ORIGINAL ARTICLE

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Camptothecin analogs with enhanced activity against human breast cancer cells. I. Correlation of potency with lipophilicity and persistence in the cleavage complex

Abstract The effect of 7-alkyl substitutions on growth inhibition in seven Camptothecin (CPT) ring systems with various groups at the ten position was evaluated in three human breast cancer cell lines that model (1) hormone-sensitive (MCF-7/wt), (2) hormone insensitive (MDA-MB-231), or (3) alkylator-resistant (MCF-7/4-hc) forms of disease. To assess the impact of persistence of cleavage complexes on antiproliferative activity, a post-exposure recovery period in drug-free medium was incorporated into the growth inhibition assay. This modification produced on average a twofold reduction in the growth inhibition endpoint (the IC50), suggesting a greater apoptotic response. The results further revealed a three log range in potency from a mean IC_{50} of 2 nM (7-butyl-10,11-methylenedioxy-CPT) to 2.5 μM (7-bromomethyl-10-hydryoxy-CPT). Increasing 7-alkyl chain length in six of the ten-substituted CPTs enhanced potency, which was directly correlated with persistence of topoisomerase I-induced DNA cleavage complexes in 10-hydroxy, 10-methoxy, and 10,11-methylenedioxy substituted CPTs. Modeling of the binding mode of 7-butyl-10-amino-CPT revealed a direct hydrogen bond contact for the 10-amino to the side chain of Glu-356 of Core Subdomain I of top1 in addition to known contacts found for other camptothecins. More important, residues 350–356 and 425–431 of Core Subdomain I may provide induced fit stabilization to the lipophilic alkyl moiety at the seven position.

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Introduction

Since the initial discovery of camptothecin by Wall et al. [26], and the elucidation of its mechanism of action [7, 9], many modifications have been made to the molecule. Modifications that improved water solubility were the primary advance that led to the first clinically useful Camptothecin (CPT) analogs, topotecan and irinotecan. Improved potency and cross-resistance profile, particularly as related to drug efflux pumps have become more recent objectives. In addition, investigators have sought to understand the connection between potency of CPT's and the stability of the cleavage complexes they create when bound to the target enzyme, topoisomerase I (top1) and DNA. Engineering greater tumor selectivity into the molecule has received relatively less attention. Instead new CPT analogs still rely for selectivity on the fact that certain human tumors tend to overexpress top1 compared to normal tissues [5, 6, 8, 17, 23]. We evaluated potency

of 39 CPT analogs against three human breast cancer cell lines and determined the effect of these structural changes on the persistence of cleavage complexes. Our results indicate that seven substitutions at C-10 as well as increasing lipophilicity at C-7 enhance potency in vitro, and that these effects correlate with increasing persistence of cleavage complexes in three of the ten-substituted series tested.

Materials and methods

Synthesis of 7-alkyl camptothecin analogs

7-alkyl derivatives of CPT, 10,11-MD, 10-OH-, 10,11-ED-, 10-OMe-, and 10,11-DFMD-CPT were all prepared by following the procedure of Sawada et al. [20]. The 7-methyl derivatives were prepared in 70–80% yield by the reaction of the appropriate CPT analog with methanol in the presence of tert-butyl hydroperoxide and ferrous sulfate in an aqueous acetic acid-sulfuric acid solution. The rest of the 7-alkyl-CPT analogs were made by treating the appropriate CPT analog with an aldehyde (propionaldehyde for 7-ethyl analogs, butyraldehyde for 7-propyl analogs, and valeraldehyde for 7-butyl analogs) in the presence of hydrogen peroxide, ferrous sulfate in aqueous sulfuric acid solution. The yields were higher for 7-ethyl analogs (75–80%) and decreased with increase in chain length [e.g., 7-propyl (55%); 7-butyl (30%)]. In the case of 7-alkyl-10-amino-CPT analogs, a slightly modified procedure was employed. First, the appropriate 10-nitro-CPT analog was synthesized by a known procedure [27], which was then converted to the corresponding 7-alkyl-10-nitro-CPT derivative by the procedure of Sawada et al. [20]. Finally the corresponding 10-amino analog was prepared by reduction with $SnCl_2$ and HCl [27]. Identity and purity of all analogs was confirmed by mass spectroscopy and high performance liquid chromatography, with purity exceeding 95%.

Cell culture

The MCF-7/wt human breast cancer cell line was obtained from the Cell Resources Core of the Barbara Ann Karmanos Cancer Institute (Detroit, MI, USA). MCF-7/hc cells were obtained from Dr. Beverly Teicher (Genzyme Corp, Boston, MA, USA), while MDA-MB-231 cells were purchased from the American Type Culture Collection (Rockville, MD, USA). All cell lines were grown in Dulbecco's Modified Eagle's Medium, Low Glucose (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 2 g/l glucose, 3.7 g sodium bicarbonate, 25 mM HEPES (GIBCO, Grand Island, NY, USA), and 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA). All incubations were at 37°C in a humidified atmosphere containing 5% CO_2/95% air.

Growth inhibition assay

Propidium iodide (Molecular Probes, Eugene, OR, USA) binding to nucleic acids was used to assess growth inhibitory activity of camptothecin analogs as described previously [3]. Breast tumor cells were seeded in black 96-well microplates (Falcon/Becton Dickinson, Franklin Lakes, NJ, USA), and incubated for 18–24 h. Harvesting cells and counting isolated nuclei determined plating efficiency and subsequent growth kinetics in each experiment. Cells were exposed to cytotoxic agents for two cell doublings, then the medium was replaced with fresh growth medium and the cells were allowed to recover for an additional population doubling. This growth kinetic-normalized exposure and recovery procedure allowed full expression of both cell cycle-dependent cytotoxicity and persistence of that toxicity under drug-free culture conditions. Following agent exposure, the media was removed and cells were fixed with cold 70% ethanol for 18–24 h at 4°C. The ethanol was then aspirated, 100 µl of propidium iodide solution (50 µg/ml in H_2O) was added, and the fluorescence was quantified in a FL-600 microplate fluorescence reader (Bio-Tek, Winooski, VT, USA) at 530/590 nm excitation/emission wavelengths, respectively. The data was processed with KC4 software (BioTek), and expressed in relative fluorescence units (RFU). Dose response curves were constructed and the respective IC_{50}'s computed using TableCurve 2D software (SPSS, Chicago, IL, USA). The IC_{50} was defined as the concentration of the agent required to reduce fluorescence by 50% compared to untreated controls. Statistical analysis of the resulting data was performed using JMP Statistical Discovery software (SAS Institute Inc., Cary, NC, USA).

