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Camptothecin analogs in malignant gliomas: comparative analysis and characterization

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Object. The authors compared and characterized several new classes of camptothecin (CPT) analogs (a total of 22 drugs) directed against human and murine glioma cell lines in vitro, trying to identify CPT analogs that can be used for local therapy in future clinical trials. Camptothecin is a naturally occurring alkaloid that inhibits the DNA-replicating enzyme topoisomerase I. Moreover, CPT and its analogs have shown promising antitumor activity against both systemic and intracranial neoplasms. Because the CPTs have poor bioavailability and are unable to cross the blood–brain barrier, they may best be delivered to the central nervous system by polymers. The authors have previously shown that local delivery of Na-CPT by implantable polymers prolongs survival in a rat intracranial glioma model. In recent years, a number of newly synthesized CPT analogs have been developed that exhibit more potency and stability than Na-CPT.

Methods. Cytotoxicities of the drugs were tested using modified clonogenic and monotetrazolium assays in three glioma cell lines. A potassium chloride–sodium dodecyl sulfate coprecipitation assay was used to determine the frequency of drug-stabilized cleavable complexes. Of the CPT analogs analyzed, the 10,11-methylenedioxy (MD) class consistently demonstrated the greatest cytotoxicity. Three of these analogs, 10,11-MD-20(RS)-CPT, 10,11-MD-20(S)-CPT-glycinate ester (Gly).HCl, and 9-amino-10,11-MD-20(S)-CPT-Gly, exhibit significantly greater antiproliferative activities than CPT, Na-CPT, or 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) against all three glioma cell lines. In addition, the 10,11-MD-20(RS)-CPT analog induces more cleavable complexes than Na-CPT at every concentration.

Conclusions. The increased potency and greater stability of CPT analogs hold promise for more effective local antitumor treatments against malignant intracranial brain tumors. The greater cytotoxicity of 10,11-MD CPTs in comparison with other CPT analogs as well as CPT, BCNU, or Na-CPT, may present an ideal candidate drug class for development against both primary and metastatic brain tumors.

KEY WORDS • camptothecin • camptothecin analog • glioma • drug delivery • polymer

CAMPTOTHECIN is a naturally occurring alkaloid that was first isolated from the Chinese tree *Camptotheca acuminata* (Nyssaceae) by Wall and coworkers in 1966.[34] Because of its potent activity in vitro and in vivo in the mouse L1210 lymphoid leukemia assay, the compound in the form of its water-soluble salt (Na-CPT) was advanced rapidly to clinical trial. Unsuccessful results in three Phase I studies,[10,19,21] however, lessened interest in CPT for a number of years. Hsiang, et al.,[12,13] have shown that CPT specifically inhibited the enzyme topoisomerase I, a nuclear protein essential for DNA repair during DNA replication. As a result, interest in CPT and its analogs revived, and it has since been shown that many CPT analogs possess much greater activity in topoisomerase I inhibition than CPT itself.[13,14]

Two CPT analogs, topotecan[16] and CPT-11,[28] are now approved for marketing by the Food and Drug Administration; several more, including 9-amino-CPT[8,38] and Gl147211C[1], are in advanced clinical trials. Information about the synthesis, structure, and activity of a large number of novel CPT analogs has been published by Wall, et al.,[35] who have demonstrated that an intact α-hydroxy lactone ring is an absolute requirement for in vivo and in vitro activity, and for inhibition of topoisomerase I. These authors also have shown that these activities are greatly potentiated by CPT analogs with the 10,11-MD substituent.[13,14,35,38] Recently it has been found that CPT analogs combining the 10,11-MD moiety with functionality at carbon 7, such as ethyl or chloromethyl substituents, provide not only topoisomerase I inhibition, but also stabilization of the CPT DNA topoisomerase I complex.[22,32]

The effect of CPTs on malignant gliomas has not been

Abbreviations used in this paper: BBB = blood–brain barrier; BCNU = 1,3-bis(2-chloroethyl)-1-nitrosourea; CPT = camptothecin; GI_{90} = 90% growth inhibition; Gly = glycinate ester; LD_{90} = 90% lethal dose; MD = methylenedioxy; MTT = monotetrazolium; OD = optical density; SDS = sodium dodecyl sulfate.

well characterized. In an effort to improve bioavailability with systemic administration of CPTs, we have developed polymeric delivery systems that allow sustained drug delivery directly to the brain,[4,27] thus circumventing the need for drugs to cross the BBB, and minimizing systemic toxicity.[3,17,37] We have previously demonstrated that local delivery of Na-CPT by polymers significantly prolonged survival in a rat intracranial gliosarcoma model.[40] The Na-CPT may have been converted to CPT by the process that incorporated the compound into the polymer. Of particular significance, systemic delivery of Na-CPT had no survival benefit in a glioma model, whereas polymeric delivery of Na-CPT made it the most potent chemotherapeutic agent we have studied in this fashion.[37] In addition, BCNU-impregnated biodegradable polymers have now been shown to be effective also against both primary and recurrent malignant gliomas in clinical trials.[5,6,33] These results encouraged our group and others to develop more potent antiglioma agents for delivery by polymers against both primary and metastatic intracranial tumors.

