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7- and 10-Substituted Camptothecins: Dependence of Topoisomerase I-DNA Cleavable Complex Formation and Stability on the 7- and 10-Substituents

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ABSTRACT

7-Alkyl, 7-alkyl-10-hydroxy, 7-alkyl-10-methoxy, and 7-alkyl-10,11-methylenedioxy analogs of camptothecin have been synthesized and evaluated for their ability to trap human DNA topoisomerase I in cleavable complexes. The 7-alkyl chain lengths varied linearly from methyl to butyl. The concentration required to produce cleavable complexes with purified topoisomerase I in 50% of the plasmid DNA (EC_{50}) was reduced by 1 order of magnitude by the introduction of a 10-methoxy or 7-alkyl group compared with camptothecin. The EC_{50} values were reduced by 2 orders of magnitude with a 10-hydroxy or 10,11-methylenedioxy moiety compared with camptothecin. The steady-state EC_{50} concentrations for all of the analogs tested were slightly dependent on substitution at the 7-position, but this dependence was least with the 10-methoxy series. The kinetics of the reversibility of the complexes formed with all analogs was only slightly influenced by the length of the 7-substitution, with the trend that ethyl or greater lengths led to slightly reduced rate constants for cleavable complex reversal. These results were also observed for DNA-protein cross-link formation by the analogs in isolated CEM cell nuclei. Our data indicate that in vitro cleavable complex stability, as determined by the apparent rate constants for complex dissociation, does not reflect the in vitro biological activity of these camptothecin analogs. However, complex stability in vivo may be important for the antitumor activity of the compounds.

In 1966, Wall et al. (1966) discovered that camptothecin (CPT; Fig. 1) was the component in the extract from the stem of the Chinese tree *Camptotheca acuminata* active against L1210 murine leukemia cells. Early clinical trials with the sodium salt of CPT in the late 1960s showed that this plant alkaloid had activity against a variety of solid tumors (Gottlieb et al., 1970; Creaven et al., 1972; Muggia et al., 1972). However, further clinical trials were discontinued because of unpredictable and severe myelosuppression, gastrointestinal toxicity, and hemorrhagic cystitis.

Renewed interest in CPT was stimulated by the characterization of CPT as a specific topoisomerase (topo) I inhibitor (Hsiang et al., 1985, 1989). Topo I relaxes DNA supercoiling by making transient single-strand breaks (Champoux, 1990; Wang, 1991). These breaks are coupled with the transient formation of a covalent DNA-enzyme intermediate termed the cleavable complex (Hsiang et al., 1985, 1989). CPT and analogs specifically and reversibly stabilize cleavable complexes by inhibiting their religation [reviewed in Chen and Liu (1994) and Pommier et al. (1998)]. The mechanism of CPT cytotoxicity is thought to be the consequence of a collision between moving replication forks and CPT-stabilized cleavable DNA-topo I complexes (Holm et al., 1989; Hsiang et al., 1989; Pommier et al., 1994).

There are now several topo I inhibitors at various stages of clinical development. One of these, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-CPT (CPT-11; irinotecan), has recently been approved by the U.S. Food and Drug Ad-

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ABBREVIATIONS: CPT, camptothecin; topo, topoisomerase; OHC2CPT, 7-ethyl-10-hydroxycamptothecin; CPT-11, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin; MDCPT, 10,11-methylenedioxycamptothecin; CMMD, 7-chloromethyl-10,11-methylenedioxycamptothecin; OHCPT, 10-hydroxycamptothecin; OMeCPT, 10-methoxycamptothecin; C1CPT, 7-methylcamptothecin; C2CPT, 7-ethylcamptothecin; C3CPT, 7-propylcamptothecin; C4CPT, 7-butylcamptothecin; OHC1CPT, 7-methyl-10-hydroxycamptothecin; OHC3CPT, 7-propyl-10-hydroxycamptothecin; OHC4CPT, 7-butyl-10-hydroxycamptothecin; OMeC1CPT, 7-methyl-10-methoxycamptothecin; OMeC2CPT, 7-ethyl-10-methoxycamptothecin; OMeC3CPT, 7-propyl-10-methoxycamptothecin; OMeC4CPT, 7-butyl-10-methoxycamptothecin; MDC1CPT, 7-methyl-10,11-methylenedioxycamptothecin; MDC2CPT, 7-ethyl-10,11-methylenedioxycamptothecin; MDC3CPT, 7-propyl-10,11-methylenedioxycamptothecin; MDC4CPT, 7-butyl-10,11-methylenedioxycamptothecin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; DPC, DNA-protein crosslinks.

ministration for the second-line treatment of metastatic colorectal cancer. CPT-11 is a pro-drug and is converted to the active 7-ethyl-10-hydroxy-CPT (OHC2CPT; SN-38) by carboxylesterases to exert its antitumor activity (Kunimoto et al., 1987; Kawato et al., 1991).

The potent activity of OHC2CPT has led to the development of a number of 7- and 10-substituted camptothecins (Jaxel et al., 1989; Wall et al., 1993; Emerson et al., 1995; Luzzio et al., 1995; Valenti et al., 1997). These include the highly potent 10,11-methylenedioxy-CPT (MDCPT) and 7-chloromethyl-10,11-methylenedioxy-CPT (CMMD; Fig. 1). The latter compound is of particular interest because it is capable of forming a covalent complex with DNA through nucleophilic displacement of the chlorine moiety by DNA while in the cleavable complex (Pommier et al., 1995; Valenti et al., 1997).

It has been suggested that the property of the 7- and 10-substituted analogs thought to be most relevant to their potent antitumor activity is the slow reversal of the cleavable complexes formed with these drugs (Tanizawa et al., 1994; Tanizawa et al., 1995; Valenti et al., 1997). Compared with CPT, the 10-hydroxy-CPT (OHCPT), OHC2CPT, MDCPT, and CMMD all seemed to have a longer-lived cleavable complex. These compounds are all substantially more cytotoxic than CPT; therefore, it has been postulated that, because CPT toxicity is a time-dependent phenomenon, the persistence of cleavable complexes may be an essential property for increasing the likelihood of a collision between the replication fork and a cleavable complex, giving rise to lethal DNA lesions (Tanizawa et al., 1995; Valenti et al., 1997).

To understand the relationship between CPT substitution, cytotoxicity, and cleavable complex reversibility, we have systematically synthesized and evaluated a series of 7-alkyl-CPT, 7-alkyl-10-hydroxy-CPT, 7-alkyl-10-methoxy-CPT, and 7-alkyl-10,11-methylenedioxy-CPT that have incremental lengths in the 7-alkyl chains. These analogs have been characterized with respect to: 1) their ability to induce topo I-mediated cleavage of plasmid DNA and DNA-protein crosslinks in CEM cell nuclei; 2) the reversibility of the cleavable complexes formed with plasmid DNA and in CEM cell nuclei; and 3) the growth inhibitory activity of the analogs to selected cancer cell lines. Our results indicate that the potency of the 7- and 10-substituted analogs does not reflect the lifetime for reversal of the cleavable complex formed with these compounds—even using analogs capable of forming covalent complexes with DNA. We demonstrate that rate constants for reversal of cleavable complexes are similar among the analogs tested. Instead, the in vitro biological activity of the drugs seems to more closely parallel the concentration of drug required to produce cleavable complexes in plasmid DNA under steady-state conditions.

