Biochemical Pharmacology, Vol. 34, No. 8, pp. 1225–1230, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

ACTION OF CAMPTOTHECIN AND ITS DERIVATIVES ON DEOXYRIBONUCLEIC ACID

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(*Received* 13 *January* 1984; *accepted* 9 *August* 1984)

Abstract—Camptothecin, an antitumor alkaloid, induced alkali-labile linkages to supercoiled closed circular DNA. The induction was dependent on the concentration of salt, and the highest level of induction was obtained in the presence of 1.0 M NaCl. The active site was the OH group at the C-20 in the E ring. Camptothecin interacted with adenine base at the C-7 in the B ring which resulted in inhibition of hyperchromicity in heat denaturation of DNA. Replacement of the H group with a substituent whose chain length was longer than $-CH_2CH_3$, such as $-CH_2OCOCH_2CH_3$ or $-CH_2(CH_2)_4CO_2CH_3$, caused a loss of potency for the inhibition of hyperchromicity. In the presence of 1.0 M NaCl, camptothecin interacted with superhelical closed circular DNA at 37°. The apparent number of binding sites per base-pair of superhelical DNA was about one hundredth of ethidium bromide according to the equilibrium dialysis experiment. An intercalative interaction was observed with poly(dG-dC) in a 4 M NaCl concentration, but not in 0.1 M NaCl. A similar intercalation was detected in brominated poly(dG-dC) in the physiological concentration of NaCl. Camptothecin seemed to intercalate into the Z-form region which was favorably induced in a negatively superhelical closed circular DNA.

Camptothecin is an antitumor alkaloid isolated from *Camptotheca acuminata* [1]. Camptothecin rapidly and strongly inhibits the synthesis of nucleic acids in eukaryotic cells, but does not affect the activity of enzymes that participate in DNA and RNA synthesis or the pools of uridine and thymidine [2, 3]. The DNA extracted from camptothecin-treated cells or the viral DNA extracted from DNA-virus infected cells is converted into molecules of lower molecular weight by sedimentation through alkaline sucrose gradients. As no such conversion is observed on centrifugation at neutral pH, alkali-labile linkages are considered to be introduced into DNA [4–8]. However, no induction of alkali-labile linkages is observed when isolated cellular or viral DNA is incubated with camptothecin *in vitro* [4, 9]. I found that the alkali-labile linkage is induced in superhelical closed circular DNA when it is incubated with camptothecin in 1.0 M NaCl at 37° [9, 10]. The property of alkali-labile linkages *in vitro* is the same as *in vivo* [3, 10], but camptothecin does not induce alkali-labile linkages in linear or relaxed circular DNA *in vitro*. Thus, the action of camptothecin seems to be dependent on the higher-order structure of DNA and seems to indicate that cellular or viral DNA forms a specific superhelical structure *in vivo*. The actual higher-order structure of DNA *in vivo* has not been elucidated, although nucleosomes are found and superhelical, bending or kinky forms of DNA are suggested [11–13]. Therefore, analysis of the action of camptothecin will shed more light on the structure and function of DNA *in vivo*.

This paper describes the characteristics of action and active site of camptothecin on DNA *in vitro*.

MATERIALS AND METHODS

DNA. Purified closed circular DNA (DNA I) was prepared from SV40 [11], pBR 322 [14] and ΦX174 [15].

DNA-polymers were purchased from P-L Biochemicals, Inc. (U.S.A.). Poly(dG-dC) was brominated according to the method reported by Möller *et al.* [16]. The $A_{295/260}$ absorbance ratio of the product was 0.32 which corresponded to 0.91 as the degree of transition (1.0 is for fully converted) [16].

Camptothecin and its derivatives (*Fig.* 1). Crystallized forms of camptothecin and its derivatives [17, 18] were obtained from the Yakult Co., Ltd. (Tokyo, Japan). They were dissolved in 0.2 M NaOH or in demethyl sulfoxide (DMSO) at a concentration of 1–5 mg/ml and stored in light shielded bottles at −20°. ^{3}H-Labeled camptothecin which was labeled by the Wilzbach method (Amersham International Ltd., Amersham, England) was purified by thin-layer chromatography [19]. The experiments were performed in a dark room with red lamp whenever possible, and light-shielded tubes were used for elution, centrifugation, and incubation.