The newer, more potent CPT analogs appear to be ideal candidates to investigate for polymeric delivery. In particular, previous studies have shown that Na-CPT has only one tenth the activity of CPT, which in turn has much less activity than the new CPT analogs.[35] The efficacy of Na-CPT in vivo in experimental brain tumors may be due to conversion to CPT during polymer incorporation or at the tumor site. Therefore, the encouraging results after local delivery of Na-CPT in a rat glioma model indicate that CPT analogs may be of much greater benefit in treating brain tumors.

Although CPT analogs have been analyzed in non–central nervous system tumor types, the potential efficacy of this family of drugs against malignant gliomas has not been studied in a systematic fashion. We examined only Na-CPT earlier. In this report we examine the in vitro cytotoxicity of 22 CPT analogs, provide detailed characterization of the most potent ones, and compare these agents with BCNU, CPT, and Na-CPT. We show that enhanced cytotoxicity is associated with increased exposure time and with the increased formation of CPT-induced topoisomerase I DNA-cleavable complexes. We characterize a new class of CPT analogs that holds promise for future local therapy against malignant brain tumors.

Materials and Methods

Drugs and Chemicals

A total of 20 CPT analogs were developed and synthesized (Fig. 1, Table 1) by Drs. Wall and Wani (Research Triangle Institute, Research Triangle Park, NC). The Na-CPT and 10,11-MD-20(RS)-CPT were obtained from the National Cancer Institute (Bethesda, MD). The CPT analogs were kept at −20°C and solutions were prepared fresh in dimethyl sulfoxide at 200-μM concentrations before plating. The BCNU was purchased from GenCorp Aerojet (Sacramento, CA).

Tumor Cell Lines

Rat 9L gliosarcoma cells were obtained from Dr. M. Barker (San Francisco, CA), and the human glioma cell lines U87 and H80 were supplied by Dr. O. M. Colvin (Duke University Medical Center, Durham, NC). The cells were maintained in RPMI medium containing 10% fetal calf serum and penicillin/streptomycin in humidified incubators at 37°C in 5% CO_2. Cultured tumor monolayers were harvested with 0.025% trypsin, counted, and resuspended in RPMI medium before plating for in vitro analysis.

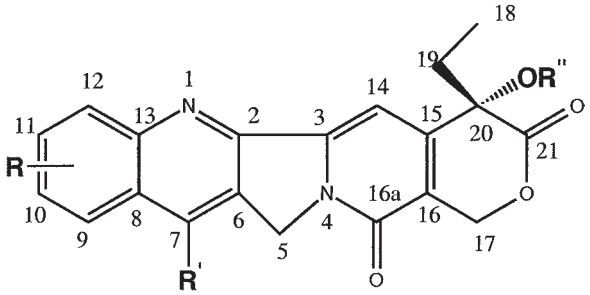


FIG. 1. Diagram of the structure of CPT showing R, R′, and R′′ substitution sites for analog synthesis.

Growth Inhibition Assays

The MTT Assay. The growth inhibition by CPT analogs was tested using a modified MTT colorimetric assay described previously.[20] In brief, cells were harvested from exponentially growing cultures, counted with a hemocytometer, and seeded in 96-well flat-bottomed microtiter plates at 10^4 cells/ml density in a 100-μl volume. After overnight incubation at 37°C in 5% CO_2, the cells were treated with various concentrations of CPT analogs for 1, 12, 24, and 48 hours. Each time exposure and drug concentration combination was performed in quadruplicate. For all plates, supernatant fractions were removed at 48 hours and 100 μl of MTT solution (0.5 mg/ml in RPMI medium) was added to each well, followed by 4 hours of incubation at 37°C. The medium was then removed and 150 μl of dimethyl sulfoxide was added to dissolve the formazan crystals produced. The OD was measured at 570 nm with a microplate reader (BioRad, Richmond, CA). Cell survival was calculated according to the following equation: Survival (%) = $(OD_{570[sample]}/OD_{570[control]}) \times 100\%$.

The LD_{90} was calculated by fitting a log linear function to a plot of survival (%) compared with drug concentration.