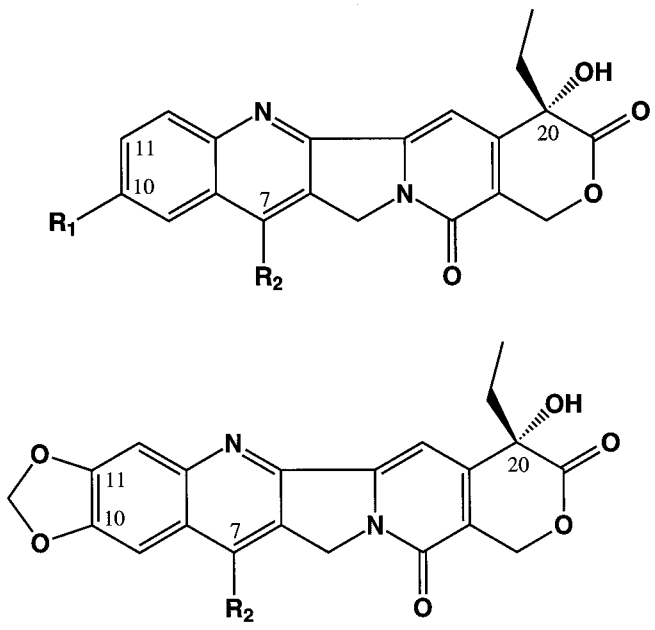

Fig. 1. The molecular structure of the camptothecin analogs used in these studies. CPT (top): R_1 = H, R_2 = H. C1CPT through C4CPT, R_1 = H, R_2 = methyl, ethyl, propyl, or butyl. OHCPT, R_1 = OH, R_2 = H. OHC1CPT through OHC4CPT, R_1 = OH, R_2 = methyl, ethyl, propyl, or butyl. OMeCPT, R_1 = OCH_3, R_2 = H. OMeC1CPT through OMeC4CPT, R_1 = OHC_3, R_2 = methyl, ethyl, propyl, or butyl. MDCPT (bottom), R_2 = H. MDC1CPT through MDC4CPT, R_2 = methyl, ethyl, propyl, or butyl.

Materials and Methods

Chemistry. The results for the CPT analogs are given in Table 1 and their structures given in Fig. 1. All CPT analogs used were the synthetic 20(*S*)-stereoisomer. CPT, OHCPT, and 10-methoxy-CPT (OMeCPT) were obtained as pure natural products (Wall et al., 1966; Wani and Wall, 1969). 7-Alkyl-CPT (C1CPT through C4CPT) were synthesized according to the method of Sawada et al. (1991). 7-Alkyl-OHCPT analogs (OHC1CPT through OHC4CPT) were synthesized from OHCPT by the procedure of Sawada et al. (1995). The 10-methoxy analogs OMeC1CPT through OMeC4CPT were synthesized according to the procedure for the synthesis of 10-methoxycamptothecin (Wani et al., 1980). The synthetic scheme for the preparation of MDCPT, MDC1CPT through MDC4CPT, and CMMD has also been reported previously (Wall et al., 1993; Luzzio et al., 1995). See the abbreviation list for definitions of these CPT analogs.

In Vitro Growth Inhibitory Activity. Human breast carcinoma lines MDA-231 and BT-20 were maintained in improved minimal essential media supplemented with 10% fetal bovine serum at 37°C in 5% CO_2 incubators until plated for use in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assays. The human cervical carcinoma line HeLa adapted for growth in serum-free media (HeLa/SF) was obtained from ATCC (Manassas, VA). The HeLa/SF cells were maintained in RPMI-1640 supplemented with the proprietary serum replacement TCH (Celox Laboratories, Hopkins, MN) as directed by ATCC. The total protein in the TCH-supplemented growth media was <0.1%.

Exponentially growing cells (1–2 × 10^3 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 at 37°C in a humidified incubator with 5% CO_2/95% air for 3 days, the plates were centrifuged briefly and 100 μl of the growth medium was removed. Cell growth was monitored by the standard MTT assay.

Cleavable Complex Formation by CPT Analogs in Plasmid DNA. CPT analog-induced cleavable complex formation was performed in 10 mM Tris-HCl, pH 7.9, 1 mM EDTA, 0.15 M NaCl, 0.1% BSA, 0.1 mM spermidine, 5% glycerol. A 250-ng sample of pBR322 plasmid DNA (Life Technologies, Gaithersburg, MD) was mixed with the drug of interest, and 4 U of human topoisomerase I enzyme (Topogen, Inc., Columbus, OH) was subsequently added to a 20-μl total mixture volume. Reaction mixtures were assembled on ice.

Each mixture was incubated at 37°C for 30 min; then the formation of complexes was terminated by the addition of 2 μl of 10% SDS and 2 μl of 0.5 mg/ml Proteinase K (Promega, Madison, WI). The

mixtures were incubated for another 30 min at 37°C, then treated with 2 μl of loading solution (25% bromphenol blue, 50% glycerol) and extracted with 20 μl of chloroform/isoamyl alcohol (24:1). After the chloroform/isoamyl alcohol extraction, the resulting sample was analyzed by electrophoresis for 16 h at 30 V on a 1% agarose gel in 1× Tris-acetate/EDTA buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0, containing 0.5 μg/ml ethidium bromide).

After electrophoresis, the gel was stained with 1:10,000 dilution of SYBR Green (Molecular Probes, Eugene, OR) in Tris/EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and photographed under transillumination with 300-nm UV light. The resulting photograph was scanned using a Polaroid Photopad scanner and the relaxed DNA (Form I DNA) band was quantified using NIH Image 1.6 software. A linear relationship between the amount of DNA present and the signal generated was established by quantifying increasing concentrations of supercoiled DNA.

Dose response data were fitted to a simple E_{max} model according to:

$$\text{Cleaved Complex} = \frac{E_{max}[Drug]}{EC_{50} + [Drug]} \quad (1)$$

where [*Drug*] is the molar concentration of analog, E_{max} is the maximal relaxed DNA signal, and EC_{50} is the concentration of drug required to produce 50% of the maximal response. Data were fitted to this equation using the nonlinear least-squares routine in Kaleidagraph (Synergy Software, Reading, PA).

Reversal of Cleavable Complex Formation in Plasmid DNA. Reversal of the topo I cleavage activity of the pBR322 plasmid DNA was accomplished by the method of Hertzberg et al. (1989). Cleavable complexes were formed in the presence of sufficient analog to induce >90% nicked DNA (as determined from EC_{50} curves). The reaction protocol consisted in the preparation of one 75-μl reaction mixture constructed as described above. The reaction mixture was incubated for 30 min at 23°C. A 100-fold excess of sonicated salmon sperm linear DNA (10 mg/ml; Life Technologies) was added to the reaction mixture. Aliquots were then removed at 0.5-, 1-, 2-, 5-, 10-, 15-, 30-, 45-, and 60-min intervals. The reaction was stopped by the addition of 11 μl of water, 2 μl of 10% SDS, and 2 μl of Proteinase K, then incubated at 23°C for another 30 min. The resulting samples were further mixed with loading solution and analyzed using 1% agarose gels, as above. The percentage of cleaved DNA with respect to time was determined based on the amount of Form I (nicked) DNA present in the 0.5-s lane.

The equilibrium established under these conditions can be described by the following simplified scheme [the detailed equilibria are given in Hertzberg et al. (1989)]:

$$T + S \underset{k21}{\overset{k12}{\rightleftharpoons}} TS \quad \text{and} \quad K_1 = \frac{[TS]}{[T][S]} \quad (2)$$

and

$$T + L \underset{k31}{\overset{k13}{\rightleftharpoons}} TL \quad \text{and} \quad K_2 = \frac{[TL]}{[T][L]} \quad (3)$$

where T is the molar concentration of topo I, S is the molar concentration of supercoiled DNA (in binding sites), and L is the molar concentration of linear competitor DNA fragments (in binding sites). The kinetic scheme for linear DNA-mediated competition for topo I follows the general derivation for any ligand exchange reaction (Wu et al., 1992). When the concentration of linear DNA fragments is large compared with the concentration of supercoiled DNA, the concentration of complexes *TS* will decay with a single exponential time constant, τ, that is approximately k_{12} in eq. 2. All the time-dependent decays of topo I-mediated cleavable complexes (*TS*) could be well fitted to a single exponential decay, indicating a simple pseudo first-order reaction:

$$\%\ \text{Cleaved DNA} = A \exp(-\tau t) + C \quad (4)$$

where t is time (in min), τ is the exponential rate constant with units of min^{-1}, and A and C are constants representing amplitude and final percentage of cleaved DNA, respectively. Eq. 4 was fit to the data with Kaleidagraph to determine the rate constants $t1$.