Treatment of DNA with camptothecin. The reaction mixture (100 μl) contained 0.1 to 1 μg of [^{14}C] thymidine-labeled DNA I in 1.0 M NaCl, 0.2 M Tris–HCl (pH 7.8), 0.1 mM EDTA and 33 μM camptothecin. The mixture was incubated for 6 hr at 37° in the dark, unless otherwise noted. Six microliters of 5 M NaOH was added to the reaction mixture, and the incubation was continued at 37° for an additional 30 min to complete breakage of the alkali-labile linkages.

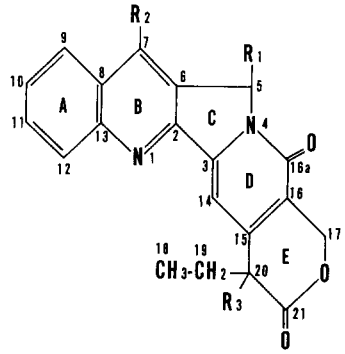

Fig. 1. Structures of camptothecin and its derivatives. R_1, R_2 and R_3 represent the site of substituents in the derivatives. R_1, R_2 and R_3 are H, H, and OH for camptothecin respectively.

Measurement of alkali-labile linkage. After the alkali treatment, the reaction mixture was layered on a 5 ml linear gradient from 5 to 20% in 1.0 M NaCl, 0.2 M NaOH, 1 mM EDTA, 15 μM camptothecin, and centrifuged in a Hitachi RPS 50T rotor at 130,000 *g* for 120 min at 18° to separate 53S DNA (closed circular DNA, I) and newly produced 16-18S DNA (nicked circular and linear DNA, II and III). After centrifugation, gradients were collected on glass fiber filters (Whatman GF/C), dried, washed with 70% and 95% ethanol successively, and counted by liquid scintillation spectrometry after drying.

To express the degree of induction of alkali-labile linkage, the percentage of newly produced 16-18S DNA was obtained by dividing the radioactivity of its peak by the total radioactivity on the gradients.

RESULTS AND DISCUSSION

Induction of alkali-labile linkage to DNA I. As reported previously, superhelical closed circular DNA was converted to the relaxed form by incubation under alkaline conditions at pH 13 after interaction with camptothecin in 1.0 M NaCl at 37°. The reaction does not proceed in the physiological concentration of NaCl [9, 10]. The concentration of NaCl in the reaction mixture was increased from 0.1 M to 2.0 M, and the amount of newly formed relaxed DNA was measured. With the increase of the concentration of NaCl from 0.1 M to 1.0 M, the amount of newly formed relaxed DNA increased (Table 1). As the density of the superhelix increases with the increase of NaCl [20], DNA structure of higher superhelical density seems to be favored for action of camptothecin. However, in 2.0 M NaCl, induction of alkali-labile linkage was reduced to the same level as in 0.1 M NaCl. This means that either a specified superhelical structure of DNA is necessary for interaction with camptothecin or NaCl affects interaction of camptothecin at a critical concentration. The latter seems to be less likely, as over 70% of the maximal reaction proceeded in the presence of 1.5 M NaCl.

Table 1. Effect of NaCl concentration on induction of alkali-labile linkage*

Concn of NaCl (M)	Conversion of DNA I to 16-18S DNA after alkali treatment (%)
0.1	4.5
0.3	18.6
0.6	27.0
1.0	32.6
1.5	23.8
2.0	6.9

* [^{14}C]Thymidine-labeled SV40 DNA I was treated with camptothecin as described in Materials and Methods except that the concentration of NaCl in the reaction mixture was varied as indicated.

