Structure-Activity Study of the Actions of Camptothecin Derivatives on Mammalian Topoisomerase I: Evidence for a Specific Receptor Site and a Relation to Antitumor Activity

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ABSTRACT

Twenty-two compounds related to camptothecin, a known inhibitor of eukaryotic topoisomerase I, were studied. The following effects on the actions of topoisomerase I were observed and were well correlated among most of the compounds studied: (a) inhibition of the first-order rate of relaxation of supercoiled DNA; (b) conversion of supercoiled DNA to nicked circles; and (c) single-strand cleavage of linear DNA at specific sites. The locations of the stimulated cleavage sites were the same for all of the active derivatives. Stereochemistry and the positions of substituents were found to be crucial for the presence or absence of effects on topoisomerase I, indicating that the compounds interact with an asymmetrical receptor site on the enzyme or enzyme-DNA complex. From the structure-activity relations, the regions of interaction between the camptothecin ring system and the receptor site were inferred. Striking correlations were observed between activity against topoisomerase I and reported activity against murine leukemias, indicating that an action on topoisomerase I is responsible for the antitumor activity of the camptothecins.

INTRODUCTION

A variety of antitumor drugs, including several types of DNA intercalators and epipodophyllotoxins, have been found to inhibit eukaryotic topoisomerase II by stabilizing complexes that form when the enzyme generates a DNA strand break and binds covalently by way of a tyrosine residue to the 5' terminus of the strand break (1-3). Topoisomerase II catalyzes the passage of DNA strands through a double-strand break formed when the enzyme produces double-strand cleavage complexes. This type of reversible complex is a normal intermediate in the strand passage reaction catalyzed by the enzyme. The strand breaks and covalent DNA-enzyme links are revealed when the enzyme produces double-strand cleavage complexes.

Eukaryotic topoisomerase I-mediated DNA breaks differ from those mediated by topoisomerase II in that only a single DNA strand is cleaved and the covalent linkage is to the 3' terminus of the DNA strand break (6, 7). Drug-stabilized cleavable complexes can, however, occur in the case of topoisomerase I, indicating that the compounds interact with an asymmetrical receptor site on the enzyme or enzyme-DNA complex. Comparison with previously reported antitumor data for these derivatives provides strong evidence that the action on topoisomerase I is responsible for the antitumor activity.

MATERIALS AND METHODS

Materials. SV40 DNA, BanI and HpaII restriction enzymes, and agarose were purchased from Bethesda Research Laboratories (Gaithersburg, MD). Klenow polymerase was purchased from Pharmacia. Polyacrylamide and [α-32P]dGTP were purchased from Bio-Rad (Richmond, CA) and New England Nuclear (Boston, MA), respectively. Autoradiography was performed with XAR-5 films (Eastman Kodak Company, Rochester, NY).

Type I DNA topoisomerase was purified from mouse leukemia L1210 cells, as described previously (3). One unit of topoisomerase I was defined as the activity yielding 70% of closed circular-relaxed SV40 DNA (0.14 µg) in 10 min at 37°C.

The syntheses of the camptothecin derivatives have been reported previously (11-16) (Table 1 and Fig. 7 for compound identification). The RS derivatives are synthetic products, whereas the S derivatives are modifications of natural products. All drugs were dissolved in dimethyl sulfoxide at 10 mM, except the two water-soluble camptothecins (compounds 9 and 16; see Table 1), which were dissolved in distilled water at 10 mM. Drug stock solutions were kept frozen at -20°C for no more than 2-3 weeks. Further drug dilutions were made in distilled water. The final dimethyl sulfoxide concentration, which was never greater than 1% (v/v), had no effect on topoisomerase I activity.

Chloroquine diphosphate salt was purchased from Sigma Chemical Co. (St. Louis, MO).

DNA Relaxation and Cleavage Assays with Supercoiled DNA. All reactions were performed in 20 µl reaction buffer (0.01 M Tris-Cl, pH 7.5; 0.05 M KCl; 5 mM MgCl2; 0.1 mM EDTA; 15 µg/ml bovine serum albumin). Reactions were carried out for 10 min at 37°C and were stopped by adding 3 µl of a mixture containing sodium dodecyl sulfate, EDTA, and proteinase K (Boehringer Mannheim, Indianapolis, IN). After an additional 30 min incubation at 37°C, 2 µl loading buffer (10X solution consisting of 0.3% bromophenol blue, 16% Ficoll, and 10 mM Na3PO4) were added to the samples, which were loaded into 1% agarose gels containing 10X solution of chloroquine. Gels were run at 1.4 V/cm for 15 h and contained 2 µg/ml chloroquine in order to separate nicked, relaxed, and supercoiled DNA molecules. Negatives of the gel pictures were scanned with a Beckman DU-8B spectrometer interfaced with a computer in order to quantify enzyme inhibition and DNA cleavage (17).