Oligonucleotide religation assay

Recombinant DNA top1 was produced in baculovirus as described [18]. The oligonucleotide religation assay was the one that was employed previously [14, 15, 18, 22]. Briefly, a duplex oligonucleotide (see Fig. 6) was 3′-end labeled with $\alpha-^{32}P$-cordycepin (New England Nuclear, Boston, MA, USA), and reacted with human top1 in the presence and absence of drug at 25°C. After 15 min, reactions were either stopped directly with 0.5% sodium dodecyl sulfate (SDS, Sigma Chemical Co.) or treated with 0.35 M NaCl for the indicated times at 25°C to force top1 religation, and then stopped with 0.5% SDS. Loading buffer (89 mM Tris–HCl, pH 8.0, 89 mM boric acid, 2 mM EDTA) was then added to the samples, which were electrophoresed into 16% polyacrylamide gels containing 7 M urea at 40 V/cm, 50°C, for 2–3 h. Imaging and quantitation were performed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA).

Molecular modeling

Molecular modeling was performed with the commercially available modules on Insight II 2000 (Insight, San

Diego, CA, USA). All of the computational work was performed on a Silicon Graphics O2 workstation (R120000). Partial atomic charges for BACPT were calculated using the semi-empirical program MOPAC under Insight II. They were energy minimized on cvff force field with a distance-dependent dielectric and the Powell conjugate gradient algorithm, with a convergent criterion of 0.001 Kcal/(mole*A). The aromatic skeleton in the lactone form of BACPT was superposed to that of topotecan in the 2.1 Å refined X-ray crystal structure of the ternary complex of topotecan/topo1/DNA [21]. The disposition of the A-ring was adjusted keeping BACPT in the same plane as TPT and minimizing any steric interactions. The final orientation of the A-ring of BACPT deviates very little from that of topotecan. The contacts for TPT were kept for the E-ring of BACPT.

Results

Potency is increased with post-exposure incubation in drug-free medium

The CPT analogs whose syntheses and structure are summarized in Fig. 1 were evaluated in three human breast cancer cell lines that represent hormone-sensitive (MCF-7/wt), hormone-insensitive (MDA-MB-231), and alkylator-resistant forms of the disease (MCF-7/hc). To reveal the impact of persistence of CPT analogs in the cleavage complex on growth inhibition, we incorporated a post-exposure incubation period in drug-free medium to provide time for CPT's to exit the cleavage complex, for the complex to be resolved by ubiquitination and subsequent proteosomal degradation, and for cells to repair any associated DNA damage. Alternatively, highly persistent complexes and resulting DNA strand breaks could lead to initiation of apoptosis. The results shown in Fig. 2 reveal that inclusion of such a "recovery" period equal to one cell doubling time uniformly lowered the IC50 endpoint by an average of twofold compared to cells assayed immediately following drug exposure with no recovery period. The effect was statistically significant, occurred in each cell line and could be as high as 4.5-fold. Hence, this assay format was chosen to evaluate the effect of 7-alkyl chain length on
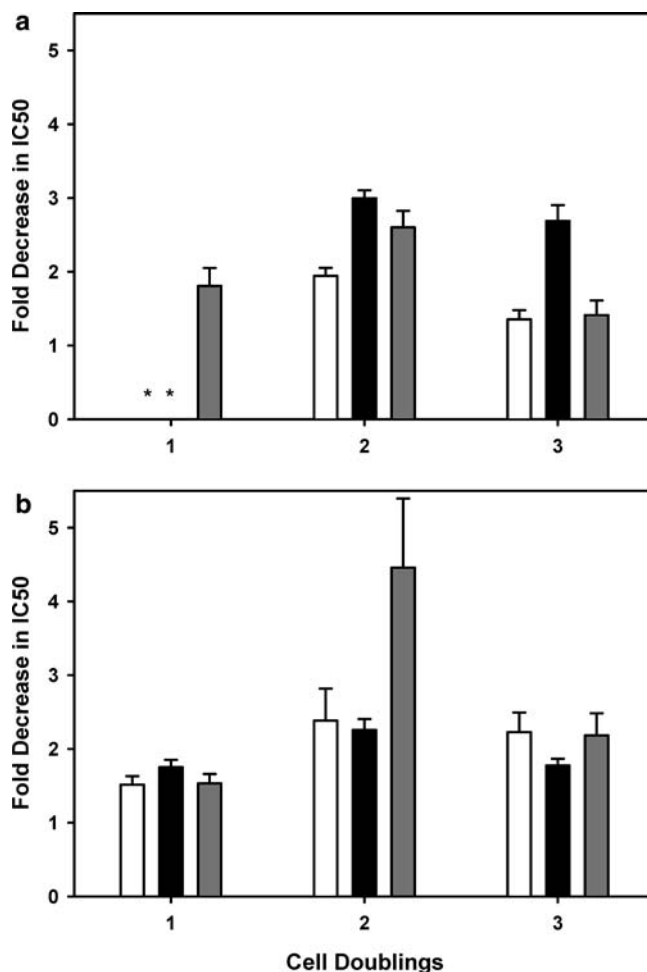
Fig. 2 Effect of post-exposure incubation in drug-free medium on antiproliferative activity. **a** MCF-7/wt (*open bars*), MDA-MB-231 (*black*), and MCF-7/hc (*gray*) cells were exposed to varying concentrations of 7-ethyl-10-hydroxy-CPT (SN-38) at physiological pH for one, two or three cell doubling times. One set of samples was then immediately fixed and stained with propidium idiode as described in Methods for growth inhibition assay (set 1). Replicate samples were switched to drug-free medium and incubated for an additional cell doubling time prior to assay (set 2). The IC50 concentrations were determined and the IC50 from set 1 divided by that from set 2. *Error bar* is the standard error of the mean from three replicate experiments. *Asterisks* indicate an IC50 that was not achieved in the concentration range examined. **b** The study in panel **a** was repeated for the CPT analog 10,11-methylenedioxy-CPT(20S) glycinate ester. The mean fold decrease in IC50 for both analogs in all three cell lines was 2.2 ± 0.2 (range 1.4–4.5)

