Hydrophilic Camptothecin Analogs That Form Extremely Stable Cleavable Complexes with DNA and Topoisomerase I

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ABSTRACT
Camptothecin (CPT) analogs that form more stable ternary complexes with DNA and topoisomerase I (termed cleavable complexes) show greater activity in their ability to inhibit tumor cell line growth in preclinical studies. Based on our earlier work, we hypothesized that analogs bearing hydrogen bonding moieties at the 7- through 10-position of CPT would result in more stable cleavable complexes. Consequently, we synthesized analogs with 7-mono-, 7-di-, and 7-trihydroxymethylaminomethyl groups. These analogs showed increasing cleavable complex stability as the number of hydroxyl groups was increased. The 7-trihydroxymethylaminomethyl analog of 10,11-methylenedioxy camptothecin (THMAM-MD) showed remarkable ternary complex stability with a half-life of 116 minutes. This is an order of magnitude more stable than any previously examined analog. Our in vitro analysis demonstrated that these analogs were all potent topoisomerase I poisons and could inhibit tumor cell growth in culture. We studied the effects of THMAM-MD in vivo in severe combined immunodeficient mice bearing HT-29 colon cancer and MiaPaCa-2 pancreatic cancer tumors. The THMAM-MD analog showed excellent, persisting activity in inhibiting tumor growth with both lines. Taken together, our results suggest that CPTs with hydrophilic, hydrogen-bonding groups at the 7-position hold the promise of excellent clinical activity.

INTRODUCTION
The activity of camptothecin (CPT) and its analogs may be improved on through development of analogs that form more stable cleavable complexes with topoisomerase (topo) I and DNA (1, 2). The topo I-DNA-CPT ternary complex is in an equilibrium state and, as such, is readily reversible (3). As concentrations of CPT decrease, the drug bound in the ternary complex is lost, and the topo I is able to continue in its normal function of religating the single-stranded DNA breaks. Thus, ternary complexes and their subsequent induction of cell death can be lost before cellular toxicity is established.

The relationship between cleavable complex stability and cellular cytotoxicity is shown in Fig. 1. A simple model of logarithmic cell growth for 100 cells is shown, with an assumption that after an initial bolus of CPT analog, 50% of cells in S phase are killed, whereas no cells in G0-G1 or G2-M are affected. The 50% cell kill decays with a single exponential function to mimic drug clearance from the cells. The reversal of cleavable complex formation is given by the rate constant kapp. If kapp is large (solid line in Fig. 1), representing fast drug exit from the cleavable complex, there is an initial cell kill followed by a rapid regrowth of tumor cells. However, if kapp is small (on the order of the cell doubling time), cell growth is inhibited (dashed line) or eliminated (dotted line). Hence, whereas rapidly growing tumors with a high S-phase fraction are the most sensitive to current CPT analogs, novel analogs that form long-lived cleavable complexes may be effective against more slowly growing tumors.

In a previous report (4), we examined the molecular origins of complex stability induced by CPT analogs by measuring the temperature dependence of the rate of reversal of the complexes induced by CPT analogs with different substituents. This allowed some insight into the underlying physical mechanism of complex stability as a function of CPT analog composition. We found that the rate of reversal is controlled by the entropy (ΔS*) of the reversal reaction. Entropic control is often the case for small molecule-macromolecule interactions in aqueous media, and it usually implies that water reorganization around the small molecule is an important part of the rate process. Our data suggested that specific interactions of water or other hydrogen bond donors/acceptors might be important for stabilized cleavable complexes. This would be consistent with emerging data on a number of small molecules that interact with DNA that are now thought to gain additional binding affinity by hydrogen bonding with the first hydration layer of DNA (e.g., Refs. 5 and 6). Indeed, at least one ordered water molecule, which is located adjacent to the active site tyrosine of the enzyme, has been observed in the crystal structure of human topo I bound to DNA (7), and a recent structure of the cleavable complex stabilized by topotecan shows extensive hydration at the drug-DNA-topo I interface (Ref. 8; see Fig. 5). Molecular dynamics calculations on the topo I-DNA complex indicate that the protein-DNA interface is a water attractor, with a number of water molecules residing for extended times within the active site region (9). It is likely that various substituted CPT analogs must interact with and/or perturb water molecules associated with the cleavable complex.

Based on this evidence for water participation in the topo I-DNA-CPT cleavable complex, we interpreted the data from our previous work on entropic control of reversal as evidence that CPT forms contacts with the water shell around the cleavable complex. If water or hydrogen bonding interactions are, in fact, responsible for stabilizing cleavable complexes, then analogs that can form extensive hydrogen bonding networks at the interface should provide very stable cleavable complexes. Consequently, we synthesized a hydrophilic series of CPT analogs with such hydrogen bonding capabilities and examined their antitumor activity.