Table 2. Induction of alkali-labile linkage by derivatives of camptothecin*

Derivatives	Conversion of DNA I to 16-18S DNA after alkali treatment (%)
R_1: —CH_3	17.2
—CHO	15.1
—CH(OH)	13.4
—CH(OCH_3)	15.2
R_2: —COOH	17.0
—CH_2OH	12.3
R_3: —OH	21.3
—H	16.7
—Cl	4.1
—$OCOCH_3$	3.9

* R_1, R_2 and R_3 corresponded to the site in Fig. 1. [^{14}C]Thymidine-labeled SV40 DNA I was treated with the indicated derivatives as described in Materials and Methods except that the incubation time was 2 hr.

The OH-group at the C-20 in the E ring was necessary for the induction of alkali-labile linkage [11]. The new derivatives [17, 18] containing substituents at the C-7 in the B-ring or at the C-5 in the C ring were tested (Table 2). The induction of alkali-labile linkage was most inhibited in the derivatives containing the substituent at the C-20 in the E ring and not at the C-7 in the B ring or at the C-5 in the C ring. Thus, camptothecin seems to interact with DNA I at the C-20 in the E ring and the OH or the H group is active for induction of alkali-labile linkages.

Inhibition of hyperchromicity. According to Li *et al.* [21], camptothecin inhibits the increment of hyperchromicity of DNA. DNA of the higher [A + T] content is affected more than the lower [A + T] content. To clarify the specificity in nucleotide, DNA-homopolymers and DNA-alternating copolymers were used instead of natural DNA. Inhibitions of 62 and 48% were observed for poly(dA-dT) and poly(dA)·poly(dT), respectively, but no inhibition was detected for either poly(dG)·poly(dC) or poly(dG-dC) (Table 3). The extent of inhibition was dependent on the concentration of the drug, and 50% inhibition was attained at a concentration of 50 μg/ml. The concentration was fairly high when it was compared with 0.035 ~ 0.35 μg/ml at which 50% inhibition of SV40 DNA synthesis is caused in BSC-1 cells *in vivo* [22]. The effective concentration in the inhibition of hyperchromicity was close to the value in the induction of alkali-labile linkage. The activity of derivatives was compared with camptothecin. The length of the substituent at

Table 3. Inhibition of increment of hyperchromicity*

Polymer	% Inhibition				
	Camptothecin (μg/ml)				
	100	50	25	10	1
Poly(dA-dT)·Poly(dA-dT)	62	54	50	20	4
Poly(dA)·Poly(dT)	48				
Poly(dG-dC)·Poly(dG-dC)	−2				
Poly(dG)·Poly(dC)	0				

* The reaction mixture contained 7.5 μg of polymer and the indicated amount of camptothecin in 50 μl of 1 × SSC. After incubation at 37° for 30 min, the reaction mixture was heated at 100° for 15 min. Then it was rapidly cooled to 0° in an ice bath, and at the same time cold water (450 μl) was added to reduce the concentrations of salt and polymer. The optical density was measured at 260 nm. The change of hyperchromicity was expressed as a percentage of the increment of hyperchromicity in the control solution without camptothecin.

Table 4. Inhibition of hyperchromicity by derivatives*

Derivatives	Inhibition (%)	Derivatives	Inhibition (%)
R_1: —CHO	70	R_2: —NO_2	70
—C(OH)H	50	—COOH	72
		—CH_2OH	48
—C(OCH_3)H	38	—CH_2CH_3	50
		—$CH_2CH_2CH_3$	5
—C($OCOCH_3$)H	80	—$CH_2OCOCH_2CH_3$	−5
		—$CH_2(CH_2)_4CO_2CH_3$	20
R_3: —OH	60		
—Cl	65		

* R_1, R_2 and R_3 corresponded to the site in Fig. 1. The reaction mixture (50 μl) contained 7.5 μg of Poly(dA-dT) and 5 μg of the indicated derivative. The other conditions were the same as in Table 3. The inhibition was shown as a percentage of the increment of hyperchromicity in the control solution without alkaloids. The minus (−) sign means that hyperchromicity was increased over the control.