Fig. 1 Synthesis and structure of camptothecin analogs. Structures and an abbreviated synthetic route are shown for the CPT analogs used in this study. Details of syntheses are in Materials and methods and in associated references
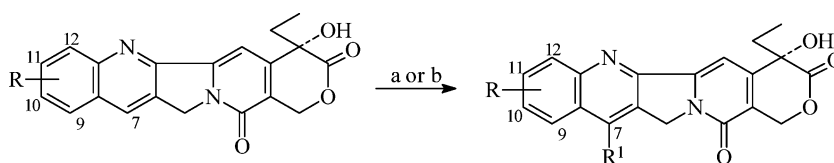
R = H, 10,11-OCH$_2$O-, 10,11-OCF$_2$O-, 10,11-O-CH$_2$-CH$_2$-O-, 10-NO$_2$, 10-OH

R^1 = CH$_3$, C$_2$H$_5$, n-C$_3$H$_7$, n-C$_4$H$_9$

a = CH$_3$OH (for 7-CH$_3$ analogs), t-butyl hydroperoxide, FeSO$_4$, aqueous AcOH-H$_2$SO$_4$

b = appropriate aldehyde (C$_2$H$_5$-CHO for 7-C$_2$H$_5$, n-C$_3$H$_7$-CHO for 7-n-C$_3$H$_7$, n-C$_4$H$_9$-CHO for 7-n-C$_4$H$_9$ analogs); H$_2$O$_2$, FeSO$_4$, aqueous H$_2$SO$_4$

potency and persistence of CPT analogs in the top1-DNA cleavage complex.

Substitutions at the 10 position and increasing 7-alkyl chain length increase potency

Table 1 shows the effects of substitution at the seven and ten positions on antiproliferative activity. The results confirm that this is an activating position, since potency increased from 10- to 60-fold for the seven substituents compared to CPT itself. On average, for the three cell lines, the 10,11-methylenedioxy- and 10,11-difluoro-methylenedioxy-CPT analogs were the most potent with the rank order of activities: 10,11-MD > 10,11-DFMD > 10,11-ED > 10-MeO ≥ 10-OH > 10-NH2 > > CPT.

Increasing lipophilicity at the seven position also enhanced antiproliferative activity in the breast cancer cell lines. We observed a linear increase in activity when the 7-alkyl chain length was varied from one to four carbons in six of the 10-substituted CPT analogs with a median slope = 0.68, and median linear regression correlation coefficient of 0.86 (Fig. 3). On average, CPT

itself was the most potentiated (up to 14-fold). The IC_{50}'s spanned 2.7 logs at pH 7.4, ranging from 1 nM for 7-butyl-10,11-MD-CPT in MCF-7/wt cells to 2 μM for CPT in MDA-MB-231 cells (Table 1). Similar results were observed when the breast cancer cell lines were adapted to growth at acidic pH, which better reflects the solid tumor microenvironment in vivo (see companion paper). In contrast to 7-alkyl substitutions, addition of a 7-halomethyl group on 10-substituted CPTs decreased potency at pH 7.4 on average by 10 (10,11-DFMD) to 37 (10-OH)-fold in the breast cancer cell lines, with the effect somewhat more prominent for bromine versus chlorine analogs (Table 2).

Comparison of CPT analog activity in individual human breast cancer cell lines

To determine whether any of the 7- and 10-substituted CPTs had preferential activity in breast cancer cells as a function of steroid hormone receptor status or resistance to the alkylating agent 4-hydroperoxy-cyclophospha-mide (4-HC), we applied a modification of the mean graph approach [13]. We compared the growth

Table 1 Potency of 10-substituted and 7-alkyl-substituted CPT analogs against human breast cancer cells in vitro

Ring system	7-Alkyl	IC_{50} (nM)					
		MDA-MB-231	Fold[a]	MCF-7/wt	Fold[a]	MCF-7/hc	Fold[a]
CPT	[None]	1821 ± 106	1	587 ± 43	1	1021 ± 51	1
	Methyl	326 ± 56	6	98 ± 4	6	109 ± 16	9
	Ethyl	301 ± 24	6	79 ± 4	7	121 ± 10	8
	Propyl	104 ± 19	18	66 ± 3	9	85 ± 6	12
	Butyl	129 ± 10	14	41 ± 4	14	69 ± 4	15
10,11-MD	[None]	27 ± 6	67	17 ± 1	35	14 ± 1	73
	Methyl	11 ± 1	166	10 ± 1	59	6 ± 0	170
	Ethyl	6 ± 0	304	6 ± 0	98	5 ± 0	204
	Propyl	4 ± 0	455	4 ± 1	147	3 ± 1	340
	Butyl	4 ± 1	455	1 ± 0	587	2 ± 0	511
10-OH	[None]	191 ± 15	10	39 ± 2	15	20 ± 2	51
	Methyl	129 ± 8	14	21 ± 1	28	22 ± 2	46
(SN-38)	Ethyl	94 ± 16	19	10 ± 1	59	14 ± 4	73
	Propyl	77 ± 20	24	10 ± 1	59	16 ± 1	64
	Butyl	66 ± 4	28	7 ± 0	84	10 ± 2	102
10,11-ED	[None]	53 ± 6	34	34 ± 3	17	13 ± 4	79
	Methyl	40 ± 4	46	28 ± 2	21	9 ± 1	113
	Ethyl	20 ± 1	91	22 ± 2	27	8 ± 1	128
	Propyl	18 ± 3	101	20 ± 1	29	8 ± 1	128
	Butyl	18 ± 4	101	14 ± 1	42	5 ± 1	204
10-NH2	[None]	203 ± 17	9	106 ± 7	6	71 ± 4	14
	Methyl	189 ± 24	10	85 ± 4	7	64 ± 5	16
	Ethyl	150 ± 14	12	81 ± 5	7	60 ± 5	17
	Propyl	124 ± 10	15	78 ± 7	8	44 ± 3	23
	Butyl	89 ± 15	20	50 ± 3	12	30 ± 5	34
10-OMe	[None]	67 ± 4	27	51 ± 7	12	23 ± 1	44
	Methyl	50 ± 7	36	40 ± 5	15	19 ± 2	54
	Ethyl	45 ± 8	40	38 ± 3	15	21 ± 1	49
	Propyl	39 ± 5	47	30 ± 1	20	11 ± 2	93
	Butyl	30 ± 5	61	15 ± 2	39	5 ± 1	204
10,11-DFMD	[None]	78 ± 2	23	9 ± 0	65	17 ± 1	60
	Ethyl	87 ± 3	21	13 ± 1	45	25 ± 3	41