MATERIALS AND METHODS
Chemistry. The basic procedure for the total synthesis of CPT, involving the Friedlander reaction of orthoaminobenzaldehyde with a 20(S) tricyclic synthon yielding CPT (10–14), was used previously to synthesize a number of CPT analogs (15). We have accomplished the synthesis of the 7-trihydroxymethylaminomethyl analog (THMAM) of CPT (Fig. 2) by reacting CPT with FeSO4, methanol, H2SO4, and H2O2 to give 7-hydroxymethyl-CPT analog.

The latter was heated with HBr at 80°C for 18 h, leading to the formation of the 7-bromomethyl analog. The reaction of 7-bromomethyl analog with trihydroxymethylaminomethane yielded the desired analog THMAM-CPT. The corresponding THMAM-10,11-methylenedioxy camptothecin (THMAM-MD) analog was prepared by starting with MDCPT instead of CPT, and the dihydroxymethylaminomethyl analog (DMMAM) and monohydroxymethyl-
Cleavable Complex Formation by Camptothecin Analogs in Plasmid DNA. CPT analog-induced cleavable complex formation was performed as described previously (16) using pBR322 plasmid DNA (Life Technologies, Inc.) and human top I enzyme (Topogen, Inc.). The samples were analyzed by electrophoresis for 16 hours at 30 V on a 1% agarose gel in TAE buffer (containing 0.5 μg/mL ethidium bromide). After electrophoresis, the gel was stained with SYBR Green (Molecular Probes, Eugene, OR) and photographed using a Kodak Image Station 440CF (Kodak, Rochester, NY). The resulting photograph was analyzed as described previously (16).

Dose-response data were fitted to a simple $E_{\text{max}}$ model according to the following equation:

$$\text{Cleaved complex} = \frac{E_{\text{max}}[\text{Drug}]}{E_{50} + [\text{Drug}]}$$

(A)

where $[\text{Drug}]$ is the molar concentration of analog, $E_{\text{max}}$ is the maximal relaxed DNA signal, and $E_{50}$ is the concentration of drug required to produce 50% of the maximal response.

Reversal of Cleavable Complex Formation in Plasmid DNA. Reversal of the top I cleavage activity of the pBR322 plasmid DNA was accomplished by the method of Hertzberg et al. (3). Cleavable complexes were formed in the presence of sufficient analog to induce >90% nicked DNA (as determined from $E_{50}$ curves). A 100-fold excess of sonicated salmon sperm linear DNA (10 mg/mL; Life Technologies, Inc.) was added to the reaction mixture to initiate reversal of complex formation. All reactions were performed at 37°C. Aliquots were removed at selected time intervals for analysis. All of the time-dependent decays of top I-mediated cleavable complexes could be well fitted to a single exponential decay, indicating a simple pseudo first-order reaction:

$$\%\text{Cleaved DNA} = A e^{t} + C$$

(B)

where $t$ is time (in minutes), $k_{\text{app}}$ is the exponential rate constant for reversal with units of minutes$^{-1}$, and $A$ and $C$ are constants representing amplitude and final percentage of cleaved DNA, respectively.

Cell Growth Inhibition Assays. The human prostate carcinoma cell line PC-3 and the cervical carcinoma HeLa-S3 line were maintained in Dulbecco’s modified Eagle’s medium, whereas HT-29 cells were maintained in RPMI 1640 (all media were supplemented with 10% fetal bovine serum). Exponentially growing cells ($1-2 \times 10^5$ cells, unless otherwise specified) in 0.1 mL of medium were seeded on day 0 in a 96-well microtiter plate. On day 1, 0.1-mL aliquots of medium containing graded concentrations of test analogs were added in triplicate to the cell plates. After incubation for 3 days, cell growth was monitored by the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (17).

Animal Studies. Female severe combined immunodeficient mice received subcutaneous implantation with the human colorectal carcinoma cell line HT-29 or the human pancreatic cancer cell line MiaPaCa-2. Ten days after inoculation, the animals were pair-matched into treatment and control groups, with each treatment and control group containing eight tumor-bearing mice. The administration of drugs or vehicle began the day the animals were pair-matched (day 1), and all injections were intraperitoneal. The CPT analogs were formulated for injection in 0.25% methylcellulose and 2% Tween 80. Each drug was administered at 1.0 mg/kg on a once daily ×5 schedule. Mice were weighed twice weekly, and tumor measurements were taken by calipers twice weekly, starting on day 1. These tumor measurements were converted to tumor volume by using the equation $L^2 \times W/2$ ($L$ is length, and $W$ is width), and from these measurements, tumor weights were calculated. The mice were treated with two cycles of treatment. After stopping treatment, the tumor growth was determined, and these data were compared to vehicle controls.