Camptothecin-induced inhibition of topoisomerase I-mediated DNA relaxation was computed from the formula

\[
\% \text{ of inhibition} = \frac{S_{C0D} - S_{C8}}{S_{0} - S_{C8}} \times 100 
\]  

(A)

where \(S_{C0D}\), \(S_{C8}\), and \(S_{0}\) represent the fraction of supercoiled DNA measured in the presence of enzyme and drug, in the presence of enzyme alone, and in untreated supercoiled DNA, respectively. Drug-induced DNA cleavage was computed from the formula

\[
\% \text{ of stimulation} = \frac{N_{C8} - N_{C0D}}{1 - N_{C0D}} \times 100 
\]  

(B)

where \(N_{C8}\) and \(N_{C0D}\) represent the fraction of nicked DNA measured in...
Table 1 Relation between antitumor activity of camptothecin congeners and effects on topoisomerase I-mediated reactions with SV40 DNA

<table>
<thead>
<tr>
<th>Compound</th>
<th>Camptothecin derivative</th>
<th>% of inhibition of relaxation</th>
<th>% of DNA scission</th>
<th>Antitumor activity (T/C) x 100</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>Supercoiled</td>
<td>Linear</td>
<td>L1210</td>
</tr>
<tr>
<td>1</td>
<td>S-</td>
<td>52†</td>
<td>48‡</td>
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<td>10</td>
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* Mouse leukemias L1210 and P388, inoculated i.p.; drug administered i.p. on days 1 and 5. T/C, survival times of treated and control animals, respectively. In parentheses, dose (mg/kg) giving greatest reported T/C value. Antitumor data are from Refs. 11-14.

† Effect of 10 μM test compound, as percentage of control.

The presence of topoisomerase I and drug in the presence of topoisomerase I alone, respectively.

**DNA Cleavage of [32P]-End-labeled SV40 DNA.** [32P]-End-labeled SV40 DNA was prepared as described previously (10). Briefly, DNA was linearized with BanI at position 295 of the SV40 genome and its termini were labeled with [α-32P]dGTP and Klenow polymerase. This DNA was then cut with HpaII at position 347 in order to generate two 32P-3'-end-labeled fragments, one of 5191 base pairs and the other of 52 base pairs. The topoisomerase I-mediated DNA breaks generating DNA fragments longer than 53 base pairs could be localized unequivocally in the SV40 genome.

Approximately 0.04 μg of [32P]-3'-end-labeled SV40 DNA was incubated in 30 μl of reaction buffer with 220 units of topoisomerase I for 30 min at 37°C. Reactions were stopped as described above. Sample preparation and DNA sequencing gels were performed as described previously (10, 17, 18).

RESULTS

Effects of Camptothecin on the Actions of Topoisomerase I on Supercoiled DNA. At low concentrations of topoisomerase I, camptothecin inhibits the relaxation of supercoils and does not significantly increase the amount of nicked DNA (Fig. 1). Supercoiled DNA disappears with first-order kinetics, and camptothecin reduces the rate constant of the reaction (Fig. 2). The reaction is processive, because the distribution of topoisomers remains unchanged during the course of the reaction; this distribution also is unchanged by camptothecin.

Since the topoisomer positions are not shifted in the presence of camptothecin, the drug does not bind extensively to DNA by intercalation or by another mode of binding that would alter helix twist angles.

At high concentrations of topoisomerase I, the main observed effect of camptothecin is the production of nicked circular DNA (Fig. 3). Camptothecin apparently enhances the stability of cleavable states of the enzyme-DNA complex which may be similar to normal intermediates in the relaxation reaction.

Effects of Camptothecin Derivatives on the Actions of Topoisomerase I on Supercoiled DNA. The effects of various camptothecin derivatives on the relaxation and cleavage reactions are shown in Fig. 4. The relaxation and cleavage reactions were carried out over a range of concentrations of each inhibitor in the presence of 0.05 or 2.3 units of topoisomerase I per μl, respectively. It is seen that the R camptothecin isomer has little or no effect, in contrast to the natural S isomer. The racemic RS isomer mixture shows intermediate effects, and the 10-hydroxy-S-camptothecin shows enhanced potency. The effects of other derivatives were also determined over a range of concentrations; the effects at a standard concentration of 10 μM are listed in Table 1.