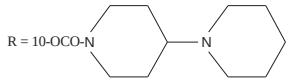
Clonogenic Assay. The sensitivity of glioma cell lines to CPT analogs and BCNU was determined by a modification of clonogenic assays described previously.[24,26] Briefly, confluent cells from our tumor bank were trypsinized and plated at 800 cells per 60-mm well. After 24 hours, fresh medium containing CPT analogs or BCNU at various concentrations was added. All cells were continuously exposed to drug-containing medium for 96 hours, at which time all plates were fixed and stained with Coomassie brilliant blue (Bio-Rad). All colonies containing more than 50 cells were identified and counted. Survival was calculated as the ratio between the number of colonies formed by the treated cells and the number of colonies formed by the untreated cells. All tests were performed in triplicate and the experiment was performed twice. The GI_{90} was calculated by fitting a log linear function to a plot of viability (%) compared with drug concentration.

The KCl-SDS Precipitation Assay to Determine Frequency of Drug-Stabilized Cleavable Complexes. Because elevated cleavable complex levels have been most consistently correlated with increased CPT cytotoxicity,[9] we measured the in vitro formation of covalent topoisomerase I DNA complexes by a modification of the SDS precipitation assay described previously by Trask, et al.,[31] and modified by Rowe, et al.[25] This assay was used to measure the formation of protein DNA complexes in the cultured human glioma cell line H80; cells were grown in RPMI medium. The DNA in logarithmically growing cells (2×10^5 cells/ml) was labeled by adding [methyl-^{3}H]thymidine (specific activity, 50 Ci/mmol) to the medium, to a final concentration of 1.4 μCi/ml.

After overnight incubation, cells were spun at 1000 rpm for 5 minutes in an International centrifuge (model CM; IEC, Needham Heights, MA). The supernatant fraction was removed, and cells were washed three times in phosphate-buffered saline and resuspended in fresh medium to a final concentration of 10^5 cells/ml. Aliquots (1 ml/well) were placed in a 24-well microtiter plate (Falcon; Fisher Co., Pittsburgh, PA) and incubated for another 2 hours at 37°C. The

TABLE 1

*Chemical structure of CPT analogs**

Drug No.	CPT Analog	MW	Structure
1	CPT	348	R = R′ = R′′ = H
2	9-amino-20(S)-CPT	363	R = 9-NH_2; R′ = R′′= H
3	10,11-MD-20(S)-CPT	392	R = 10,11-OCH_2O-; R′ = R′′= H
4	nitidine†	383	
5	6-methoxydihydronitidine†	400	
6	7-methyl,10,11-MD-20(S)-CPT	406	R = 10,11-OCH_2O-; R′ = Me; R′′ = H
7	7-ethyl,10,11-MD-20(S)-CPT	420	R = 10,11-OCH_2O-; R′ = Et; R′′ = H
8	CPT 11	622	 -HCl; R′ = Et; R′′ = H
9	topotecan	458	R = 10-OH-9-$CH_2N(Me)_2$-HCl; R′ = R′′ = H
10	9-amino-10,11-MD-20(S)-CPT	407	R = 9-NH_2-10,11-OCH_2O-; R′ = R′′ = H
11	9-amino-10,11-MD-20(S)-CPT-Gly.HCl	500	R = 9-NH_2-10,11-OCH_2O-; R′ = H; R′′ = $COCH_2NH_2$-HCl
12	10,11-MD-20(S)-CPT-Gly.HCl	485	R = 10,11-OCH_2O-; R′ = H; R′′ = $COCH_2NH_2$-HCl
13	9-amino-20(S)-CPT-Gly.HCl	456	R = NH_2; R′ = H; R′′ = $COCH_2NH_2$-HCl
14	10,11-MD-20(S)-CPT Na salt	414	R = 10,11-OCH_2O-; R′ = H; R′′ =
15	9-amino-20(S)-CPT Na salt	385	R = 9-NH_2; R′ = H; R′′ =
16	7-ethyl,10,11-MD-20(S)-CPT-Gly.HCl	513	R = 10,11-OCH_2O-; R′ = Me; R′′ = $COCH_2NH_2$-HCl
17	12-amino-20(S)-CPT	363	R = 12-NH_2; R′ = R′′ = H
18	10,11-MD-20(R)-CPT	392	R = 10,11-OCH_2O-; R′ = R′′ = H
19	9-chloro-20(S)-CPT	382	R = 9-Cl-; R′ = R′′ = H
20	9-chloro-10,11-MD-20(S)-CPT	426	R = 9-Cl-10,11-OCH_2O-; R′ = R′′ = H

* MW = molecular weight.
† Non-CPT topoisomerase I inhibitors.

