0026-895X/97/010082-06$3.00/0

MOLECULAR PHARMACOLOGY, **52:**82–87 (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

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Received November 19, 1996; Accepted March 24, 1997

SUMMARY

An alkylating camptothecin (CPT) derivative, 7-chloromethyl-10,11-methylenedioxy-camptothecin (7-CM-MDO-CPT) was recently shown to produce irreversible topoisomerase I (top1) cleavage complexes by binding to the +1 base of the scissile strand of a top1 cleavage site. We demonstrate that 7-CM-EDO-CPT (7-chloromethyl-10,11-ethylenedioxy-camptothecin) also induces irreversible top1-DNA complexes. 7-CM-MDO-CPT, 7-CM-EDO-CPT, and the nonalkylating derivative 7-ethyl-10,11-methylenedioxy-camptothecin (7-E-MDO-CPT) also induced reversible top1 cleavable complexes, which were markedly more stable to salt-induced reversal than those induced by 7-ethyl-10-hyroxy-CPT, the active metabolite of CPT-11. This greater stability of the top1 cleavable complexes was contributed by the 7-alkyl and the 10,11-methylene- (or ethylene-) dioxy substitutions. Studies in SW620 cells showed that 7-E-MDO-CPT, 7-CM-MDO-CPT, and 7-CM-EDO-CPT are more potent inducers of cleavable complexes and more cytotoxic than CPT. The reversal of the cleavable complexes induced by 7-E-MDO-CPT, 7-CM-MDO-CPT, and 7-CM-EDO-CPT was markedly slower after drug removal than that for CPT, which is consistent with the data with purified top1. By contrast to CPT, 7-E-MDO-CPT, 7-CM-MDO-CPT, and 7-CM-EDO-CPT were cytotoxic irrespective of the presence of 10 μM aphidicolin. These results suggest that 7-E-MDO-CPT, 7-CM-MDO-CPT, and 7-CM-EDO-CPT are more potent top1 poisons than CPT and produce long lasting top1 cleavable complexes and greater cytotoxicity than CPT in cells.

CPT is an antitumor alkaloid from the Asian tree *Camptotheca acuminata* (1), and derivatives are now used for cancer treatment. CPTs are selective poisons of the nuclear enzyme, DNA top1 (for review, see Refs. 2 and 3), which is essential for mammalian cell growth and plays a critical role in relieving DNA torsional tension during all transactions, including replication and transcription (4, 5). CPT stabilizes top1 cleavable complexes by inhibiting the religation step of the top1 catalytic reaction (for review, see Ref. 2). This inhibition is, however, 1) rapidly reversible upon drug removal and 2) not cytotoxic by itself. Reversible cleavable complexes need to be converted into irreversible top1 complexes by their interaction with DNA replication and possibly transcription. This explains why the cytotoxicity of CPT depends on 1) the length of drug treatment, which needs to be prolonged to obtain significant effect, and 2) cell metabolism and active DNA replication.

The present study was performed to investigate the molecular and cellular pharmacology of new lead candidates for second generation CPTs. We recently reported that 7-CM-MDO-CPT (Fig. 1) is unique among CPTs because it produces irreversible cleavable complexes (6). 7-CM-MDO-CPT alkylates the purine immediately 3′ to the top1 cleavage site (+1 base), although having no detectable effect in purified DNA. Such an alkylation (at position N3 of the purine in the DNA minor groove) blocks top1 religation. Another difference between 7-CM-MDO-CPT and CPT is that both substitutions (7-chloromethyl and 10,11-methylenedioxy) have previously been shown to enhance the top1 inhibitory activity (7–9). We

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ABBREVIATIONS: CPT, camptothecin; 7-CM-MDO-CPT, 7-chloromethyl-10,11-methylenedioxy-camptothecin; 7-E-MDO-CPT, 7-ethyl-10,11-methylenedioxy-camptothecin; 7-CM-EDO-CPT, 7-chloromethyl-10,11-ethylenedioxy-camptothecin; SN-38, 7-ethyl-10-hydroxycamptothecin; MDO-CPT, 10,11-methylenedioxy-camptothecin; SDS, sodium dodecyl sulfate; DPC, DNA-protein cross-link; SSB, single-strand break; top1, topoisomerase I.