At 0.05 unit/μl topoisomerase I, many compounds inhibited...
CAMPTOTHECIN STRUCTURE-ACTIVITY FOR TOPOISOMERASE I

Fig. 2. Effect of camptothecin on the kinetics of the relaxation of supercoiled DNA by topoisomerase I. Native supercoiled SV40 DNA (0.2 μg) was incubated at 37°C with topoisomerase I (0.05 unit/μl) with or without 5 μM S-camptothecin. Reactions were carried out and analyzed as in Fig. 1. The areas corresponding to supercoiled, relaxed, and nicked DNA in the digitized densitometer scans were determined by computer, and the percentage of supercoiled DNA remaining was plotted as a function of time.

Fig. 3. Inhibition of supercoil relaxation and formation of topoisomerase I-DNA cleavable complexes by camptothecin; dependence on topoisomerase concentration. (A) SV40 DNA (0.1 μg) was incubated with various concentrations of topoisomerase I in the presence (— — —) or absence (———) of 100 μM S-camptothecin and analyzed by chloroquine agarose gel electrophoresis as in Fig. 1. (B) Part of the chloroquine agarose gel. Lane 1, DNA only; Lane 2, DNA and 0.75 unit/μl topoisomerase I (15 units); lane 3, same with 100 μM camptothecin. N, nicked; Rel, relaxed; Sc, supercoiled.

Effects of Camptothecin Derivatives on Topoisomerase I-Induced Single-Strand Cleavage of a Linear DNA Fragment. Sites of DNA cleavage were determined in a 3'-32P end-labeled SV40 DNA fragment which allows the detection of the major topoisomerase I-mediated DNA cleavage sites induced by camptothecin (10). In the presence of 7.3 units/μl topoisomerase I, many camptothecin derivatives enhanced cleavage at specific sites in the DNA sequence, but no variation was detected among different derivatives in the locations or relative enhancement intensities of cleavage sites (Fig. 5). The major cleavage site in the SV40 genome in all cases was at position 4955, which is in agreement with the results obtained previously (10). The extent of cleavage stimulated by camptothecin derivatives at 10 μM was quantitated by cutting out the band around position 4955

Fig. 4. (A) Inhibition of topoisomerase I-mediated DNA relaxation by several camptothecin congeners. DNA relaxation reactions were carried out in the presence of 0.05 unit/μl topoisomerase I for 10 min at 37°C as in Fig. 1, but in the presence of various concentrations of 10-hydroxy-5-, 5-, RS-, or R-camptothecin. (B) Formation of topoisomerase I-DNA cleavable complexes in response to the above camptothecin congeners. SV40 DNA was reacted with 2.3 units/μl topoisomerase I; conditions otherwise as in Fig. 1.

Fig. 5. Topoisomerase I cleavage sites stimulated by camptothecin congeners in a linear SV40 DNA fragment. The BamHI-PstI fragment of SV40 DNA, with 32P end label at the BamHI site, was incubated for 30 min at 37°C with 10 μM concentrations of various camptothecin congeners. Samples were run into a 6% polyacrylamide sequencing gel. Numerical lane designations correspond to the numerical identifications of the compounds listed in Table 1. Lane C, untreated DNA fragment; Lane E, enzyme alone; Lane M, purine sequencing lane.
DISCUSSION

Camptothecin derivatives affected the actions of topoisomerase I on DNA by (a) inhibiting the rate of relaxation of supercoils and (b) stabilizing single-strand cleavable complexes, which were observed as nicked DNA circles in the reaction with supercoiled DNA or as specific cleavage sites in the reaction with linear DNA. These effects were reasonably well correlated among a variety of camptothecin derivatives (Fig. 6), suggesting that both types of actions arise from the same drug interaction process. The inhibition of relaxation at low enzyme concentration occurred in the absence of significant DNA cleavage. However, under these conditions only a small fraction of the DNA molecules are associated with active topoisomerase at any given time, and the resulting cleavable complexes may not have been detectable. The stabilization of these complexes would nevertheless impede the relaxation process to a significant extent. The stabilization or trapping of cleavable complexes therefore could be responsible for the inhibition of relaxation, as suggested by the structure-activity relationship.

This is in contrast with DNA intercalators, such as amsacrine derivatives and ethidium, which inhibit the relaxation of DNA supercoils by topoisomerase I without stimulating the production of cleavable complexes (17). Such inhibitors may act by blocking the association of DNA with the enzyme. Evidence with actinomycin suggests that this drug may act by this mechanism, although there is also evidence for some degree of stabilization of cleavable complexes (19).

Camptothecin has been found to stimulate topoisomerase I-induced cleavage at specific DNA sites (9, 10, 19, 20). All of the camptothecin derivatives that stimulated DNA cleavage by topoisomerase I in the current study produced the same pattern of specific cleavage sites. The enzyme by itself produced weak cleavage observable at most or all of these sites, but in the presence of the camptothecin derivatives the relative intensities of cleavage at different sites was altered markedly. It is not clear to what extent the stabilized or trapped cleavable complexes correspond to normal intermediates in the strand passing reaction.