[a]Fold increase in growth inhibitory activity vs CPT defined as IC50 $_{CPT}$/IC50 $_{analog}$. Cells were exposed to agents for two cell doublings, following by one cell doubling in drug-free medium before assay

Fig. 3 Correlation of 7-alkyl chain length to antiproliferative activity in human breast cancer cell lines. Breast tumor cell lines that had been adapted to growth at pH 6.8 or cultured normally at pH 7.4 were exposed to 10-substituted CPT analogs with varying 7-alkyl groups for two cell doublings, then switched to fresh, analog-free growth medium for an additional cell doubling. Growth inhibition was assessed by propidium idodide staining of nucleic acids and the IC_{50} determined as described in Materials and methods. The fold increase in activity (decrease in IC_{50}) was then plotted as a function of 7-alkyl chain length for each series, including CPT (→), 10,11-MD-CPT (*circle*), 10-OH-CPT (*inverted triangle*), 10,11-ED-CPT (*square*), 10-NH2-CPT (*up triangle*), and 10-MeO-CPT (*octagon*)

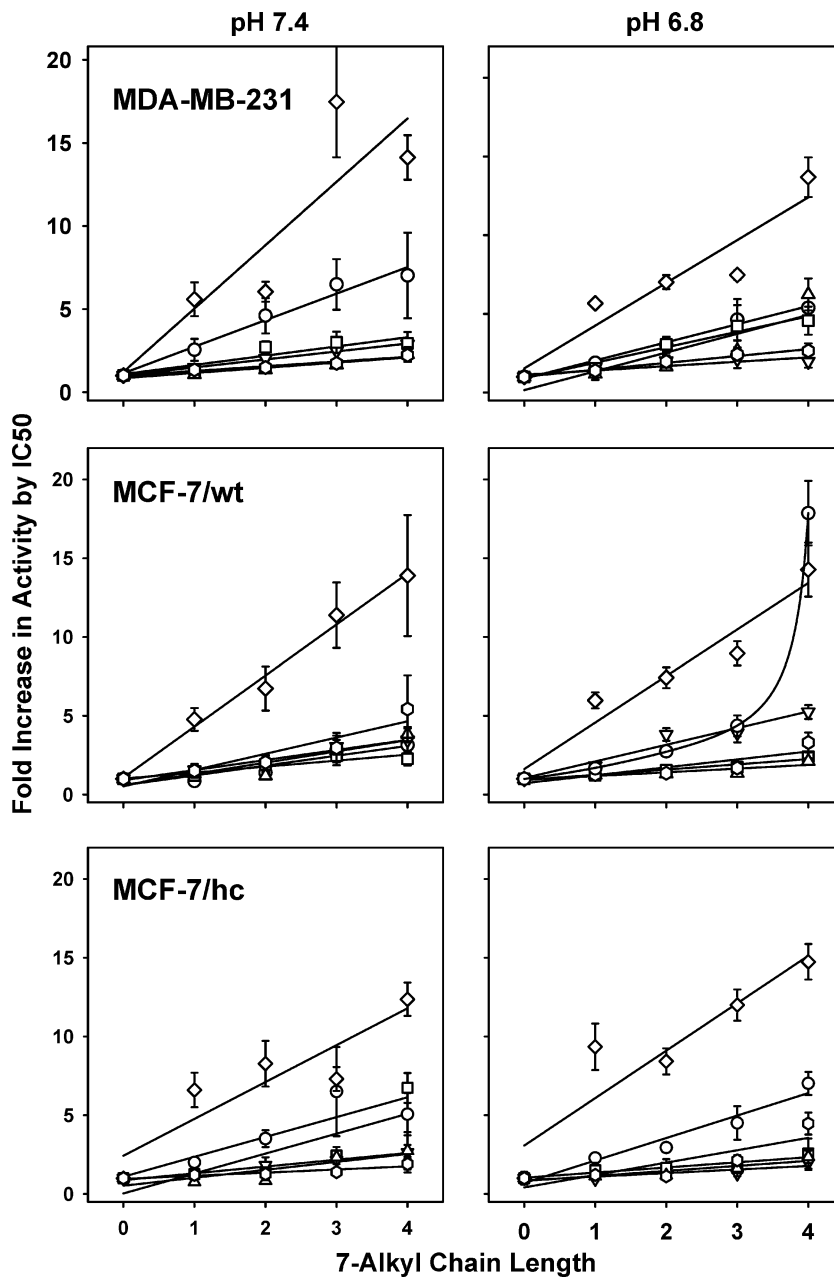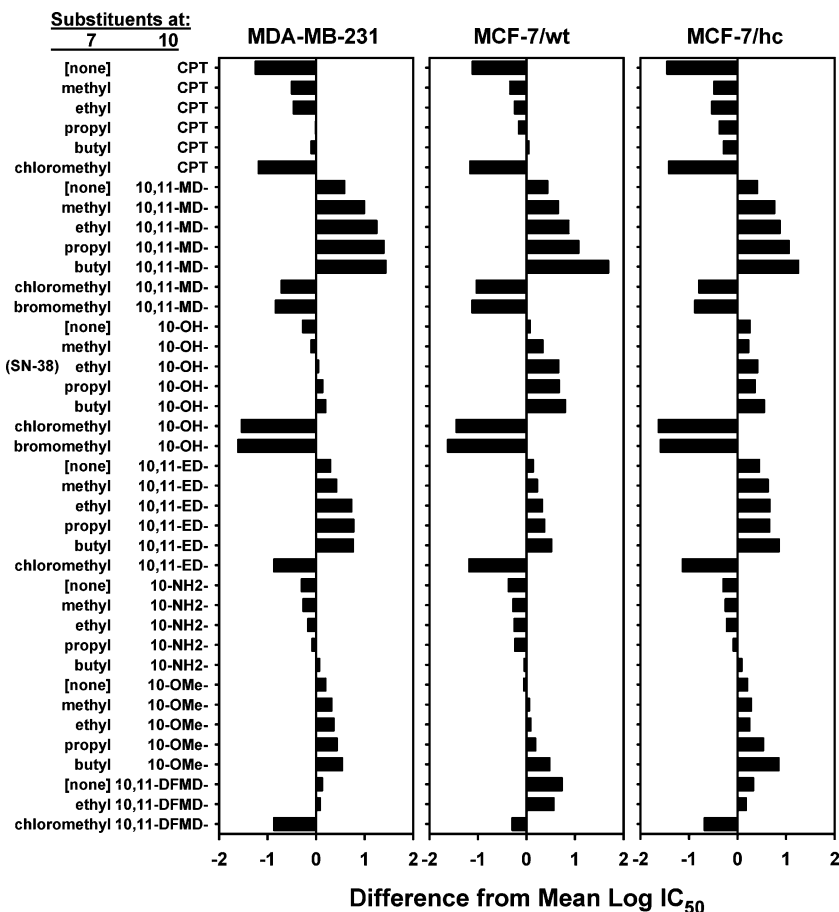**Table 2** Potency of 7-halomethyl-substituted CPT analogs against human breast cancer cells in vitro