cells were then treated with various concentrations of drugs for 60 minutes. The microtiter plate was spun in a centrifuge (Centra GP 8R; IEC) at 2500 rpm for 2 minutes at room temperature, the medium was removed from each well, and cells were lysed by adding 1 ml of prewarmed (65˚C) lysis solution (1.25% SDS, 5 mM ethylenediamine tetraacetic acid, pH 8, 0.4 mg/ml salmon sperm DNA). The lysate was transferred to a 1.5-ml tube (Eppendorf, Westbury, NY) containing 250 μl of 325 mM KCl. After vigorous mixing with a vortex for 10 seconds at the highest setting, the sample was cooled on ice for 10 minutes and centrifuged in a microfuge (Eppendorf) at 14,000 rpm for 10 minutes at 4˚C. The pellet was resuspended in 1 ml of a wash solution (10 mM Tris-HCl, pH 8, 100 mM KCl, 1 mM ethylenediamine tetraacetic acid, 0.1 mg/ml salmon sperm DNA) and incubated at 65˚C for 10 minutes. The suspension was then cooled on ice for 10 minutes and recentrifuged. The pellet was washed again before resuspending in 200 μl of H_2O at 65˚C. The suspension was then combined with 5 ml of scintillation liquid (Atomlight; DuPont, Wilmington, DE) and the scintillation counts per minute were measured to determine the level of cleavable complexes. The counts per minute, expressing cleavable complex formation, were plotted as a percentage of the control at each concentration.

TABLE 2

*Glioma cell survival after treatment with CPT analogs**

CPT Analog (drug no.)	Cell Line (Survival as % of Control)		
	9L	H80	U87
CPT (1)	55.4	50.6	54.1
9-amino-20(S)-CPT (2)	25.5	10.3	42.9
10,11-MD-20(S)-CPT(3)	79.6	23.9	61.4
nitidine (4)	44.6	7.1	43.6
6-methoxydihydronitidine (5)	63.7	8.5	25.1
7-methyl,10,11-MD-20(S)-CPT (6)	64.9	56.4	67.8
7-ethyl,10,11-MD-20(S)-CPT (7)	14.7	1.3	4.0
CPT 11 (8)	96.1	97.0	101.0
topotecan (9)	92.6	100.0	82.0
9-amino-10,11-MD-20(S)-CPT (10)	88.2	67.0	19.0
9-amino-10,11-MD-20(S)-CPT-Gly.HCl (11)	2.3	1.9	0.6
10,11-MD-20(S)-CPT-Gly.HCl (12)	34.0	27.3	3.0
9-amino-20(S)-CPT-Gly.HCl (13)	91.0	80.0	152.0
10,11-MD-20(S)-CPT Na salt (14)	66.7	86.0	34.0
9-amino-20(S)-CPT Na salt (15)	81.0	76.2	101.0
7-ethyl,10,11-MD-20(S)-CPT-Gly.HCl (16)	86.1	51.5	40.0
12-amino-20(S)-CPT (17)	77.2	80.3	92.0
10,11-MD-20(R)-CPT (18)	92.1	98.9	102.0
9-chloro-20(S)-CPT (19)	79.4	102.5	83.0
9-chloro-10,11-MD-20(S)-CPT (20)	93.6	60.8	78.0
Na-CPT†	71.8	46.1	67.8
10,11-MD-20(RS)-CPT†	74.6	22.0	51.0

* Controls were glioma cell cultures to which no drug was added.
† Drug obtained from the National Cancer Institute.

Results

Screening of Analogs

Initially, we screened the 22 CPT analogs for antiproliferative activity at 1 nM against the human glioma cell lines H80 and U87 and the rat glioma cell line 9L by using the modified clonogenic assay. Table 2 illustrates survival as a percentage of control (cells receiving no drug) and Table 3 shows the consistently most potent and less potent CPT compounds.

The CPT analogs showed significant variability in their antiproliferative activities, evinced by the greater than 25-fold difference in percentage of survival between 9-chloro-20(S)-CPT, topotecan, 10,11-MD-20(R)-CPT, and CPT-11 (the least potent analogs), and 9-amino-10,11-MD-20(S)-CPT-Gly.HCl (the most potent analog). The 10,11-MD class of drugs appeared to exhibit the greatest cytotoxicity

TABLE 3

Consistently most potent and less potent CPT analogs

CPT Analog (drug no.)	Cell Line (Survival as % of Control) 9L	H80	U87
most potent CPT compounds			
10,11-MD-20(S)-CPT (3)	79.6	23.9	61.4
7-ethyl,10,11-MD-20(S)-CPT (7)	14.7	1.3	4.0
9-amino-10,11-MD-20(S)-CPT (10)	88.2	67.0	19.0
9-amino-10,11-MD-20(S)-CPT-Gly.HCl (11)	2.3	1.9	0.6
10,11-MD-20(S)-CPT-Gly.HCl (12)	34.0	27.3	3.0
7-ethyl,10,11-MD-20(S)-CPT-Gly.HCl (16)	86.1	51.5	40.0
intermediate potency CPT compounds			
CPT (1)	55.4	50.6	54.1
9-amino-20(S)-CPT (2)	25.5	10.3	42.9
nitidine (4)	44.6	7.1	43.6
6-methoxydihydronitidine (5)	63.7	8.5	25.1
7-methyl,10,11-MD-20(S)-CPT (6)	64.9	56.4	67.8
9-amino-20(S)-CPT-Gly.HCl (13)	91.0	80.0	152.0
10,11-MD-20(S)-CPT Na salt (14)	66.7	86.0	34.0
9-chloro-10,11-MD-20(S)-CPT (20)	93.6	60.8	78.0
10,11-MD-20(RS)-CPT	74.6	22.0	51.0
less potent CPT compounds			
CTP 11 (8)	96.1	97.0	101.0
topotecan (9)	92.6	100.0	82.0
9-amino-20(S)-CPT Na salt (15)	81.0	76.2	101.0
12-amino-20(S)-CPT (17)	77.2	80.3	92.0
10,11-MD-20(R)-CPT (18)	92.1	98.9	102.0
9-chloro-20(S)-CPT (19)	79.4	102.5	83.0
Na-CPT	71.8	46.1	67.8