It is important to briefly discuss the amplitude A of the decay of cleavable complexes (*TS*) with time. At equilibrium (i.e., after an

TABLE 1
Potency of CPT analogs in inhibiting growth of cancer tumor cell lines and in poisoning topo I

CPT Analog	IC_{50} Growth Inhibition vs.			EC_{50} for topo I Poisoning
	BT-20 cells	MDA-231	HeLa/SF	
	nM			*μM*
CPT	>500	>500	>500	18.85 ± 12.82
C1CPT	33.1 ± 11.9	1.03 ± 0.71	116.9 ± 37.3	1.07 ± 0.34
C2CPT	61.3 ± 21.3	2.22 ± 1.98	77.5 ± 23.9	4.44 ± 1.86
C3CPT	120.2 ± 50.3	4.74 ± 1.34	185.7 ± 68.2	7.19 ± 1.01
C4CPT	24.8 ± 8.9	1.39 ± 0.75	388.7 ± 207.9	7.22 ± 2.85
OHCPT	34.3 ± 9.8	7.27 ± 4.37	ND	0.35 ± 0.07
OHC1CPT	18.2 ± 5.4	2.13 ± 2.05	5.23 ± 3.27	0.13 ± 0.02
OHC2CPT	50.1 ± 14.0	4.32 ± 1.97	3.70 ± 2.76	0.32 ± 0.04
OHC3CPT	35.2 ± 34.2	4.25 ± 1.23	3.57 ± 1.42	0.60 ± 0.16
OHC4CPT	8.8 ± 1.5	0.73 ± 0.38	1.09 ± 0.52	0.53 ± 0.06
OMeCPT	43.5 ± 3.8	4.12 ± 1.76	310.5 ± 44.8	0.72 ± 0.26
OMeC1CPT	17.5 ± 6.0	1.67 ± 1.24	66.8 ± 10.9	2.40 ± 0.45
OMeC2CPT	19.0 ± 1.7	2.32 ± 1.55	25.8 ± 20.1	2.82 ± 0.74
OMeC3CPT	11.3 ± 2.1	3.07 ± 0.61	22.1 ± 8.0	4.51 ± 1.59
OMeC4CPT	ND	5.33 ± 5.78	3.4 ± 0.7	2.85 ± 0.94
MDCPT	7.5 ± 2.5	1.19 ± 0.29	23.5 ± 9.9	0.29 ± 0.14
MDC1CPT	1.2 ± 0.5	0.02 ± 0.01	0.8 ± 0.7	0.03 ± 0.02
MDC2CPT	4.4 ± 1.0	0.71 ± 0.17	3.5 ± 1.5	0.66 ± 0.50
MDC3CPT	2.2 ± 0.5	0.92 ± 0.44	4.8 ± 1.6	0.45 ± 0.07
MDC4CPT	2.3 ± 1.0	0.68 ± 0.53	14.4 ± 5.7	0.51 ± 0.10
CMMD (chloromethyl)	19.3 ± 1.7	0.54 ± 0.33	ND	0.31 ± 0.05

ND, not determined.

infinite time t has elapsed), the concentration of cleavable complexes remaining is given by [see Wu et al. (1992)]:

$$[TS] = \frac{1}{2K_1}\left(1 + K_2[L]_o + 2K_1[TS]_o - \sqrt{(1 + K_2[L]_o)(1 + K_2[L]_o + 4K_1[TS]_o)}\right) \quad (5)$$

where K_1 and K_2 are given in eqs. 2 and 3, $[L]_o$ is the initial amount of linear competitor DNA added, and $[TS]_o$ is the initial amount of cleavable complexes present. The amplitude of the decay ($[TS]_o - [TS]$) is therefore dependent on the ratio of the binding constants K_1 and K_2. Although not explicitly shown in eqs. 2 and 3, these binding constants are in turn dependent on the concentration of CPT analog present. The CPT analogs enhance K_1 by reducing the k_{21} value, but this reduced k_{21} depends on the concentration of analog present and the EC_{50} ($\sim K_d$) value for the analog being examined. Hence, after introduction of competing linear DNA, the cleavable complexes will decay from the initial steady state to a new steady state. We use a fixed amount of topo I, plasmid DNA, and linear DNA in the experiments. However, because of the differences in EC_{50} values for poisoning of topo I (see below), the experiments required the use of differing concentrations of CPT analogs to achieve >90% cleavable complex formation. Thus, the amplitudes of the decay are the differences in the steady-state concentrations of complexes (TS) in the presence of varying concentrations of CPT analogs, and therefore the amplitudes of the decay do not necessarily reflect the rate constants τ for the decay. In general, we would expect that those analogs with lower EC_{50} values would have smaller amplitudes of decay, because of the CPT analog's high affinity for the topo I-DNA binary complex. The amplitudes were normalized to the initial (at 0.5 sec) and final (at 60 min) amounts of cleavable complex remaining. We report here only the values of τ, because these, not the amplitudes, reflect the true kinetic stability (or lifetime) of the cleavable complex.

DNA-Protein Crosslinks (DPC) in Isolated Nuclei. Trapping of the cleavable complexes of endogenous topoisomerase I in nuclei were assessed based on DPC induction measured by a standard K^+/dodecyl sulfate precipitation technique (McHugh et al., 1989; Woynarowski et al., 1994). Briefly, human leukemia CEM cells, grown in Jokklik's minimal essential medium with 10% fetal calf serum were prelabeled with [^{14}C]thymidine as described elsewhere (Woynarowski et al., 1997). Cells were lysed in isotonic Nuclei Isolation Buffer (2 mM KH_2PO_4, 5 mM $MgCl_2$, 150 mM NaCl, 1 mM EGTA, pH 6.9) with 0.3% (v/v) Triton X-100 followed by centrifugation (300g for 13 min) and resuspension in the isolation buffer at 0.5×10^6 nuclei/ml. Aliquots of nuclear suspension were treated with drugs as indicated. In the reversal experiments, samples were centrifuged (500g for 5 min), the nuclear pellets resuspended in a fresh nuclei isolation buffer followed by the additional incubation as indicated. All the reactions were terminated by addition of equal volume of 3% SDS, 40 mM EDTA, and 0.4 mg/ml DNA, pH 8.0, preheated to 65°C. Further steps were performed exactly as described elsewhere (McHugh et al., 1989; Woynarowski et al., 1994). Separate aliquots of nuclear suspension were used to determine total radioactivity in each individual suspension (McHugh et al., 1989; Woynarowski et al., 1994). The results are expressed as percentage of total DNA that coprecipitated with proteins corrected for the background precipitation in control samples. The latter value typically amounted to 2 to 5% of total radioactivity. For the reversal experiments, the results are normalized for the DPC levels at the end of drug treatment.

Results

Cell Growth Inhibition by Camptothecin Analogs. The IC_{50} values for growth inhibition of the synthesized compounds are given in Table 1. The CPT analogs were examined for their ability to inhibit growth of two breast carcinoma cell lines, BT-20 and MDA-231. Because of concerns about the partitioning of CPT analogs onto serum proteins and the effect of this phenomenon on availability of the compounds in solution, growth inhibition was also determined using a human ovarian carcinoma cell, HeLa/SF, line grown under serum-free conditions.