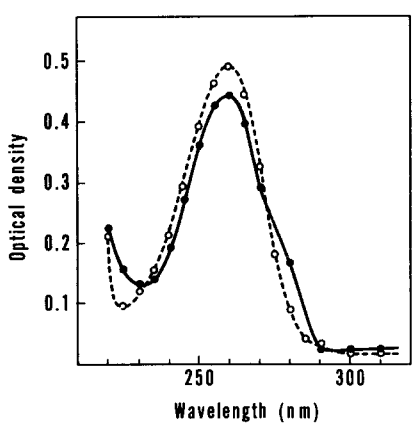

Fig. 2. Ultraviolet spectrum of adenosine after interaction with camptothecin. The reaction mixture contained 5 μg adenosine and 5 μg camptothecin in 500 μl of 1 × SSC, and it was incubated at 37° for 30 min. After cooling to room temperature, the ultraviolet spectrum was measured in the reaction mixture without nucleotide as a control, which was treated under the same conditions as the samples. Key: (○---○) adenosine; and (●——●) adenosine treated with camptothecin.

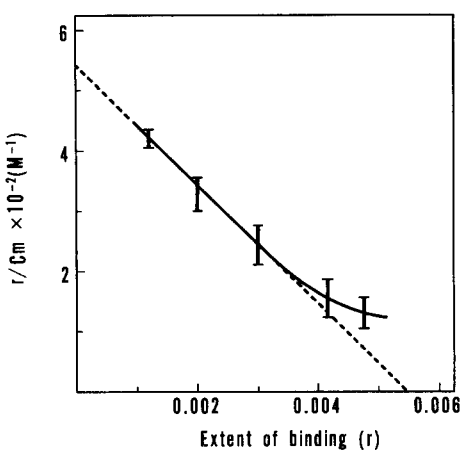

Fig. 3. Scatchard analysis of camptothecin binding to DNA. Aliquots (1 ml) of ΦX174 RF DNA (25 μg/ml) in 1 M NaCl, 1 mM EDTA and 10 mM Tris–HCl (pH 7.8) were placed in a dialysis bag together with ^3H-labeled camptothecin at an initial concentration equal to that outside the dialysis bag (20 ml total volume). The system was stirred vigorously in the dark at 37° for 96 hr, after which the concentrations of camptothecin inside and outside the membrane were determined by radioactivity measurements.

the C-7 in the B ring was critical for the inhibition of hyperchromicity, and the C-5 in the C ring and the C-20 in the E ring were not concerned in the reaction (Table 4). The mechanisms by which camptothecin caused the inhibition of hyperchromicity was unclear. Li *et al.* [21] reported that the u.v. spectrum of DNA was the same before and after interaction of camptothecin and that the melting temperature was not altered. When the melting curve of poly(dA-dT) was measured, the optical density at 260 nm started to decrease between 35 and 40° and continued to decrease to 60°. The optical density stayed constant and then started to increase at 80°. The degree of hyperchromicity from 60 to 100° was the same as the control; however, because of the initial decrease at 35–60°, the inhibition of increment was eventually observed in hyperchromicity.

The u.v. spectrum of adenosine changed after the interaction with camptothecin (Fig. 2); no such deviation from the original spectrum was observed in the other nucleotides. Therefore, camptothecin interacted with DNA between the C-7 in the B ring and adenine base to inhibit the increment of hyperchromicity.

Interaction of camptothecin with DNA. As reported in the previous paper [10], the $S^{\circ}_{20,w}$ value of SV40 DNA I is unchanged after interaction with 5×10^{-4} to 100 μg/ml of camptothecin, and the fluorescence spectrum of the reaction mixture on excitation at 370 nm is also unchanged with the mole ratio of nucleotide of DNA and the drug ranging from 0.03 to 30.0.

With the use of highly radioactive camptothecin, binding was studied by equilibrium dialysis. The reaction mixture contained 25 μg/ml of closed circular DNA and radioactive camptothecin of which the concentration was varied from 3 to 300 μM. For the calculation of the amount of bound camptothecin, the difference between radioactivity inside and outside of the dialysis membrane was used. Therefore, the error increases as this difference diminishes. The actual counts, for example, at 24×10^{-6} M camptothecin were 4908 cpm for inside and 4574 cpm for outside obtained by counting for 10 min.