(Table 3). Therefore, four of these analogs, 10,11-MD-20 (RS)-CPT, 10,11-MD-20(S)-CPT, 10,11-MD-20(S)-CPT-Gly.HCl, and 9-amino-10,11-MD-20(S)-CPT-Gly.HCl, were chosen for further analysis, based on their consistently elevated antiproliferative activities against all three glioma lines.

Time-Dependent Cytotoxicity

We investigated the antiproliferative effect of drug exposure time with CPT, Na-CPT, and the four 10,11-MD analogs discussed earlier. Viability was calculated by using the MTT assay after 1-, 12-, 24-, 48-, or 72-hour exposures to these compounds at various concentrations. Figure 2 *upper* illustrates the dramatically increased antiproliferative effect of prolonged exposure to CPT, Na-CPT, 10,11-MD-20 (RS)-CPT, and 10,11-MD-20(S)-CPT against the U87 cell line; it also demonstrates the markedly increased potency of these two 10,11-MD-CPT analogs compared with CPT or Na-CPT. Figure 2 *lower* compares the increased antiproliferative effect of prolonged exposure to four 10,11-MD-CPT analogs against the U87 glioma line. Similar time-dependent responses were observed with the other glioma lines, H80 and 9L (Table 4). The 10,11-MD analogs demonstrated significantly greater antiproliferative activities than either CPT or Na-CPT against all three glioma cell lines.

Comparison With Na-CPT and BCNU

We next used the modified clonogenic assay to compare the antiproliferative activities of three of the potent CPT analogs described earlier with those of Na-CPT and BCNU, both drugs that have previously demonstrated efficacy against experimental brain tumors. Table 5 presents the GI_{90} for each drug and cell line. All five compounds clearly show a dose-dependent effect, but the CPT analogs displayed markedly greater (> 100-fold) antiproliferative activities than did BCNU against all three glioma lines. Moreover, the 10,11-MD compounds had an approximately 10-fold greater antiproliferative activity than Na-CPT.