All 10-substituted analogs inhibit cell growth more than CPT, as did the 7-alkyl CPT. For the CPT, OHCPT, and MDCPT series, the 7-methyl analogs were among the most potent in inhibiting cell growth, but longer alkyl chain lengths were also effective. The activity of the longer-chained compounds may be attributable to enhanced cellular accumulation of these more hydrophobic compounds. The presence of serum proteins played little or no role in the relative potency of the compounds, because the HeLa/SF line (grown under serum-free conditions) gave data that paralleled those observed with the BT-20 and MDA-231 lines, both of which were grown with serum.

Interestingly, the MDCPT analogs inhibited cell growth substantially more than the other analogs examined, including the OHCPT. The MDCPT growth inhibitory activity against the cell lines was 2- to 40-fold higher than observed with CPT or the other analogs. This is also probably caused by the enhanced cellular accumulation, because the MDCPT analogs are likely to be more hydrophobic than the OHCPT analogs.

Induction of Cleavable Complexes by Camptothecin Analogs. Similar trends seen in the growth inhibition assays were also seen with the ability of the CPT analogs to act as topo I poisons. All compounds examined exhibited a simple hyperbolic dose response in their ability to induce cleavable complexes (Fig. 2A), and this response that could be fitted well by a simple E_{max} model (eq. 1). The EC_{50} values obtained from the curve fits, such as that shown for OHCPT in Fig. 2A, are recorded in Table 1 for each compound tested.

In agreement with previous reports (Jaxel et al., 1989; Wall et al., 1993; Luzzio et al., 1995; Tanizawa et al., 1995; Valenti et al., 1997), substituting the 10-position of camptothecin with a hydroxy group, or by making the 10,11-methylenedioxy analog yielded compounds with significantly higher potency in inducing cleavable complex formation (Table 1), compared with CPT. Substitution at the 7-position of CPT (7-alkyl CPT) also yielded compounds with more potency than CPT in inducing cleavable complex formation.

For the 7,10-disubstituted compounds, the 7-alkyl-10-methoxy compounds were only slightly more (~2-fold) potent than the 7-alkyl-CPTs in this function. Both the 10-hydroxy CPT and 10,11-methylenedioxy CPT were substantially more potent than CPT or OMeCPT as topo I poisons. Likewise, the 7-alkyl-10-hydroxy CPT and 7-alkyl-10-methylenedioxy CPT series were all highly potent compounds.

Interestingly, in the 7-alkyl CPT, OHCPT, and MDCPT series, the potency of the analogs were maximized by the presence of a 7-methyl group. Longer alkyl chains at this position were either as potent as or slightly less potent than the parent compound, with the exception of the 7-alkyl CPT, which were all more potent than CPT. This data was consistent with the IC_{50} data for growth inhibition (see above). The 7-methyl group was not the most potent analog of the OMeCPT series, where the length of the 7-alkyl chain did not seem to affect the potency of these compounds.

Induction of Protein-DNA Crosslinks in CEM Cell Nuclei. To extend the findings with purified enzyme and plasmid DNA, we examined the effects of selected CPT analogs (CPT, MDCPT, MDC2CPT, and CMMD) in isolated human leukemic CEM cell nuclei. Drug-induced trapping of the cleavable complexes in nuclei was assessed based on induction of DNA-protein crosslinks (DPC). This system allows one to monitor drug effects on endogenous topo I on its natural target—nuclear chromatin (McHugh et al., 1989).

The results indicate that all the examined analogs are more potent inducers of DPC than CPT. For example, 1 μM CPT was needed to produce a significant DPC level (35.3 $\pm$ 11% total DNA) after a 30-min incubation of nuclei with drug. In contrast, similar or higher DPC levels were induced by the other drugs at 0.1 μM (53.6 $\pm$ 8.2, 36.3, and 55.8 $\pm$ 1.9% total DNA for MDC2CPT, MDCPT, and CMMD, respectively; Fig. 3). Also, whereas DPC induced by CPT plateau after a few minutes' incubation, DPC by CMMD continue to increase until 30 min.

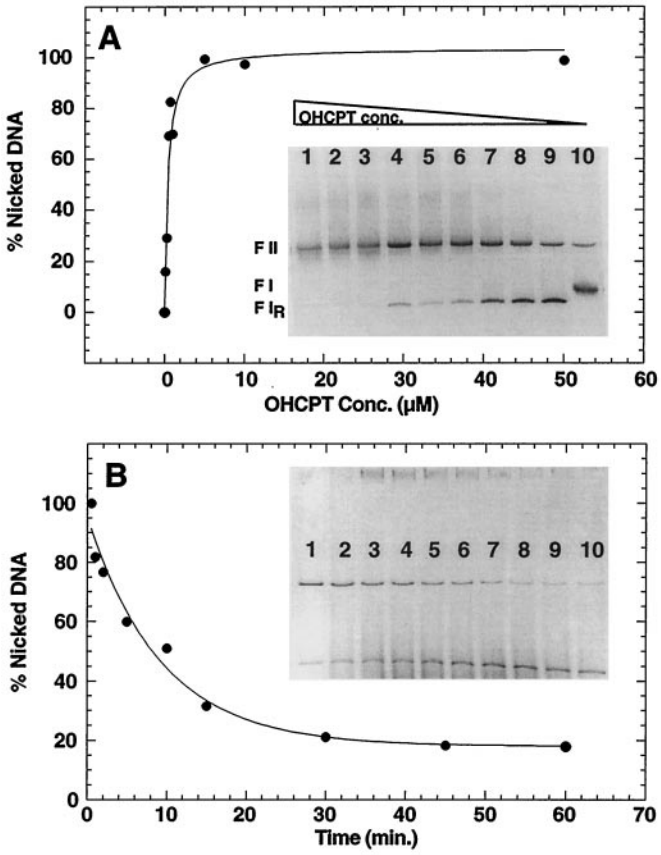

Fig. 2. Induction of cleavable complexes and their reversal by 10-hydroxycamptothecin. A, concentration dependence of cleavable complex formation. Human topo I and pBR322 supercoiled plasmid DNA were incubated with 10-hydroxycamptothecin at concentrations of 50, 10, 5, 1, 0.7, 0.5, 0.3, 0.1, and 0 μM concentrations (lanes 1–9, respectively). Lane 10 contained no drug or topo I. Form I (supercoiled) and Form I_R (relaxed, closed circular) DNA are indicated by F I and F I_R, respectively. The upper DNA band (nicked; Form II or F II) was quantified and expressed as percentage of total DNA after subtracting the amount of nicked DNA observed in the absence of drugs or topo I (i.e., lane 10). The solid curve is to the EC_{50} equation (eq. 1 in the text). The EC_{50} values determined in this manner for all analogs are recorded in Table 1. B, time course for reversal of cleavable complexes formed with 10-hydroxycamptothecin (OHCPT). Complexes were formed with 20 μM OHCPT, and then 100-fold linear salmon sperm DNA was added. Aliquots were withdrawn before DNA addition (lane 1) and 0.5, 1, 2, 5, 10, 15, 30, 45, and 60 min after DNA addition (lanes 2–10, respectively). Nicked DNA (Form II) was quantified and plotted versus time, with the solid line being the fit to eq. 4 in the text. The rate constants, τ, were determined for all analogs by this method, and are given in Table 2.