Table 1  Activity of CPT analogs with hydrogen bonding moieties at the 7-position

<table>
<thead>
<tr>
<th>CPT analog</th>
<th>$E_{50}$ for topo I (μmol/L)</th>
<th>$k_{\text{app}}$ for complex stability (min$^{-1}$)</th>
<th>$I_{50}$ vs. HeLa-S3 (μmol/L)</th>
<th>$I_{50}$ vs. PC-3 (μmol/L)</th>
<th>$I_{50}$ vs. HT-29 (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPT</td>
<td>0.35 ± 0.01</td>
<td>0.149 ± 0.010</td>
<td>117.2 ± 30.5</td>
<td>102.8 ± 7.6</td>
<td>2.0 ± 1.5</td>
</tr>
<tr>
<td>SN-38</td>
<td>0.32 ± 0.04</td>
<td>0.061 ± 0.010</td>
<td>25.7 ± 2.0</td>
<td>25.9 ± 2.1</td>
<td>26.8 ± 14.4</td>
</tr>
<tr>
<td>Topotecan</td>
<td>7.10 ± 4.66</td>
<td>0.169 ± 0.009</td>
<td>180.5 ± 50.3</td>
<td>287.1 ± 7.1</td>
<td>402.8 ± 248.6</td>
</tr>
<tr>
<td>MDCPT</td>
<td>0.05 ± 0.02</td>
<td>0.058 ± 0.003</td>
<td>9.6 ± 2.8</td>
<td>12.8 ± 1.0</td>
<td>8.3 ± 6.7</td>
</tr>
<tr>
<td>7-Aminomethyl-MD</td>
<td>0.20 ± 0.19</td>
<td>0.051 ± 0.002</td>
<td>32.7 ± 13.1</td>
<td>19.7 ± 3.0</td>
<td>11.7 ± 8.3</td>
</tr>
<tr>
<td>7-Isopropyl-aminomethyl-MD</td>
<td>0.51 ± 0.02</td>
<td>0.047 ± 0.007</td>
<td>13.9 ± 2.7</td>
<td>19.2 ± 4.0</td>
<td>2.4 ± 1.5</td>
</tr>
<tr>
<td>7-MHMAM-MD</td>
<td>3.00 ± 0.86</td>
<td>0.029 ± 0.007</td>
<td>124.1 ± 5.5</td>
<td>108.9 ± 40.2</td>
<td>105.9 ± 26.7</td>
</tr>
<tr>
<td>7-DHMAM</td>
<td>4.44 ± 0.49</td>
<td>0.059 ± 0.007</td>
<td>1207.9 ± 63.4</td>
<td>807.3 ± 128.8</td>
<td>831.4 ± 79.6</td>
</tr>
<tr>
<td>7-MDMC</td>
<td>0.04 ± 0.02</td>
<td>0.023 ± 0.002</td>
<td>178.2 ± 21.2</td>
<td>206.8 ± 31.5</td>
<td>182.0 ± 76.7</td>
</tr>
<tr>
<td>7-THMAM</td>
<td>2.8 ± 1.86</td>
<td>0.037 ± 0.013</td>
<td>3379.8 ± 94.1</td>
<td>2078.2 ± 405.4</td>
<td>5025.6 ± 993.0</td>
</tr>
<tr>
<td>7-THMAM-MD</td>
<td>0.11 ± 0.07</td>
<td>0.006 ± 0.001</td>
<td>230.9 ± 17.3</td>
<td>90.8 ± 24.5</td>
<td>404.5 ± 185.9</td>
</tr>
</tbody>
</table>

Synthesis and properties of camptothecin analogs with hydroxymethyl aminomethane substituents at the 7-position, manuscript in preparation.
RESULTS

Hydrogen Bonding Moieties at the 7-Position of Camptothecin Increase Cleavable Complex Stability. To test the hypothesis that CPT interacts with or affects the water layer in the cleavable complex, a number of new analogs were synthesized bearing increasing hydrogen bond-donating groups at the 7-position. These are the analogs given in Fig. 2 and designated MHMAM, DHMAM, and THMAM. The corresponding MDCPT analog of each of these was synthesized as well. For comparison, 7-aminomethyl-MDCPT and 7-isoproplaminomethyl-MDCPT were also examined. The results are recorded in Table 1. As shown in Fig. 3, the increasing number of hydroxyl groups on the 7-aminomethyl moiety resulted in progressively more stable cleavable complexes. Whereas our earlier work (4) identified the 10-amino-CPT as the analog that formed the most stable cleavable complex (with a half-life of 17.8 minutes), the 7-THMAM-MDCT analog showed a remarkably stable complex with a half-life of 116 minutes.