Ring system	7-Halogen	IC_{50} (nM)		
		MDA-MB-231	MCF-7/wt	MCF-7/hc
CPT	Chloro	$1,561 \pm 73$	658 ± 40	929 ± 31
10,11-MD	Chloro	533 ± 24	486 ± 22	225 ± 13
10,11-MD	Bromo	701 ± 39	591 ± 47	272 ± 22
10-OH	Chloro	$3,499 \pm 176$	$1,259 \pm 173$	$1,524 \pm 124$
10-OH	Bromo	$4,160 \pm 124$	$1,894 \pm 130$	$1,387 \pm 149$
10,11-ED	Chloro	761 ± 39	688 ± 22	488 ± 18
10,11-DFMD	Chloro	759 ± 32	88 ± 4	172 ± 7

inhibitory activity of each analog to that of the mean activity for all analogs within each individual cell line (Fig. 4). Analogs with relatively higher activity are shown as horizontal bars to the right of the mean value and vice versa. Overall, data from the mean graphs indicated that the pattern of response to CPT analogs

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Fig. 4 Comparison of CPT analog activity in individual human breast cancer cell lines. CPT analog activity was compared across three breast tumor cell lines that represent steroid hormone-sensitive, hormone-insensitive, and alkylator-resistant forms of the disease by in vitro growth inhibition assay as described in Materials and methods. The results are presented in a mean graph format. The mean log IC_{50} was determined for the 39 analogs, then the individual agent log IC_{50} was subtracted from the mean value. The difference was plotted relative to the mean ($= 0$), such that analogs with greater than average activity in a particular cell line produce bars to the right of zero and those with less activity to the left. Results represent the average of 3–5 experiments per cell line

Substituents at:		MDA-MB-231	MCF-7/wt	MCF-7/hc
7	10			
[none]	CPT			
methyl	CPT			
ethyl	CPT			
propyl	CPT			
butyl	CPT			
chloromethyl	CPT			
[none]	10,11-MD-			
methyl	10,11-MD-			
ethyl	10,11-MD-			
propyl	10,11-MD-			
butyl	10,11-MD-			
chloromethyl	10,11-MD-			
bromomethyl	10,11-MD-			
[none]	10-OH-			
methyl	10-OH-			
(SN-38) ethyl	10-OH-			
propyl	10-OH-			
butyl	10-OH-			
chloromethyl	10-OH-			
bromomethyl	10-OH-			
[none]	10,11-ED-			
methyl	10,11-ED-			
ethyl	10,11-ED-			
propyl	10,11-ED-			
butyl	10,11-ED-			
chloromethyl	10,11-ED-			
[none]	10-NH2-			
methyl	10-NH2-			
ethyl	10-NH2-			
propyl	10-NH2-			
butyl	10-NH2-			
[none]	10-OMe-			
methyl	10-OMe-			
ethyl	10-OMe-			
propyl	10-OMe-			
butyl	10-OMe-			
[none] 10,11-DFMD-				
ethyl 10,11-DFMD-				
chloromethyl 10,11-DFMD-				

Difference from Mean Log IC_{50}

was highly correlated among the breast tumor cell lines with Pearson pairwise correlation coefficients exceeding 0.90. However, individual differences were observed. For example, sensitivity to the 10-hydroxy-CPTs differed among the cell lines, in the order of MCF-7/wt > MCF-7/hc > MDA-MB-231. In contrast, analogs in the 10,11-ED series tended to be more active in MDA-MB-231 and MCF-7/hc cells than in MCF-7/wt cells.