The results are shown in Fig. 3 as a conventional Scatchard plot [23] where r = (concentration of bound camptothecin)/(total concentration of DNA in base pairs) and C_m = concentration of free camptothecin. Following the nomenclature of Bresloff and Crothers [24], the Scatchard plot for the independent binding site is expressed by the equation $r/C_m = K_{ap}(B_{ap} - r)$ where B_{ap} is the apparent number of binding sites per base-pair and K_{ap} is the association constant for the formation of bound camptothecin from the free state. In this convention, K_{ap} is given by the equation $K_{ap} = r/C_m(1/1-r)$ and the intrinsic binding constant to an isolated potential binding site, $K(0)$, described in Bresloff and Crothers [24] is equal to K_{ap} at $r = 0$, $K(0) = \lim r/C_m$. The intercept of the nearly linear portion of the curve on the r/C_m axis yields an intrinsic binding constant, $K(0)$, of $5.4 \times 10^2\ M^{-1}$ with the intercept on the r axis occurring at $B_{ap} = 0.0055$. This means 0.55% of the base-pairs act as binding sites for camptothecin. An apparent binding constant, K_{ap}, for the base-pair of the 0.55% is 9.8×10^4 ($K_{ap} = K(0)/B_{ap}$). As these values are fairly low compared with the corresponding values for ethidium bromide [25], camptothecin will be unable to cause measurable change in the physicochemical property of DNA. The supposition is consistent with the previous findings that the T_m of DNA is not affected by camptothecin [21], and the $S^{\circ}_{20,w}$ of SV40 DNA I is unaltered after interaction with camptothecin under the conditions in which alkali-labile linkage is inducible [10]. The observed convexity toward the r axis suggests that there is more than one type of binding site on the DNA, with the lower affinity sites becoming occupied as r increases. As mentioned in the previous section, the C-7 in the B ring of the drug bound with adenine residue of DNA at higher concentrations of camptothecin, and, if the DNA was in an appropriate superhelical structure, the C-20 in the E ring at the same time presumably interacted with DNA. Isocamptothecin in which the site of C-20 in the E ring is reversed against the C-7 in the B ring is inactive in degradation of high molecular weight DNA by alkaline treatment *in vivo* [3]. These findings suggest that camptothecin interacts with DNA at the C-7 and the C-20, and, at the same time, a secondary or tertiary structure of camptothecin influences the interaction with DNA.

In the study of photoactivated camptothecin, Lown and Chen [26] suggested a weak intercalative interaction of camptothecin into the G, C regions of DNA. In equilibrium dialysis, binding of camptothecin was not detected with linear or relaxed circular DNA. The possibility exists, therefore, that camptothecin may intercalate into the Z-form region which is a favored form for alternating purine and pyrimidine sequences in supercoiled DNA resulting in the

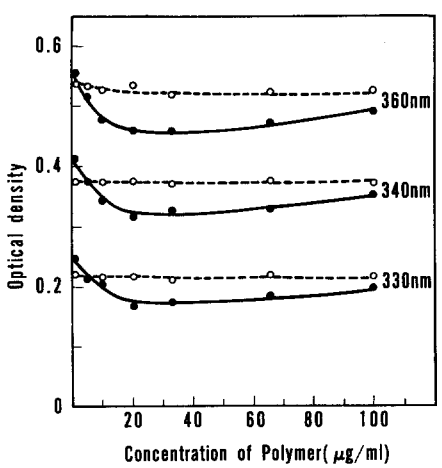

Fig. 4. Change of optical density of camptothecin in the presence of DNA-homopolymers and DNA-alternating copolymers. The reaction mixture contained 33 μM camptothecin and an indicated amount of poly(dG-dC) (●——●) or poly(dG)·poly(dC) (○- - -○) in 4 M NaCl, 10 mM Tris–HCl (pH 7.8) and 1 mM EDTA. The reaction mixture was incubated for 30 min at 37°. After cooling to room temperature, the optical density was measured. The reaction mixture without camptothecin was used as a control.