Reversal of Cleavable Complexes Formed with CPT Analogs on Plasmid DNA. We used the competitive DNA approach (Hertzberg et al., 1989) to measuring the religation of DNA single-strand breaks on plasmid DNA. An example of the data obtained for OHCPT and its analysis is given in Fig. 2B. The assumption of this method is that once topo I is dissociated from the cleavable complex, the excess of linear DNA competes for the topo I and inhibits it from reattaching to the same or other plasmids (eqs. 2 and 3). Hence, the process should be a pseudo first-order dissociation of topo I from the cleavable complex, and therefore well described by a single exponential decay. This approach characterizes the stability of the complex induced by the drug, because drug dissociation is the limiting step in the process [i.e., religation in the absence of drug is rapid (Wang et al., 1998)]. Other groups have used salt-induced denaturation of the DNA to produce similar decay curves (Tanizawa et al., 1994, 1995; Pommier et al., 1995; Valenti et al., 1997; Wang et al., 1998), where the salt acts to affect the equilibria given in eq. 2 by decreasing k_{12}. However, because of the unknown solubilities of the hydrophobic CPT analogs in 0.35 to 0.5 M NaCl, we chose to keep the ionic strength constant and use competitive DNA. We show below that the data obtained here is consistent with that obtained using the salt-induce release of cleavable complexes.

There was only a minor dependence of the reversibility of the cleavable complex on the structural composition of the CPT analogs. The reversal of cleavable complexes formed with 7-alkyl CPT is shown in Fig. 4A. Despite the enhanced potency in poisoning topo I and inhibiting cell growth by the 7-substituted CPT, the reversal kinetics for complexes formed by these compounds are essentially identical with those formed with CPT and all followed a simple single exponential decay. The fitted decay curve for CPT is shown by the solid line in Fig. 4A, but the τ values determined for all the analogs is given in Table 2.

When the 10-hydroxy compounds were examined, there was also only minor, if any, dependence of kinetics of reversal

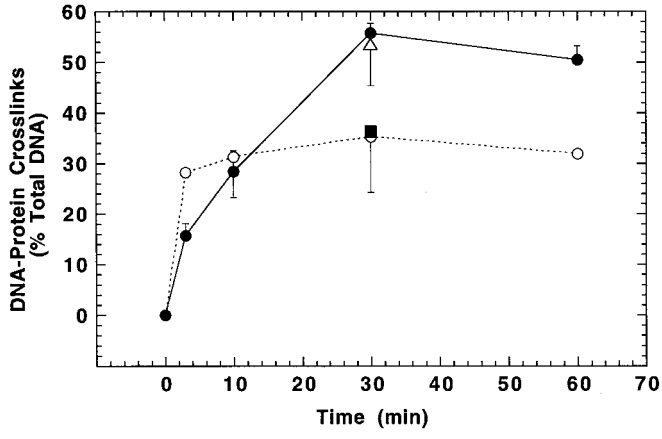

Fig. 3. Time course for DNA-protein crosslinks induced in CEM nuclei by CPT analogs. CPT at 1 μM (○) is shown and compared with 0.1 μM CMMD (●). Crosslinks induced by MDCPT (△) and MDC2CPT (■) at 30 min are shown for comparison. Error bars are positive for filled symbols and negative for open symbols.

on the 7-position (Fig. 4B). The solid line in Fig. 4B is for OHCPT, whereas the dashed line is shown for OHC2CPT. The rate constants governing the decay are similar between OHCPT and all the 7-alkyl OCHPT (recorded in Table 2). Furthermore, despite the high potency of the OHCPT series versus CPT in poisoning topo I and inhibiting cell growth, the rate constants governing religation by topo I in the presence of the 7-alkyl OHCPT are actually slightly larger (i.e., religation is faster) than with those of the corresponding 7-alkyl CPT analogs.

The decay of cleavable complexes stabilized with the OMeCPT was faster than that observed with CPT (Fig. 4C; Table 2). All of the 7-alkyl OMeCPT had identical decay curves; hence, they were independent of 7-alkyl chain length. The dashed line drawn in Fig. 4C is shown for OMeC2CPT, and all the τ values are recorded in Table 2.

Reversal of complex formation formed with MDCPT analogs also displayed little dependence on the 7-substitution (Fig. 4D). All cleavable complexes formed with the MDCPT analogs had similar rate constants for decay (Table 2). Although not apparent in the normalized plots in Fig. 4D, all MDCPT analogs did maintain a higher number of cleavable complexes after 1 h (~60%) compared with the CPT, OHCPT, or OMeCPT series of analogs (20 to 40% of complexes remaining after 1 h). In other words, the amplitude for the decay with the MDCPT analogs was lower than for the other series examined (see the discussion of Figs. 5 and 6 below).

Reversal of Protein-DNA Crosslinks in CEM Cell Nuclei. A comparison of reversal data from plasmid DNA versus cell nuclei is given in Fig. 5 for selected CPT analogs. These data are not normalized to the final concentration of cleavable complexes to indicate the relative amplitudes for complex decay. CMMD, the MDCPT analog with the potential for alkylation of DNA, shows an initially rapid, exponential reversal in plasmid DNA (Fig. 5A). However, only approximately 40% of the CMMD complexes religate during this decay compared with almost complete reversal by CPT. This reduced reversal for CMMD may not be caused by covalent linkage of the drug, because the data for the nonalkylating analog MDC2CPT reveal a slower relaxation of the complexes formed by this drug—even slower than those observed for CMMD (Fig. 5A). MDC2CPT does not seem to alkylate DNA (Pommier et al., 1995; Valenti et al., 1997).

Reversal of protein-DNA crosslinks in cell nuclei on washing with drug-free media is shown in Fig. 5B. The analogs used were those in Fig. 5A. Decay curves for each analog in cell nuclei on washing out of drug are similar to those seen with plasmid DNA (τ values recorded in Table 2). For both