Increased 7-alkyl chain length correlates with increased persistence of DNA damage

Previous work has demonstrated that introduction of an alkylating group at the seven position in CPT produces slowly reversible and irreversible top1 cleavage complexes [14, 22]. We sought to determine whether the increased potency observed as a function of 7-alkyl chain length could be due to increased persistence of associated cleavage complexes. Results from oligonucleotide religation assays indicated that increasing lipophilicity at the seven position was equal if it is not more effective than an alkylating moiety at producing more stable complexes (Fig. 5). Analyses of the salt reversal half times for the 10-hydroxy, 10-methoxy, and 10,11-methylenedioxy substituted CPTs also indicated that

there was a direct correlation between 7-alkyl chain length and persistence of cleavage complexes (Fig. 6).

Binding mode of 7-butyl-10-amino-CPT to the cleavage complex

The finding that increasing 7-alkyl length correlates with the persistence of the cleavage complex led us to assess the binding mode of BACPT relative to recent structural studies of camptothecin-top1-DNA complexes. BACPT is of particular interest, because it also exhibits increased activity at the acidic extracellular pH common to many solid tumors (see companion paper), and is highly fluorescent. To define the binding mode of BACPT, we superposed its aromatic skeleton in the lactone form to that of topotecan in the 2.1 Å refined X-ray crystal structure of the ternary complex of topotecan/topo1/DNA [21]. BACPT intercalates at the site of DNA cleavage, forming base-stacking interactions with both the -1 (upstream) and $+1$ (downstream) base pairs. In Fig. 7a the orientation of BACPT is shown with base-stacking to the -1 base pair. The rest of the DNA molecule was deleted for clarity. BACPT is also stabilized by E-ring hydrogen bond contact with Asp-533, water-bridged contacts to the active site

Fig. 5 Stability of top1 cleavage complexes induced by seven-substituted camptothecin analogs. A 23-mer oligonucleotide was used as a substrate for top1. The sequence of this oligonucelotide, the top1 cleavage site, and the resultant cleavage product are shown at the *top*. Cleavage reactions were performed for 15 min at 25°C as described in Methods. An aliquot was then removed (reversal time zero) and 0.35 M NaCl was added for the times indicated before reactions were stopped by an addition of 0.5% sodium dodecyl sulfate. Samples were then separated by polyacrylamide gel electrophoresis and analyzed on a phosphoimager. The doublet observed for the 13-mer is due to the presence of some dATP in the commercial cordycepin, which results in the addition of another adenine nucleotide
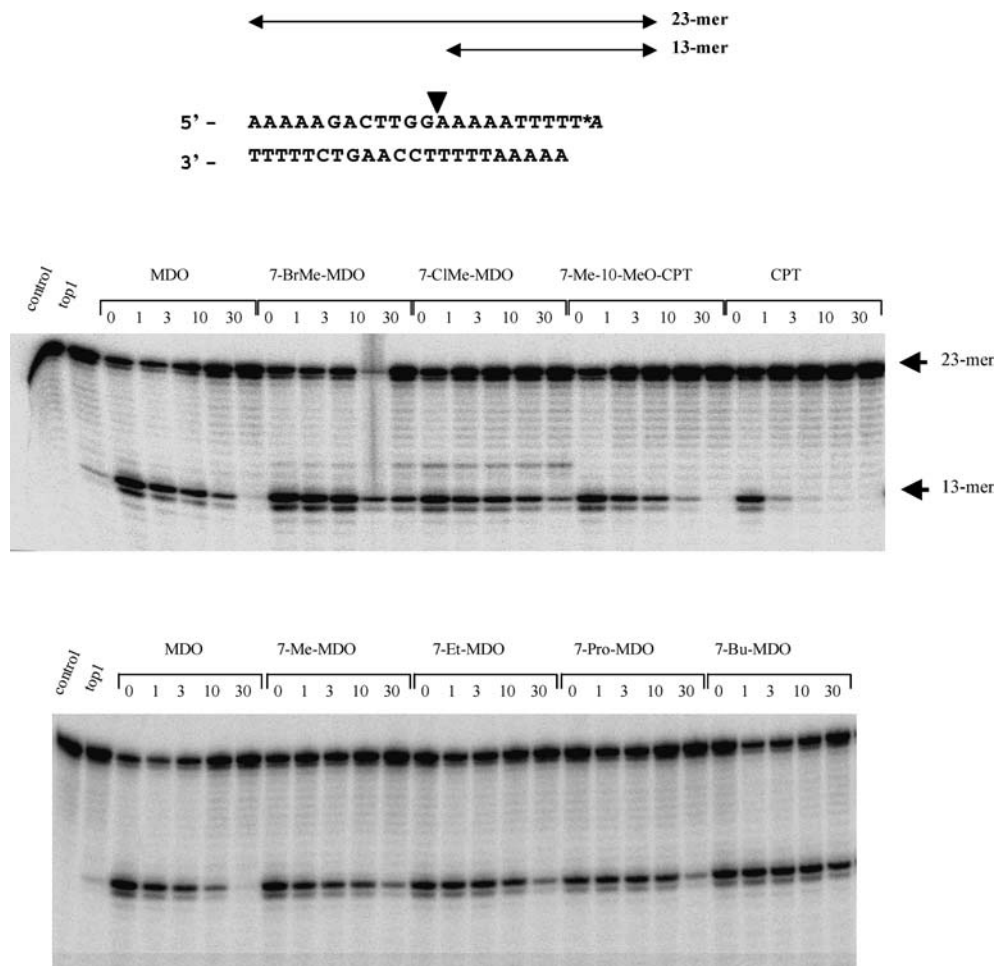
phosphotyrosine and Asn-722 as for topotecan. Notably, the A-ring 10-NH$_2$ forms a hydrogen bond to an oxygen atom of the side chain of Glu-356. Figure 7b includes the four Subdomains derived from the X-ray crystal structure of top1 [19], and reveals a lack of secondary structure in Subdomain I that can provide induced fit stabilization to the 7-alkyl chain. Indeed, the 7-butyl moiety of the lowest energy conformation of BACPT shows a very good steric fit to the structural environment provided by the side chain of Met428, the phenolic moiety of Tyr426, Pro431 and other residues (Fig. 7c).