Table 5. Optical density of camptothecin in the presence of brominated poly(dG-dC)*

Wavelength (nm)	Optical density of camptothecin					
	Concentration of brominated poly(dG-dC) (μg/ml)					
	0	2.0	4.0	8.0	16.0	25.0
340	0.470	0.450	0.430	0.415	0.410	0.402
360	0.560	0.546	0.535	0.526	0.520	0.510
380	0.487	0.480	0.475	0.470	0.466	0.460

* The reaction mixture contained 33 μM camptothecin and the indicated amount of brominated poly(dG-dC) in 0.1 M NaCl, 10 mM Tris–HCl (pH 7.8) and 1 mM EDTA. The other conditions are the same as in Fig. 4.

decrease of the free energy level [27]. The degree of intercalation was measured by the decrease of optical density of the reaction mixture in which the concentration of the drug was constant and the concentration of polynucleotide was varied from 5 to 100 μg/ml. In the presence of 0.1 M NaCl, no decrease of optical density was observed in DNA-homopolymers, DNA-alternating copolymers, calf thymus DNA, pBR322 DNA I or III. In high NaCl concentration, poly(dG-dC) undergoes a salt-induced cooperative transition and the polymer adopts the Z conformation in 4 M NaCl (for a general review, see Ref. 28). Although the optical density of camptothecin was unchanged with the various amounts of poly(dG)·poly(dC) in 4 M NaCl, a decrease of optical density was observed with poly(dG-dC) in the presence of 4 M NaCl (Fig. 4). Bromination of poly(dG-dC) is known to stabilize this polymer in the Z-form under low-salt conditions [16]. To avoid high-salt concentration, intercalation of camptothecin was tested with brominated poly(dG-dC) in the physiological concentration of salt. With the increase of brominated poly(dG-dC), optical density of camptothecin decreased (Table 5). These results show that camptothecin specifically intercalated into the Z-form region of DNA. At high concentrations of the polymer, a slight increase in optical density is observed in Fig. 4, although this does not fit the present understanding of intercalation. The reason is unknown now, but a similar tendency was observed repeatedly.

As negative supercoiling induces the Z form in alternating purine-pyrimidine regions [27], intercalation of camptothecin into the Z form is compatible with the result that binding of camptothecin was observed with closed circular DNA of high superhelical density in high concentration of salt (Fig. 3), but not with linear or relaxed form (data not shown). In Fig. 3, 0.55% of the base-pairs in ΦX174 RFDNA acted as binding sites for camptothecin. This value corresponds to about 30 base-pairs with the total base of 5386 [29]. The alternating pyrimidine-purine regions over 10 bases in length are known to form the Z conformation in plasmid DNA of moderate amounts of superhelicity [30]. By computer analysis, the alternating pyrimidine-purine regions over 10 bases in length are located at 763 ~ 776(TGCGTGTACGCGCA), 3497 ~ 3506(TATATGCACA) and 4184 ~ 4193 (ATATGTATGT) according to the numbering of Sanger *et al.* [29]. The total length of these regions is 34 base-pairs, which is close to the 30 base-pairs obtained by equilibrium dialysis experiments as binding sites of camptothecin.

The conditions for the intercalation of camptothecin into superhelical closed circular DNA were the same as for induction of alkali-labile linkages. At present it is unclear whether the former and the latter correspond to cause and effect, respectively, but it is quite probable. Nordheim and Rich [31] reported that negatively supercoiled SV40 DNA contained the Z form within transcriptional enhancer sequences that is located close to the origin of replication. As reported previously, alkali-labile linkage was induced close to the origin of replication of SV40 DNA I [9].

The analytical results that camptothecin inhibited the synthesis of nucleic acids *in vivo* by inducing alkali-labile linkage in DNA and introduced alkali-labile linkage in only DNA of a certain specific superhelical density *in vitro* suggest that a tertiary structure of DNA plays an important role in the function of DNA as a template *in vivo*. A superhelical model for regulation of gene expression [32, 33] was developed based on this specific activity of camptothecin. There may be natural or artificial products which are apparently inactive in direct action on DNA *in vitro* under the ordinary conditions, but possess activity similar to that of camptothecin. Analysis of these substances will provide a new clue in research on interrelation between the biological function and the tertiary structure of DNA.

Acknowledgements—I thank Miss Y. Chiba for preparing the manuscript. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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