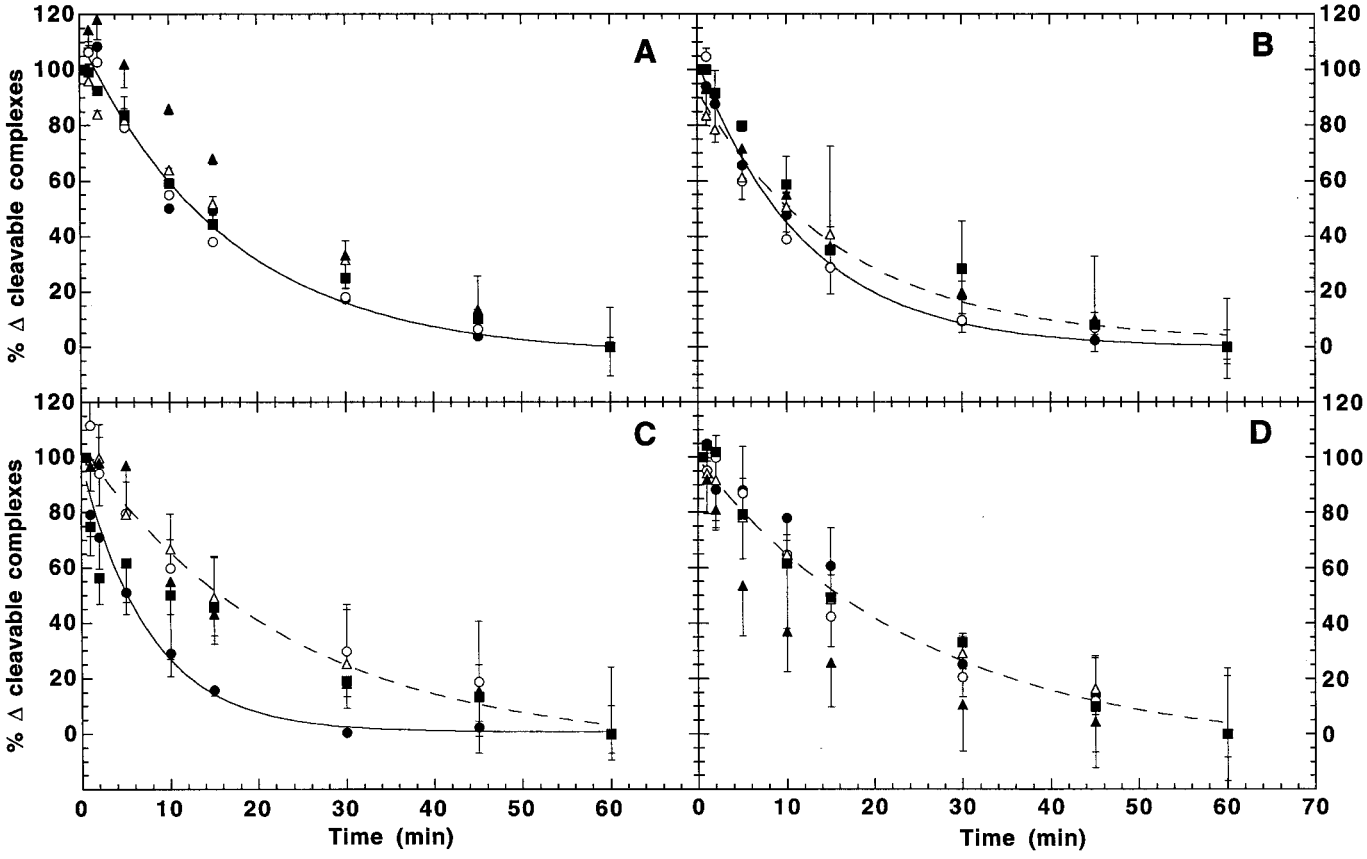

Fig. 4. Kinetics of competitive DNA-induced religation of cleavable complexes formed on plasmid DNA with human topoisomerase I and CPT analogs. The rate constants for the disappearance of cleavable complexes for all of the compounds are given in Table 2. In all panels, error bars are negative for filled symbols and positive for open symbols. A, CPT (●) and the 7-alkyl CPT analogs C1CPT (○), C2CPT (△), C3CPT (▲), and C4CPT (■). The solid line is for the decay in the presence of CPT. B, 10-Hydroxy-CPT OHCPT (●), and the 7-alkyl-10-hydroxy-CPT analogs OHC1CPT (○), OHC2CPT (△), OHC3CPT (▲), and OHC4CPT (■). The solid line is for the decay of OHCPT and the dashed line is for OHC2CPT. C, 10-Methoxy CPT OMeCPT (●) and the 7-alkyl-10-methoxy CPT analogs OMeC1CPT (○), OMeC2CPT (△), OMeC3CPT (▲), and OMeC4CPT (■). The solid line is for the decay of OMeCPT, whereas the dashed line is for OMeC2CPT. D, 10,11-Methylenedioxy CPT analog MDCPT (●), and the 7-alkyl-10,11-methylenedioxy-CPT analogs MDC1CPT (○), MDC2CPT (△), MDC3CPT (▲), and MDC4CPT (■). The dashed line is for MDC2CPT.

MDCPT and MDC2CPT, the rate constants for reversal in both plasmid DNA and cell nuclei are (within error) identical, indicating that the DNA competition method is effectively similar to drug washout experiments. CPT and CMMD had slightly higher rate constants for complex reversal in nuclei than in plasmid DNA.

In both the plasmid and nuclei, cleavable complexes with CPT are extensively reversible; however, the complexes formed with MDC2CPT remained at high levels even after 1 h in the presence of competitor salmon sperm DNA (Fig. 5A) or after washing of the nuclei (Fig. 5B). CMMD showed similar behavior in nuclei as in plasmids, with an initial decay followed by stable complexes. However, CMMD-induced complexes reversed to a greater extent in nuclei than with plasmid DNA. As a control, the effects of formaldehyde, used as a compound forming direct covalent DPC, were completely irreversible under the same conditions (118 and 95% of the initial DPC after 60 min with 100 and 500 μM formaldehyde, respectively; data not shown).

TABLE 2

Pseudo first-order rate constants for decay of cleavable complexes stabilized by 7,10-disubstituted CPTs

Camptothecin Analog	Rate Constant τ	
	From plasmid[a]	From nuclei[b]
	min^{-1}	
CPT	0.061 ± 0.010	0.130 ± 0.013
C1CPT	0.066 ± 0.008	
C2CPT	0.034 ± 0.006	
C3CPT	0.024 ± 0.009	
C4CPT	0.050 ± 0.005	
OHCPT	0.082 ± 0.004	
OHC1CPT	0.108 ± 0.012	
OHC2CPT	0.061 ± 0.013	
OHC3CPT	0.064 ± 0.006	
OHC4CPT	0.058 ± 0.010	
OMeCPT	0.131 ± 0.017	
OMeC1CPT	0.054 ± 0.012	
OMeC2CPT	0.044 ± 0.006	
OMeC3CPT	0.054 ± 0.014	
OMeC4CPT	0.045 ± 0.024	
MDCPT	0.028 ± 0.007	0.036 ± 0.011
MDC1CPT	0.049 ± 0.008	
MDC2CPT	0.040 ± 0.006	0.045 ± 0.019
MDC3CPT	0.114 ± 0.012	
MDC4CPT	0.045 ± 0.009	
CMMD (chloromethyl)	0.277 ± 0.048	0.088 ± 0.011

[a] Disappearance of cleavable complexes with time after addition of 100-fold excess sheared salmon sperm DNA.

[b] Disappearance of PDCs in CEM nuclei after suspension of nuclei in drug-free buffer.

Discussion

The evaluation of the CPT analogs reported here has been performed to advance the development of "third generation" CPT analogs, with topo I poisoning activities higher than topotecan (TPT), OHC2CPT, or MDCPT. Recent reports (Tanizawa et al., 1995; Valenti et al., 1997) have suggested that CPT analogs with greater potency than CPT or TPT in forming cleavable complexes might produce this enhanced activity by forming complexes that are slow to reverse. Such persistent complexes would be more likely to be present during replication of a given DNA segment and would therefore be more toxic to cells. Thus, structural features of CPT analogs lending themselves to slowly-reversing cleavable complexes are important for generating improved CPT analogs with activities that might surpass CPT-11 and TPT much as CPT-11 and TPT surpassed the parent CPT.

In this report, we examined 7-alkyl- and 7-alkyl-10-substituted CPT analogs for their mechanism of in vitro topo I poisoning and their ability to inhibit tumor growth in cell culture. Our data indicate that all 7- or 7, 10-substituted CPT analogs are more potent than CPT in inducing topo I-mediated cleavable complexes with purified enzyme and plasmid DNA (Table 1). The MDCPT analogs of CPT are among the most potent compounds known for poisoning topo I. However, the kinetic stability of the ternary complexes is independent of the potency of the compounds to both poison topo I on plasmid DNA or inhibit cell growth (compare Tables 1 and 2). CPT and its 7-alkyl derivatives show rapid, exponential reversibility of the complexes formed (Fig. 4A), despite the much higher potency of the 7-alkyl derivatives to poison topo I and to inhibit tumor cell growth. Similar results were observed for the OHCPT, OMeCPT, and MDCPT analogs of CPT: no correlation between the potency of the drug to trap topo I cleavable complexes or inhibit cell growth and its ability to kinetically stabilize cleavable complexes. Our work is in agreement with a recent paper on 9,10-disubstituted