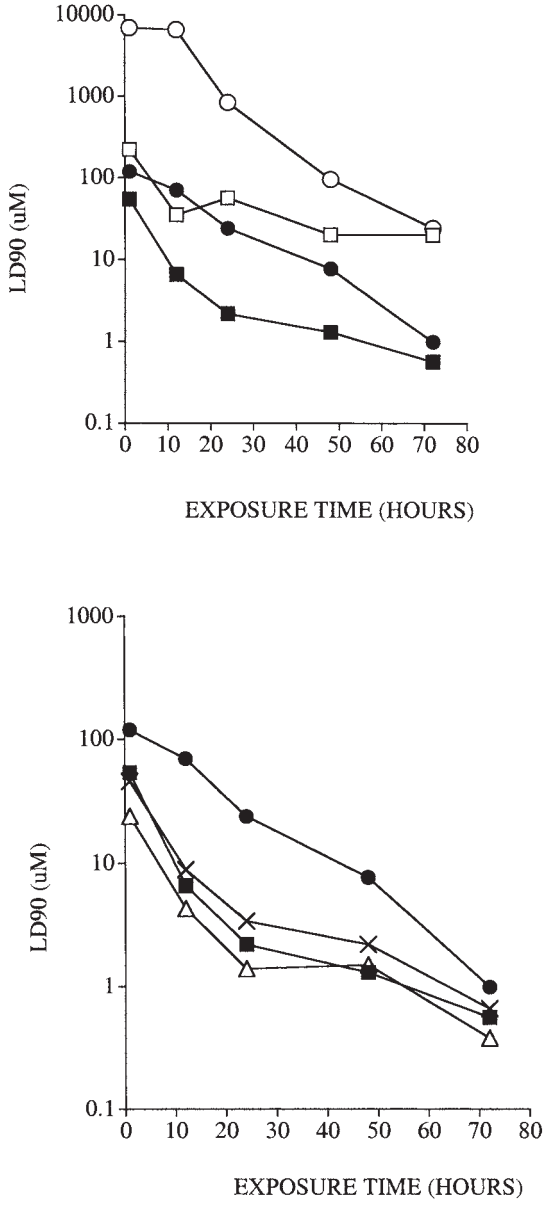


FIG. 2. Graphs showing time-dependent cell viability for the U87 human glioma line after treatment. *Upper:* Results of treatment with CPT *(white squares)*; Na-CPT *(white circles)*; 10,11-MD-20(S)-CPT *(black squares)*; or 10,11-MD-20(RS)-CPT *(black circles)*. *Lower:* Results of treatment with 10,11-MD-20(S)-CPT; 10,11-MD-20(RS)-CPT; 10,11-MD-20(S)-CPT-Gly.HCl *(×)*; or 9-amino-10,11-MD-20(S)-CPT-Gly.HCl *(triangles)*.

Levels of Na-CPT–Induced and 10,11-MD-20(RS)-CPT–Induced Cleavable Complexes

After exposure to various concentrations of Na-CPT and

TABLE 4

Time-dependent cell viability (LD_{90}, nM) after treatment with CPT analogs in glioma cell lines

Cell Line & Treatment Time (hrs)	CPT	Na-CPT	10,11-MD-20(RS)-CPT	10,11-MD-20(S)-CPT	10,11-MD-20(S)-CPT-Gly.HCl	9-amino-10,11-MD-20(S)-CPT-Gly.HCl
9L						
1	69.0	81.0	7.5	9.6	13.0	13.0
12	5.1	34.0	0.42	0.68	1.2	0.97
24	2.8	9.4	0.47	0.44	0.55	0.53
48	2.1	3.3	0.36	0.96	1.0	0.89
72	1.7	0.85	0.12	0.18	0.19	0.15
H80						
1	49.0	37.0	3.4	2.5	2.0	2.2
12	18.0	6.5	0.46	0.15	0.15	0.16
24	2.0	1.1	0.35	0.12	0.14	0.12
48	1.5	0.67	0.20	0.12	0.11	0.091
72	0.85	0.44	0.09	0.045	0.08	0.059
U87						
1	220.0	6950.0	119.0	54.0	46.0	24.0
12	35.0	6601.0	70.0	6.6	8.9	4.3
24	56.0	834.0	24.0	2.2	3.4	1.4
48	20.0	95.0	7.7	1.3	2.2	1.5
72	20.0	24.0	0.99	0.56	0.66	0.38

10,11-MD-20(RS)-CPT, CPT-induced topoisomerase I DNA-cleavable complexes in whole H80 cells were measured using the KCl-SDS precipitation assay. A dose-dependent increase in cleavable complexes is clearly shown for both compounds (Fig. 3). In addition, the 10,11-MD-20(RS)-CPT compound induced more cleavable complexes than did the less cytotoxic Na-CPT at all concentrations. It is likely that the 10,11-MD-20(S)-CPT stereoisomer would have been more potent than the racemic 20(RS) analog.

Discussion

We analyzed several different classes of CPT analogs and compared their cytotoxicity against glioma cell lines in vitro. We then took the most potent analogs and characterized them in detail by varying the exposure time and concentration, demonstrating that their enhanced cytotoxic effect is due to an increase in the formation of drug-stabilized cleavable protein complexes. Our results show convincingly that the 10,11-MD class of CPTs are more cytotoxic than other CPT analogs, including topotecan and CPT-11, which are now in clinical use. In addition, 10,11-MD-20(S)-CPT analogs exhibit markedly greater cytotoxicity than either CPT, Na-CPT, or BCNU. We conclude that 10,11-MD-20(S)-CPT analogs may be excellent candidate drugs for future development as local delivery agents against both primary and metastatic brain tumors.

This study was designed to examine the antiproliferative effects of a variety of CPT analogs, with a view to developing their use for local delivery against malignant gliomas. The role of CPTs in the treatment of central nervous system tumors has not been well established. Despite promising results in early studies, systemically administered CPTs penetrated the BBB poorly, and effective systemic levels were toxic. Pharmacological studies revealed that the water-soluble Na-CPT formulation is strongly protein bound (> 97%) when administered intravenously,[29] and it is not detectable in the cerebrospinal fluid.[10] This indicates that poor bioavailability is a critical factor in the lack of efficacy of systemically administered CPTs against brain tumors.