Although camptothecin has been reported to bind to DNA, the apparent number of binding sites per base pair was too low to cause measurable change in the physicochemical property of DNA (21). It is more likely therefore that camptothecin acts by binding to the enzyme or to the DNA-enzyme complex (22).

The camptothecin molecule has a single asymmetrical carbon, located at position 20 (Fig. 7); in natural camptothecin the configuration is S. Since the synthetic optical isomer, R-camptothecin, has almost no effect on topoisomerase I, camptothecin must bind to an asymmetrical receptor site on the enzyme or enzyme-DNA complex. The structure-activity relationship (Table 1) suggests other inferences regarding the general nature of the drug-receptor interaction geometry. An interaction of the E ring, in the vicinity of position 20, with the receptor is further supported by the loss of activity resulting from substitution of the 20-hydroxy group (compound 16) and by replacement of the 21-lactone by a 21-lactam (compound 5; Fig. 7).

The 20-hydroxy-21-lactone structure undergoes facile ring opening and closure reactions under mild conditions, and ring opening does not occur easily in the lactam or in 20-deoxy-camptothecin. This suggests that opening of the lactone may be an essential part of the drug action mechanism. It seems possible that the ring-opened drug may form a reversible covalent bond with the enzyme via an ester exchange reaction which might be facilitated by hydrogen bonding of the 20-hydroxyl to an electronegative atom on the enzyme.

Substitutions on the A ring indicate an interaction of this ring near position 12 with the receptor. Whereas addition of an amino group to position 9 (compound 12) enhanced the actions on topoisomerase I, the addition of an amino group at position 12 (compound 15) abolished activity. Similarly, nitro substitution at position 9 or 10 yielded active compounds, whereas...
substitution at position 11 or 12 caused inactivation (compounds 18–22). These results suggest that substituents that encroach upon space in the vicinity of position 12 block interaction with the receptor.

This hypothesis is strikingly supported by the results with the 10,11-dimethoxy and 10,11-methylenedioxy derivatives (compounds 10 and 11, respectively). Whereas the former is inactive, the latter has enhanced activity. Steric repulsion between the two methoxy groups at positions 10 and 11 in compound 10 may cause the 11-methoxy to encroach upon a critical region in the vicinity of position 12. In the case of compound 11 (Fig. 7), which has substituents that are chemically very similar to those of compound 10, the substituent atoms are tied together in a ring which prevents encroachment upon the critical region.

The effects on topoisomerase I determined in the current work were compared with previously published in vivo antitumor data (14–16). Although the available in vivo data are incomplete and include assays in two different murine leukemia systems, the correlations between topoisomerase I inhibition and antitumor activity were remarkable (Table 1; Fig. 6). The R-camptothecin stereoisomer, which had little effect on topoisomerase I, was inactive in the antitumor assays. The racemic mixture RS was reported to have antitumor activity comparable to that of the natural S isomer, but requiring twice the dose, consistent with the expected 2-fold reduction in potency of an equal mixture of an active and an inactive isomer, the actions on topoisomerase I were correspondingly weaker relative to the S isomer. Several antitumor-inactive camptothecin derivatives exhibited little or no topoisomerase inhibition, including compounds 4, 6, 10, and 15. Compounds 7, 8, 11, and 12 were highly active in vivo at low dosage and exhibited marked inhibitions in all three topoisomerase I assays.

It is noteworthy that configurational alterations that would be expected to cause little change in general chemical properties produced marked changes in antitumor activity and topoisomerase I inhibition in parallel. The major examples are (a) S-versus R-camptothecin, (b) 9-amino versus 12-amino camptothecin and (c) 10,11-methylenedioxy versus 10,11-dimethoxy camptothecin (compounds 11 and 10).

Perfect agreement between topoisomerase I inhibition and antitumor activity was not obtained and is not to be expected, because drug metabolism and barriers to cell penetration may in some cases markedly alter biological effectiveness. Compounds 13 and 17 were reported highly active in vivo at moderate dosage but exhibited relatively low degrees of topoisomerase I inhibition. Compound 5 was reported to produce a significant antitumor response at high dosage but failed to show significant effects on topoisomerase I even at 100 μM. These were the only significant discrepancies among 21 compounds (Table 1).

The results strongly indicate that the antitumor activity of the camptothecins may be due to an action on topoisomerase I. The possibility, however, is not excluded that the specific camptothecin receptors may exist on other enzymes or proteins upon which the drug may act.

REFERENCES

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