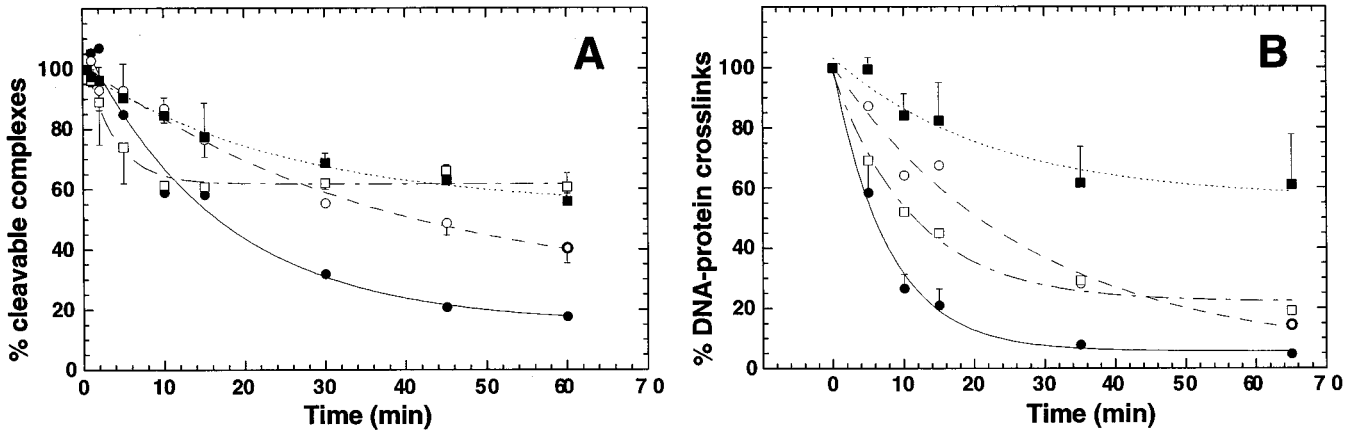

Fig. 5. Comparison of cleavable complex reversal on plasmid DNA with reversal of DNA-protein crosslinks in CEM cell nuclei. A, reversal of cleavable complexes formed on pBR322 plasmids with CPT (●), MDCPT (○), CMMD (□), and MDC2CPT (■). B, reversal of DNA-protein crosslinks induced by the drugs in CEM cell nuclei (same symbols as in A). Rate constants for reversal of DNA-protein crosslinks with the four compounds shown are given in Table 2.

CPT analogs by Hecht and colleagues (Wang et al., 1998). Using salt-induced religation and dissociation of topo I from a defined oligonucleotide, these authors also found no correlation between the potency of these analogs in poisoning topo I and their ability to kinetically stabilize cleavable complexes.

Our results, and those of Hecht and colleagues (Wang et al., 1998), point out a critical facet of topo I-DNA poisoning that may not be readily apparent when considering "longer-lived" cleavable complexes. In Fig. 6, we have recast data from Fig. 4 on a semilog plot using the CPT analogs and symbols taken from Fig. 3B of (Valenti et al., 1997). Comparison of our data, using competitive DNA, and their data, using salt-induced topo I dissociation, indicates that the data are remarkably similar with regard to the "stability" of the cleavable complexes. That is, in both of our studies, after a 1-h period, more complexes exist when MDC2CPT and CMMD are used; these in turn are greater in number than MDCPT- and OHC2CPT- induced complexes, with CPT-induced complexes even lower. However, note from Table 2 that the rate constants τ for all of these compounds are similar, and the τ value for OHC2CPT is identical with that for CPT. Hence, the "stability" of the complexes, as defined by the kinetic rate constants, are *not* correlated with the potency of the analogs in poisoning topo I or inhibiting cell growth. It should be acknowledged that our data represent average topo I cleavage sites on plasmid DNA. The possibility that selected topo I-binding DNA sequences have different kinetic behavior with more potent CPT analogs cannot be entirely ruled out.

Both the competitive DNA method used here and the salt-induced religation methods used elsewhere are examples of chemical relaxation experiments, where a steady-state is rapidly perturbed by addition of some component, and the system under study relaxes to a new steady-state [these methods are exhaustively reviewed in (Gutfreund, 1995)]. The concentration difference in components between the new steady state and the old gives rise to the amplitude of the perturbation (i.e., A in eq. 4). The decay time is given by τ. Hence, the highly potent CPT analogs may have a smaller A, because they poison topo I at very low concentrations, but generate cleavable complexes with identical kinetic "stabilities." In both the pBR322 cleavage assays and the cell growth assays, we are examining steady-state situations (i.e., constant amounts of drug present). Hence, it is not surprising that, in these assays, the potency of the drug in inhibiting cell growth more closely relates to the potency in poisoning topo I on plasmid DNA. In both situations, there is little or no dependence on kinetic parameters of the drug-DNA-topo I ternary complex. It should be noted, however, that although the EC_{50} values for topo I poisoning are more reflective of the in vitro growth inhibition IC_{50} values by the CPT analogs (that is compounds with lower EC_{50} values also had lower IC_{50} values), the actual correlation coefficient for these two parameters is low ($r = 0.5$ for BT-20 cells) or nonexistent ($r = 0.1$ for MDA-231 cells). This is most likely caused by differences in accumulation of the analogs within cells. In any event, our data with purified topo I and plasmid DNA, as well as isolated cell nuclei, suggest that the reason that OHCPT and MDCPT analogs are more active than CPT in inhibiting cell growth is that these compounds are highly potent under steady-state conditions. That is, at equivalent concentrations, the OHCPT and MDCPT analogs generate *more* cleavable complexes than CPT and OMeCPT analogs, not more stable cleavable complexes.

In contrast to the usual topo I poisoning assays and cell-growth inhibition assays described above, the clinical use of CPT analogs does involve a kinetic component. During a typical administration of CPT-11 or TPT, the blood concentration of OHC2CPT or TPT will rise then fall with time. Because cell kill in S-phase is also time-dependent (i.e., it requires collision of the replication fork with topo I-DNA complexes), clearly CPT analogs that form slow decaying ternary complexes might be desirable to maximize cancer cell killing. As suggested recently (Tanizawa et al., 1994, 1995; Valenti et al., 1997), development of a new generation of CPT analogs with antitumor activities greater than CPT-11 or TPT may include drugs designed to stabilize cleavable complexes to a much greater extent than the 7- and 10-substituted analogs examined here. Ideally, compounds such as the CMMD analog, which can alkylate DNA, may be most useful in forming stabile, irreversible cleavable complexes. However, only a small fraction of the available CMMD has been shown to covalently bind to DNA (Tanizawa et al., 1995; Valenti et al., 1997) and therefore these complexes rapidly reverse (Fig. 5). Hence, new, more reactive alkylating CPT analogs may prove more effective in their cytotoxic activity. We anticipate that the data presented here should aid in development of such compounds.

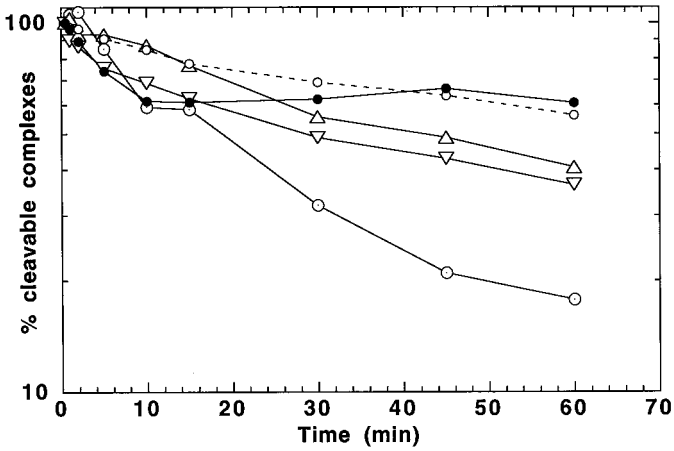

Fig. 6. Persistence of cleavable complexes on plasmid DNA after addition of competitive DNA. Data are shown for CPT (⊙), OHC2CPT (▽), MDCPT (△), MDC2CPT (○), and CMMD (●). Note that although the percentage of cleavable complexes remaining after 1 h is markedly different, the rate constants for the complex reversal process are similar for all the analogs examined (Table 2).

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References

Champoux J (1990) Mechanistic aspects of type-I topoisomerases, in *DNA Topology and Its Biological Effects* (Wang JC and Cozarelli NR eds) pp 217–242, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Chen AY and Liu LF (1994) DNA Topoisomerases: Essential enzymes and lethal targets. *Annu Rev Pharmacol Toxicol* **94:**194–218.

