNA. The drug does not prevent uptake of thymidine or uridine or their subsequent conversion to their respective phosphorylated forms. Uptake and metabolism of guanosine, adenosine, and cytidine were not tested in these experiments, but it seems unlikely that these precursors were selec-

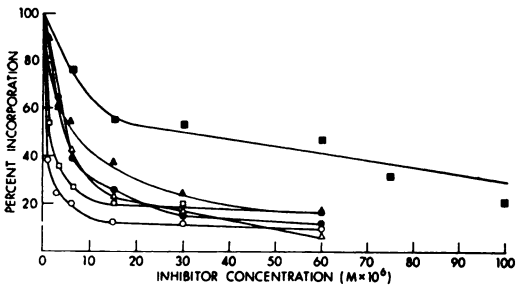

FIG. 8. *Effects of analogues of camptothecin on RNA synthesis in HeLa cells*

The experiment was performed as described in the legend to Fig. 2. Incorporation of radioactivity into trichloracetic acid-insoluble material was used to calculate the rate of RNA synthesis, which, in the control reaction, was 2492 cpm/ml. The percentage incorporation shown is expressed relative to this control. Camptothecin (○——○), camptothecin methyl amide (□——□), sodium camptothecin (●——●), α-deoxycamptothecin (△——△), 10-methoxycamptothecin (▲——▲), and camptothecin lactol (■——■) were present at the final concentrations indicated.

tively affected, since camptothecin does not prevent synthesis of viral RNA in poliovirus-infected HeLa cells.[5]

Simultaneous inhibition of DNA and RNA polymerase activity would account for the observed effects of camptothecin on DNA and RNA synthesis in HeLa cells. Under the conditions of our experiments, camptothecin failed to inhibit significantly the incorporation of nucleoside triphosphates into RNA or DNA, as tested with crude preparations of HeLa cell polymerases. Such assays measure primarily rates of chain extension and do not detect agents that affect the binding of template to polymerase or other processes involved in chain initiation. We were also unable to inhibit the activity of purified *E. coli* RNA polymerase by camptothecin, even when the enzyme was exposed to the drug prior to chain initiation.

The most plausible explanation of our results is that camptothecin inhibits RNA and DNA polymerase activity by interfering with the template function of DNA. The template appears to be altered in camptothecin-treated HeLa cells even though total DNA, measured as acid-insoluble material, is unchanged. Horwitz and Horwitz (35) observed that fragments of viral DNA are present in adenovirus-infected HeLa cells treated with camptothecin and that low molecular weight DNA appears in uninfected cells. We have also analyzed HeLa cell DNA on alkaline sucrose density gradients, a procedure that separates strands of double-helical DNA. After addition of the drug to a suspension of intact cells, sedimentation of DNA in alkaline gradients decreased from its normal value of over 90 S (27) to a form sedimenting at approximately 40 S. This conversion occurs within 10 min, even at 0°, and is observed at the same concentrations of camptothecin that inhibit nucleic acid synthesis.

[5] S. B. Horwitz, C. Chang, and A. P. Grollman, manuscript in preparation.

TABLE 6

Effects of camptothecin analogues on sedimentation of HeLa cell DNA

Experiments were performed as described in the legend to Fig. 7, except as indicated in the table. Camptothecin analogues were present at a final concentration of 20 μM. Radioactivity recovered from the bottom of the gradient is expressed as "high molecular weight DNA"; radioactivity in fractions 1–16 obtained from the gradient is expressed as "low molecular weight DNA." Since the total radioactivity in each set of experiments reflects the ability of various cell cultures to incorporate thymidine, a percentage of "high molecular weight DNA" and "low molecular weight DNA" is shown, based on the radioactivity found in the gradient fractions combined with that recovered from the "bottom" of the gradient. Each value represents the average of two experiments.

Inhibitor added	Radioactivity in DNA	
	"High molecular weight"	"Low molecular weight"
	%	%
None[a]	93	7
Sodium camptothecin	12	88
Camptothecin methyl amide	11	89
α-deoxycamptothecin	16	84
10-Methoxycamptothecin	5	95
10-Methoxycamptothecin[b]	8	92
Camptothecin lactol	78	22

[a] A total of 224,000 cpm was recovered.

[b] Incubated at 0° for 10 min.

The preceding results suggest that DNA can be partially degraded in camptothecin-treated cells, presumably as a result of endonuclease activity. If template DNA is degraded in a similar manner, this effect could account for the observed inhibition of DNA and RNA synthesis in animal cells.

The mechanism by which the postulated degradation of DNA occurs remains unclear. The drug may inhibit one of the enzymes involved in repair of DNA, such as DNA ligase. In this case, the apparent increase in degradation would reflect normal catabolism of DNA in the absence of repair. Alternatively, camptothecin or a metabolic product may bind to DNA, thus rendering it susceptible to the action of nucleases. Preliminary results indicate that camptothecin binds to DNA under certain experimental conditions; further studies to characterize this interaction are in progress.

Several inhibitors of nucleic acid synthesis, including bacterial colicins (28) and various antibiotics, are known to induce degradation of DNA. Streptonigrin produces single-strand breaks in HeLa cell DNA, as demonstrated by alkaline sucrose density gradient centrifugation (29). Phleomycin induces degradation of DNA to acid-soluble fragments in *E. coli* (30) and has recently been shown to fragment supercoiled DNA during the synthesis of polyoma virus in mouse embryo cells (31). Closed-circular mitochondrial DNA is partially fragmented in the presence of ethidium bromide (32). The precise mechanism by which these drugs induce their effects has not been established.

The structure-activity relationships of camptothecin and its analogues, as determined by their effects on nucleic acid synthesis in HeLa cells, correspond closely to their ability to suppress the growth of tumors in experimental animals (4). Camptothecin, its sodium salt, and camptothecin methyl amide are equally effective as inhibitors of RNA synthesis in HeLa cells. Methylation of the hydroxyl group at position 10 (methoxycamptothecin) does not decrease activity. Methoxycamptothecin could, conceivably, have been converted to camptothecin by cellular enzymes. If such is the case, demethylation must occur within 10 min at 0° to account for the observed conversion of DNA to a lower molecular weight form. Since deoxycamptothecin is an active inhibitor, the α-hydroxyl group of camptothecin is apparently not required for optimal biological activity. The keto group appears more essential, since reduction to the lactol significantly decreases activity *in vitro* (see above) and *in vivo* (4).

A more detailed description of the structure-activity relationships must await the isolation of other analogues and the development of methods for the synthesis of compounds related to camptothecin (33, 37). Determining the effects of such analogues and derivatives on DNA and RNA synthesis would provide a useful estimate of their potential activity. Any compound found active in such preliminary tests should be examined for its ability to induce degradation of DNA, as this effect is also correlated with the reported antitumor activity of camptothecin analogues. At least one compound that we have tested, an intermediate in the chemical synthesis of camptothecin (34), was equal to camptothecin as an inhibitor of RNA synthesis but, at a concentration of 20 μM, was much less effective in inducing partial degradation of DNA. This analogue had no activity when tested against tumors in experimental animals.[6]

ACKNOWLEDGMENTS

We are grateful to Drs. M. Wall and M. C. Wani for providing us with the samples of camptothecin and its analogues. We are indebted to Drs. M. Wall, A. S. Kende, and J. Hurwitz for helpful discussions, and thank Drs. D. Kessel and M. Horwitz for informing us of their experimental results prior to publication. Dr. M. T. Huang determined the effects of camptothecin on leucine incorporation in lysates prepared from rabbit reticulocytes.

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