Chemotherapeutic agents, given systemically, require very high concentrations to penetrate the BBB, thereby causing systemic toxicity. Certain agents such as Taxol and CPT have difficulty passing into the brain at tolerable systemic doses.[36,40] To treat brain tumors effectively with chemotherapy, we have developed a controlled-release polymer delivery system that is able to apply sustained high concentrations of antineoplastic agents directly to a brain tumor.[4] The feasibility of this approach has been demonstrated by delivering BCNU in polyanhydride polymers. We showed experimentally that BCNU delivered directly to the brain was safer and more effective than when administered systemically.[30] In two Phase III studies,[6,33] significantly improved survival has been demonstrated in patients when BCNU is delivered directly to the site of a craniotomy. The present study of CPT analogs was undertaken to improve upon these results, and to determine whether drugs such as CPTs, which cannot be effectively delivered systemically, would be of benefit against gliomas if administered in a polymer delivery form. Moreover, selected CPT analogs possess greater cytotoxic activity than does BCNU against malignant brain tumors. Because they act by topoisomerase I inhibition, there is the possibility of synergy if these drugs are used in combination with an alkylating agent such as BCNU.

We have previously shown that local delivery of Na-CPT effectively prolonged survival in a rat intracranial gliosarcoma model.[40] Nevertheless, Na-CPT is not an ideal drug to pursue for further clinical development for several reasons. First, Na-CPT was initially selected because its water solubility makes it easily incorporated into polymer matrices. The recently synthesized glycinated forms of 10,11-MD-20(S)-CPT analogs are also water soluble, making their incorporation into polymers equally feasible. Second, Na-CPT exists in equilibrium between the open (inactive) lactone form and the (active) closed ring form,[35] with the inactive form favored at a more alkaline pH. Because many

TABLE 5

The GI_{90} (nM) derived from a clonogenic assay for glioma cell lines treated with CPT analogs

Drug	Glioma Cell Line		
	9L	H80	U87
9-amino-10,11-MD-20(S)-CPT-Gly.HCl	8.82	4.77	7.88
10,11-MD-20(S)-CPT-Gly.HCl	8.8	5.89	0.87
10,11-MD-20(S)-CPT	8.79	5.91	9.01
Na-CPT	78.5	48.76	45.53
BCNU	>1000	>1000	>1000

malignant brain tumors exhibit tissue necrosis and high metabolic activity, which lower pH, the activity observed with Na-CPT may have been caused by local tissue conditions or by conversion to CPT during polymer preparation. A more stable CPT analog would be less unpredictable and it would possess reproducible pharmacokinetics. Third, as demonstrated in this study, both CPT and Na-CPT are markedly less cytotoxic than are the 10,11-(MD)-CPT derivatives. Theoretically, if a more potent drug were to be incorporated into a polymer, therapeutic drug concentrations in the same time period posttreatment might be detectable farther away from the polymer, enhancing the antitumor effect.

Of all 22 CPT analogs tested, the 10,11-MD analogs, 9-amino,10,11-MD-20(S)-CPT-Gly.HCl, 10,11-MD-20(S)-CPT-Gly.HCl, and 7-ethyl,10,11-MD-20(S)-CPT, had the most consistent antiproliferative activity in all three cell lines. The 10,11-MD-20(S)-CPT analogs are synthesized by substitutions at the 10,11 carbons on the A ring of the parent compound. Previous work has shown that both an intact lactone ring and the S stereoisomer of the 20-hydroxyl group are essential for activity.[38,39] Our results confirm the relative inactivity of the R stereoisomer (Drug 18). Furthermore, CPT-11 (Drug 8) shows limited activity against malignant gliomas, which was expected because this compound is a prodrug, requiring metabolism by the liver to 7-ethyl,10-hydroxy-CPT (SN-38), for activity.[15] In contrast, the glycinate esters of 10,11-MD (Compounds 11, 12, and 16), which are also prodrugs, do not require enzymatic intervention for hydrolysis. These glycinate esters are slowly hydrolyzed at physiological pH (unpublished data). Interestingly, topotecan (Drug 9) also shows limited cytotoxicity.

When we examined the cytotoxicities of the three most potent 10,11-(MD) compounds, CPT, Na-CPT, and 10,11-MD-20(RS)-CPT, against the U87 cell line (Fig. 2), we found that viability drops exponentially with time for each compound: a 1-hour exposure yielded an LD_{90} approximately 100-fold greater than a 72-hour exposure. A similar time dependence was also observed with the 9L and H80 cell lines (Table 4). Thus, local polymeric delivery, which provides a very high local dose of drug at the tumor site for prolonged periods of time, should be particularly advantageous for delivery of CPT analogs. Although CPT and Na-CPT were much less active than the 10,11-MD-20(S) and 10,11-MD-20(RS) analogs (Fig. 2 *upper*), the activities of the four 10,11-MD derivatives were roughly equivalent at all exposure times (Fig. 2 *lower*). We demonstrate (Fig. 2 *lower* and Table 4), however, that the pure S stereoisomer 10,11-MD-20(S)-CPT had a greater antiproliferative activity than did the RS form 10,11-MD-20(RS)-CPT, with an LD_{90} roughly 50% that of its racemic form at every exposure time for the U87 and H80 glioma lines. This was expected, given that the R stereoisomer has been demonstrated previously,[39] and was shown in this study (Drug 18), to be markedly less active.