The addition of the hydrophilic groups at the 7-position did not significantly affect the ability of the CPT analogs to effectively poison topo I in assays with purified enzyme and DNA (compare MDCPT and THMAM-MDCPT in Table 1). Hence the new compounds are very effective topo I poisons. However, one disadvantage of the increased hydrophilicity of the analogs is apparent from their low activity in steady-state culture experiments. The THMAM analog has very low activity in inhibiting cell growth under these conditions. This is presumably due to low cellular permeation by this analog, which is approximately 2 orders of magnitude more water soluble than CPT itself (data not shown). It must be noted, however, that this type of assay is not necessarily predictive of in vivo or clinical activity of the compounds (see the data for topotecan in Table 1, for example). These data merely reflect the approximate plasma concentrations that would be necessary to begin to see antitumor activity. Hence, the
THMAM-MD and related MD analogs should show activity at concentrations near or lower than those obtained with topotecan or the active metabolite of CPT-11 (SN-38).

**In vivo Activity of 10,11-Methylenedioxycamptothecin.** The stability of the cleavable complex in *in vitro* assays encouraged testing of the compound *in vivo*. This is a better test of the effects of ternary complex stability on antitumor activity than standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays (Table 1), which only provide data as to the relative cellular toxicity of the compounds. Our animal studies (Fig. 4) indicate that, as predicted, the THMAM-MD analog is more active against HT-29 tumor xenografts than any previously examined CPT analog, suppressing tumor growth for a remarkable 15 days after cessation of treatment with THMAM-MD and reducing tumor growth versus controls up to 36 days after cessation of treatment. A single dosing regimen was tested with the pancreatic cancer cell line MiaPaCa-2. Whereas this tumor is responsive to CPT-11 at high doses, moderate doses of THMAM-MDCPT produced regression and inhibition of tumor growth somewhat greater than that for CPT-11. These data indicate the potent *in vivo* activity that is possible by selecting CPT analogs that form stable cleavable complexes. It is noteworthy that the substantial *in vivo* activity of the THMAM-MD is achieved with virtually no weight loss to animals, indicating that the dose of THMAM-MD could be further increased from those used here.

**DISCUSSION**

Based on our previous data with a number of CPT analogs, as well as literature reports indicating the participation of water in the topo I-DNA cleavable complex, we designed and synthesized a series of CPT analogs with hydrogen bonding donors and acceptors at the 7-position. These stabilized the cleavable complex as the number of hydroxyl groups at this position increased. The THMAM-MD analog showed a remarkably stable cleavable complex and was chosen for *in vivo* testing. Our results indicate that this compound was extremely active against both HT-29 and MiaPaCa-2 tumor lines.
The most likely reason for the increased stability of the cleavable complex is through formation of hydrogen bonding networks by the CPT analog in the complex. In Fig. 5, we have replaced topotecan with THMAM using the recent crystal structure of the topotecan-induced cleavable complex (8). In Fig. 5A, the crystallographic water molecules within 10 Å of the drug binding site are shown to illustrate how closely associated water is with this site. Fig. 5B shows the effect on this network that the 7-THMAM group may have. Several of the crystallographic water molecules are close enough to the CPT analog to form hydrogen bonds with THMAM. Also nearby are Asn352 and Lys436 of topo I. Although this model is somewhat speculative, if the THMAM were bound in this position, some effect on the water shell around the cleavable complex would be expected. Our preliminary data using osmotic stress techniques indicate that ~18 water molecules are released on THMAM-MD binding (data not shown). Hence, another effect of the THMAM group may be replacement of water in the cleavable complex. Whatever the final mechanism, it is clear that hydrogen bonding moieties at the 7-position of CPTs result in remarkably stable cleavable complexes, and the prototype analog THMAM-MD shows excellent activity in preclinical models. The hydrophilic nature of the molecule also holds advantages for formulation and administration, due to its enhanced water solubility. Hence, we anticipate that the CPT analogs reported here may form the basis of a new group of clinically active molecules.

ACKNOWLEDGMENTS

The authors thank Ruth Jarbadan and Natalie Young for their contributions to the experimental data.

REFERENCES

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