Discussion

Work from several laboratories has indicated that the seven position in camptothecin is available for substitutions that modulate activity (reviewed in [11, 16]). Increasing lipophilicity at this position produces analogs with greater potency and stability in human plasma [2, 12, 24]. Similarly, certain substitutions at the 10 position increase potency of CPT [9, 10, 16, 25]. Our results are consistent with those of others, who found that C-10 in CPT can be an activating position and will tolerate small

substituents, while the seven-position appears to be open [9–11] and can tolerate bulky modifications, which are either hydrophilic or lipophilic. Moreover, extension of the CPT A ring with a methylenedioxy group significantly enhances activity [9, 14, 22].

Our results contrast with those of Vladu and coworkers [25], who evaluated 7-alkyl substitution in four of the ten-substituted analog series examined here, including CPT, 10-OH-CPT, 10-MeO-CPT and 10,11-MD-CPT. They found that the EC50 for inhibition of top1, the kinetics of reversibility of cleavage complexes and DNA-protein cross-link formation were only slightly dependent on 7-alkyl chain length. In addition, there was no effect of 7-alkyl chain length on potency in two human breast cancer cell lines, including the MDA-MB-231 line. These authors concluded that in vitro stability of the cleavage complexes did not correlate with in vitro biological activity, which more closely paralleled the concentration of analog required to produce cleavage complexes in plasmid DNA under steady-state conditions. Our conclusion that 7-alkyl chain length does in fact correlate with potency in six of the 10-substituted CPT analog series is likely due to the inclusion of a recovery period in our cytotoxicity assay. This recovery period allows capable cells time to efflux the

cytotoxic agent and repair any associated DNA damage, which permits better discrimination between CPT's that generate cleavage complexes with varying degrees of

persistence [1]. A similar approach has been used by others to study apoptotic events in cells exposed to anthracycline. Our results indicate that cytotoxicity increases with an additional incubation in drug-free
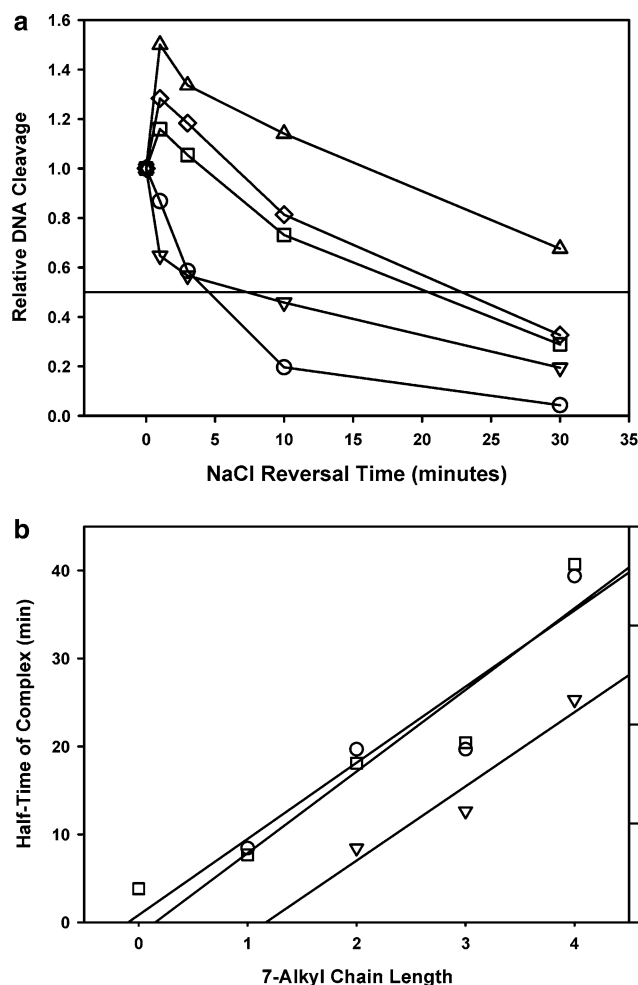