Creaven PJ, Allen LM and Muggia FM (1972) Plasma camptothecin (NSC-100880) levels during a 5-day course of treatment: Relation to dose and toxicity. *Cancer Chemother Rep* **56:**573–578.

Emerson DL, Besterman JM, Brown HR, Evans MG, Leitner PP, Luzzio MJ, Shaffer

JE, Sternbach DD, Uehling D and Vuong A (1995) In vivo antitumor activity of two new seven-substituted water-soluble camptothecin analogues. *Cancer Res* **55:**603–609.
Gottlieb JA, Guarino AM, Call JB, Oliverio VT and Block JB (1970) Preliminary pharmacologic and clinical evaluation of camptothecin sodium (NSC 100880). *Cancer Chemother Rep* **54:**461–470.
Gutfreund H (1995) *Kinetics for the Life Sciences: Receptors, Transmitters, and Catalysts,* Cambridge University Press, New York.
Hertzberg RP, Caranfa MJ and Hecht SM (1989) On the mechanism of topoisomerase I inhibition by camptothecin: Evidence for binding to an enzyme-DNA complex. *Biochemistry* **28:**4629–4638
Holm C, Covey JM, Kerrigan D and Pommier Y (1989) Differential requirement of DNA replication for the cytotoxicity of DNA topoisomerase I and II inhibitors in Chinese hamster DC3F cells. *Cancer Res* **49:**6365–6368.
Hsiang Y-H, Hertzberg R, Hecht S and Liu LF (1985) Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. *J Biol Chem* **260:** 14873–14878.
Hsiang Y-H, Lihou MG and Liu LF (1989) Arrest of DNA replication by drug-stabilized topoisomerase I-DNA cleavable complexes as a mechanism of cell killing by camptothecin. *Cancer Res* **49:**5077–5082.
Jaxel C, Kohn KW, Wani MC, Wall ME and Pommier Y (1989) 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. *Cancer Res* **49:**1465–1469.
Kawato Y, Aonuma M, Hirota Y, Kuga H and Sato K (1991) Intracellular roles of SN-38, a metabolite of the camptothecin derivative CPT-11, in the antitumor effect of CPT-11. *Cancer Res* **51:**4187–4191.
Kunimoto T, Nitta K, Tanaka T, Uehara N, Baba H, Takeuchi M, Yokokura T, Sowada S, Miyasaka T and Mutai M (1987) Antitumor activity of 7-ethyl-10-[4-piperidino)-1-piperidino]carbonyloxy-camptothecin, a novel water-soluble derivative of camptothecin against murine tumors. *Cancer Res* **47:**5944–5947.
Luzzio MJ, Besterman JM, Emerson DL, Evans MG, Lackey K, Leitner PL, McIntyre G, Morton B, Myers PL, Peel M, Sisco JM, Sternbach DD, Tong WQ, Truesdale A, Uehling DE, Vuong A and Yates J (1995) Synthesis and antitumor activity of novel water soluble derivatives of camptothecin as specific inhibitors of topoisomerase I. *J Med Chem* **38:**395–401.
McHugh MM, Woynarowski JM, Sigmund RD and Beerman TA (1989) Effect of minor groove binding drugs on mammalian topoisomerase I activity. *Biochem Pharmacol* **38:**2323–2328.
Muggia FM, Creaven PJ, Hansen H, Cohen MH and Selawry OS (1972) Phase I clinical trial of weekly and daily treatment with camptothecin (NSC 100880): Correlation with preclinical studies. *Cancer Chemother Rep* **56:**515–521.
Pommier Y, Kohlhagen G, Kohn KW, Leteurtre F, Wani MC and Wall ME (1995) Interaction of an alkylating camptothecin derivative with a DNA base at topoisomerase I-DNA cleavage sites. *Proc Natl Acad Sci USA* **92:**8861–8865.
Pommier Y, Leteurtre F, Fesen M, Fujimori A, Betrand R, Solary E, Kohlhagen G and Kohn KW (1994) Cellular determinants of sensitivity and resistance to DNA topoisomerase inhibitors. *Cancer Invest* **12:**530–542.
Pommier Y, Pourquier P, Fan Y and Strumberg D (1998) Mechanism of action of eukaryotic DNA topoisomerase I and drugs targeted to the enzyme. *Biochim Biophys Acta* **1400:**83–106.
Sawada S, Nokata K, Furuta T, Yokokura T and Miyasaka T (1991) Chemical modification of an antitumor alkaloid camptothecin: Synthesis and antitumor activity of 7-C-substituted camptothecins. *Chem Pharm Bull (Tokyo)* **39:**2574–2580
Sawada S, Yokokura T and Miyasaka Y (1995) A-ring or E-lactone modified water soluble prodrugs of 20(*S*)-camptothecin. *Current Pharm Design* **1:**113–132.
Tanizawa A, Fujimori A, Fujimori Y and Pommier Y (1994) Comparison of topoisomerase I inhibition, DNA damage, and cytotoxicity of camptothecin derivatives presently in clinical trials. *J Natl Cancer Inst* **86:**836–842.
Tanizawa A, Kohn KW, Kohlhagen G, Leteurtre F and Pommier Y (1995) Differential stabilization of eukaryotic DNA topoisomerase I cleavable complexes by camptothecin derivatives. *Biochemistry* **34:**7200–7206.
Valenti M, Nieves-Neira W, Kohlhagen G, Kohn KW, Wall ME, Wani MC and Pommier Y (1997) Novel 7-alkyl methylenedioxy-camptothecin derivatives exhibit increased cytotoxicity and induce persistent cleavable complexes both with purified mammalian topoisomerase I and in human colon carcinoma SW620 cells. *Mol Pharmacol* **52:**82–87.
Wall ME, Wani MC, Cook CE, Palmar KH, MacPhail AT and Sim GA (1966) Plant antitumor agents. 1. The isolation and structure of camptothecin, a novel alkaloidal leukemia and tumor inhibitor from *Camptotheca acuminata. J Am Chem Soc* **88:**3888–3890.
Wall ME, Wani MC, Nicholas AW, Manikumar G, Tele C, Moore L, Truesdale A, Leitner P and Besterman JM (1993) Plant antitumor agents. 30. Synthesis and structure activity of novel camptothecin analogs. *J Med Chem* **36:**2689–2700.
Wang JC (1991) DNA topoisomerases: Why so many. *J Biol Chem* **266:**6659–6662.
Wang X, Wang L-K, Kingsbury WD, Johnson RK and Hecht SM (1998) Differential effects of camptothecin derivatives on topoisomerase I-mediated DNA structure modification. *Biochemistry* **37:**9399–9408.
Wani MC, Ronman PE, Lindley JT and Wall ME (1980) Plant antitumor agents. 18. Synthesis and biological activity of camptothecin analogs. *J Med Chem* **23:**554–560.
Wani MC and Wall ME (1969) Plant Antitumor Agents. II. The structure of two new alkaloids from *Camptotheca acuminata*. *J Org Chem* **34:**1364–1367.
Woynarowski JM, McCarthy K, Reynolds B, Beerman TA and Denny WA (1994) Topoisomerase II mediated DNA lesions induced by acridine-4-carboxamide and 2-(4-pyridyl)quinolone-8-carboxamide. *Anticancer Drug Des* **9:**9–24.
Woynarowski JM, Napier C, Koester SK, Chen SF and Troyer D (1997) Effects on DNA integrity and apoptosis induction by a novel antitumor sesquiterpene drug, 6-hydroxymethylacylfluvene (HMAF, MGI 114). *Biochem Pharmacol* **54:**1181–1193.
Wu X, Gutfreund H and Chock PB (1992) Kinetic method for differentiating mechanisms for ligand exchange reactions: Application to test for substrate channeling in glycolysis. *Biochemistry* **31:**2123–2128.

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