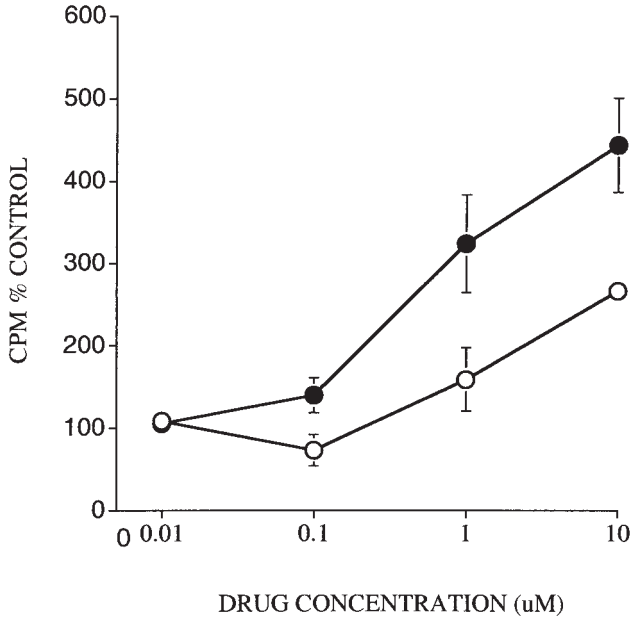


FIG. 3. Graph showing stabilized cleavable complex formation in the human glioma cell line H80 after treatment with Na-CPT *(white circles)* or 10,11-MD-20(RS)-CPT *(black circles)*.

Because of interest in using the active CPT analogs for polymeric delivery, we directly compared the effects of the 10,11-MD CPT analogs with those of Na-CPT, which was previously shown to be effective in a brain tumor model, and with the currently used BCNU polymer. We used a clonogenic assay for this comparison because it not only more closely resembles in vivo efficacy, but it also provides extended drug exposure (as would polymeric delivery) and measures cell proliferative capacity, not simply viability. We found an order of magnitude increase in antiproliferative effect (expressed as GI_{90}) in the three 10,11-MD-20(S)-CPT derivatives when compared with Na-CPT; the effect was several orders of magnitude higher than that of BCNU in all three cell lines (Table 5).

In an effort to elucidate CPT's antiproliferative mechanism of action in gliomas and to explain the differential activities of the analogs, we quantified CPT-induced topoisomerase I DNA-cleavable complexes in whole H80 cells after exposure to various concentrations of Na-CPT and 10,11-MD-20(RS)-CPT. The mechanism of action of CPT and its derivatives is thought to involve inhibition of topoisomerase I; specifically, it is thought to be the result of the collision between CPT-induced cleavable complexes and moving replication forks.[11,12] Of the cell properties that have been examined in attempts to determine what affects differential CPT action, including topoisomerase I messenger RNA levels, topoisomerase I protein levels, and CPT uptake, CPT-induced topoisomerase I–cleavable complex levels have most consistently correlated with increased CPT cytotoxicity.[9,23]

We found a dose-dependent increase in cleavable complexes and also increased cleavable complex levels at every concentration for 10,11-MD-20(RS)-CPT when compared with Na-CPT (Fig. 3), indicating that increased cytotoxic activity of the 10,11-MD analogs in gliomas might be due to their increased ability to stabilize reversible covalent topoisomerase I DNA complexes. The replication collision model indicates that the cytotoxicity of topoisomerase I inhibitors is linked not so much to drug concentration as to the time of exposure.[11,12,23] The topoisomerase I DNA-cleavable complexes are reversible and are repaired after CPT removal.[7] Therefore, a sufficiently long exposure time is essential for a significant number of collisions to occur between the replication forks and drug-stabilized cleavable complexes, converting them to potentially lethal DNA double-strand breaks. This indicates superior cytotoxicity with continuous drug exposure, because topoisomerase I DNA-cleavable complexes cannot be repaired. This is consistent with our finding that prolonged exposure to CPT analogs leads to enhanced cytotoxicity, and further strengthens the rationale for pursuing polymeric delivery rather than bolus infusion of these drugs.

Conclusions

We find that increased potency of CPT analogs may hold great promise for more effective local treatments against intracranial brain tumors. Our results demonstrate that 10, 11-MD CPTs exhibit greater cytotoxicity than other CPT analogs, BCNU, or Na-CPT. Prolonged exposure to these agents provides greater antitumor effects than shorter exposure times. This is borne out not only in direct measurement of cell growth, but also by the fact that CPT analogs exert their cytotoxic effect through the formation of DNA-cleavable complexes in gliomas. Therefore, 10,11-MD-20(S)-CPT derivatives may be ideal candidate drugs for future development using polymeric technology against both primary and metastatic brain tumors. Further in vivo preclinical investigation is warranted.

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