Fig. 6 Correlation of 7-alkyl chain length to persistence of DNA damage and salt reversal half time. **a** Data from Fig. 5 representing the major 13-mer cleavage product were quantified and plotted as a function of salt incubation time for 7-alkyl-substituted analogs of MD-CPT (*circle*), 7-methyl-MD-CPT (*inverse triangle*), 7-ethyl-MD-CPT (*square*), 7-propyl-MD-CPT (*diamond*), and 7-butyl-MD-CPT (*triangle*). Values were normalized relative to that at reversal time zero. **b** The time required for half of the complexes to religate was then plotted against the number of alkyl groups in each analog for the 10-OH-CPT (*circle*), 10-methoxy-CPT (*inverse triangle*), and 10,11-MD-CPT (*square*) series
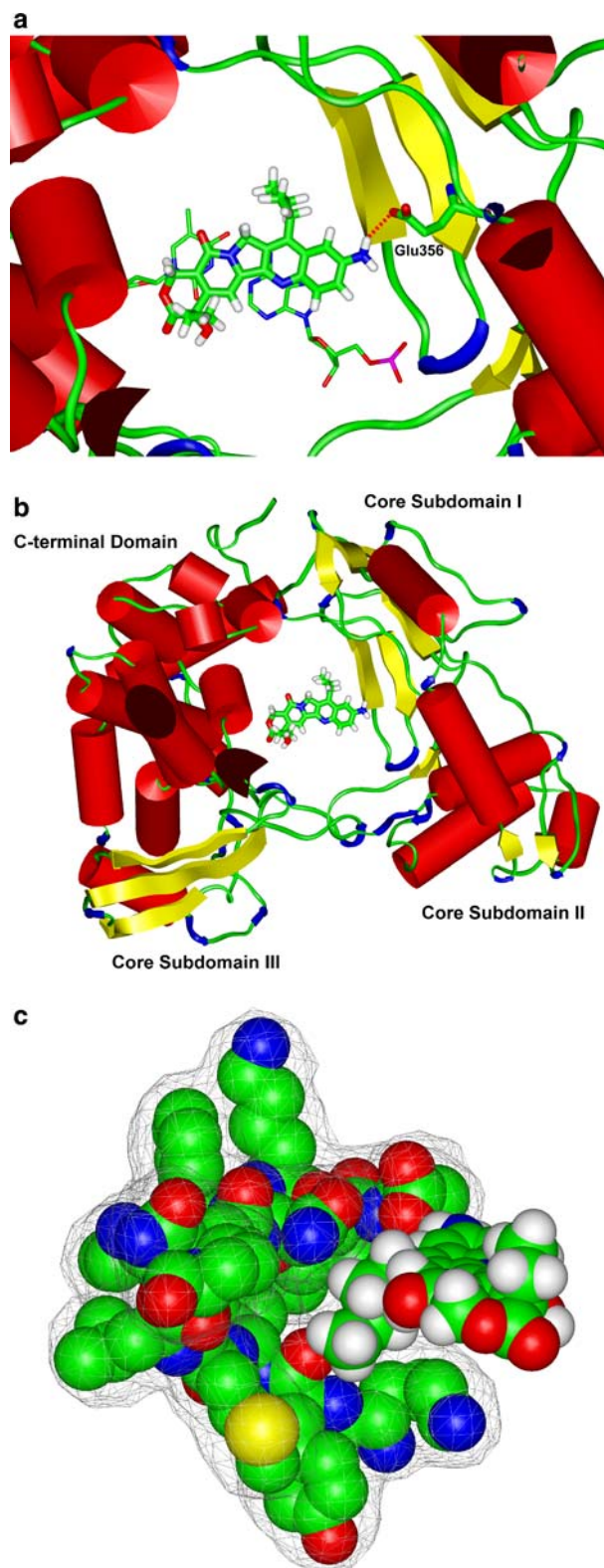
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Fig. 7 Binding mode of BACPT to the top1-DNA cleavage complex. **a** A view down the DNA stem of the accomodation of BACPT in the topo1/DNA complex. Depicted are the butyl moiety of the drug pointing into the major groove and the h-bond alignment of the 10-amino moiety and residue Glu-356 of Core Subdomain I of the enzyme. The figure also shows juxtaposition of BACPT to −1 base pair of the DNA stem. **b** Depiction of the orientation of BACPT within the secondary structural elements for the topo1/DNA complex. The DNA molecule was deleted for clarity. **c**. Depiction of the steric fit of the 7-butyl moiety of BACPT to residue regions 350 to 356 and 431 to 425 of the Core Subdomain I of topo1

medium, consistent with augmentation of the apoptotic response.

Correlation of 7-alkyl chain length with salt reversal half time in the religation assay is also at variance with the results of Vladu and colleagues. Wang and colleagues [28] used a salt-induced religation assay, and found no correlation between potency in inhibiting top1 and ability to stabilize cleavage complexes for a group of 9,10-disubstituted CPT analogs. Our recent results with 7-peptidyl-substituted CPTs [4] suggests that biological potency in this group also fails to correlate with stability of cleavage complexes, but since these analogs incorporate a charged, hydrophilic moiety, cell permeability may be a complicating factor. We would agree with the suggestion of Wang et al. that such structural modifications may change susceptibility to cellular efflux mechanisms or to interaction with other cellular targets or loci in addition to nuclear top1. Increasing lipophilicity by addition of alkyl groups at the seven position can increase persistence of several ten-substituted CPT analogs, a result that parallels an increase in antiproliferative activity against a variety of human breast cancer cell lines.

To provide a structural basis for the observed increase in persistence for 7-alkyl modifications, we substituted BACPT in the X-ray crystal structure of the ternary top1/DNA complex for TPT [21]. By superposing BACPT to TPT, all E-ring contacts were maintained. Asp-533 forms a hydrogen bond to the 20(S)-hydroxyl and water-bridged contacts to the active site phosphotyrosine and Asn-722. BACPT is further stabilized by a direct hydrogen bond with the enzyme. A hydrogen of the 10-amino moiety serves as hydrogen bond donor to an oxygen atom of the side chain of Glu-356. Thus, the planar ring system of the drug mimics a DNA base-pair and occupies the same space as the +1 base pair in the structure without drug bound. In addition, this orientation indicates that substitutions at position 7 adjust in the major groove of DNA and provide stabilization through hydrophobic interactions with residues in Core Subdomain I. Indeed, there is ample space for the accommodation of substituents, since Core Subdomain I is the least structured of the four (Fig. 7b). In fact, only the two lips of the clamp surrounding the DNA (residues 367 to 369 of Subdomain I and residues 497–499 of Subdomain II) show such stretches of random coil. Since most residues in Core Subdomain I do not fold into secondary structure they are appropriate to provide stabilization through induced fit. Specifically, residues 350–356 and 425–431 can provide induced fit stabilization to the 7-alkyl chain as exemplified by the 7-butyl moiety of BACPT. Although Tyr426 in this binding pocket is part of a small beta sheet, it is oriented in the crystal structure of the ternary top1/DNA/TPT so that its benzene ring provides hydrophobic stabilization of alkyl moieties. Thus, in general substitutions at the seven position of CPTs have ample space for accommodation and stabilization through induced fit. One can speculate that alkyl substituents at position 7 will also have the effect of orienting the CPT into a base pair mimicking orientation if bulky enough. Any other disposition of bulky groups other than major groove orientation would result in steric clashes within the DNA stem. This ternary complex model thus helps clarify the binding mode of 7-alkyl BACPT, and predicts that a point mutation at Glu356 could result in less persistence of the analog by removing an important stabilizing interaction.

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