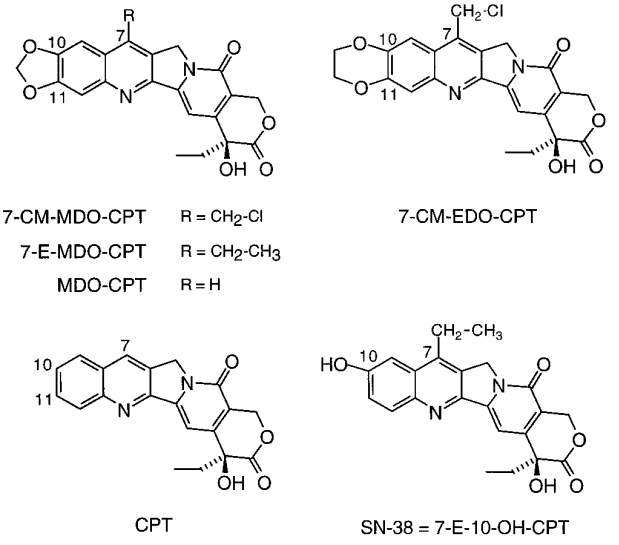

Fig. 1. Molecular structure of the CPTs studied.

also tested 7-E-MDO-CPT, the nonalkylating derivative of 7-CM-MDO-CPT, and 7-CM-EDO-CPT, a closely related analog of 7-CM-MDO-CPT (Fig. 1). The present data demonstrate that both the alkylating and nonalkylating CPT analogs induce persistent cleavable complexes and exhibit increased cytotoxicity in SW620 human colon carcinoma cells.

Materials and Methods

Cell Culture, DNA Radiolabeling, Drugs, and Treatments

SW620 colon cancer cells were grown in RPMI 1640 supplemented with 5% fetal calf serum and 2 mM glutamine without antibiotics at 37°. For alkaline elution experiments, cells were labeled with [^{14}C]thymidine (0.04 μCi/ml for 15–24 hr) and chased in drug-free medium for at least 15 hr. 7-CM-MDO-CPT and 7-CM-EDO-CPT were synthesized by published procedures (9, 10). Aphidicolin was obtained from Sigma, St. Louis, MO. All drugs were dissolved in dimethyl sulfoxide, aliquoted, and stored at −80°. Cell treatments were performed with fresh drug aliquots for 1 hr and were terminated by removing the drug-containing medium, washing cultures with an excess volume of phosphate-buffered saline twice, and adding drug-free medium at 37°.

Oligonucleotide Assays

Cleavage assays were performed as previously described (6). Briefly, a duplex oligonucleotide (Fig. 2B) was 3′-end-labeled with α-^{32}P-cordycepin and reacted with top1 (GIBCO-BRL, Gaithersburg, MD) in the absence or in the presence of different drug concentrations at 25°. After 15 min, reactions were either stopped directly with SDS (0.5% final concentration) or treated with 0.5 M NaCl for 1 hr at 25° to force top1 religation (8) and then stopped with 0.5% SDS at indicated times. Maxam/Gilbert loading buffer (98% formamide, 0.01 M EDTA, 1 mg/ml xylene cyanol, and 1 mg/ml bromphenol blue) was then added to samples, which that were loaded and electrophoresed into 16% polyacrylamide gel containing 7 M urea. Imaging and quantitation were performed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Clonogenic Assays

Approximately 2 × 10^5 cells/T25 flask were seeded and allowed to grow exponentially for 24 hr before drug treatments. After treatment, cells were harvested, counted, and seeded in 6-well plates at a density of 400 cells/well. After 14 days, colonies were fixed with 100% methanol, colored with 0.05% methylene blue, and counted. Plating efficiency was determined as the ratio between the colonies counted at day 14 and the number of cells seeded at day 0. The survival fraction of treated samples was normalized to control and expressed as percent.

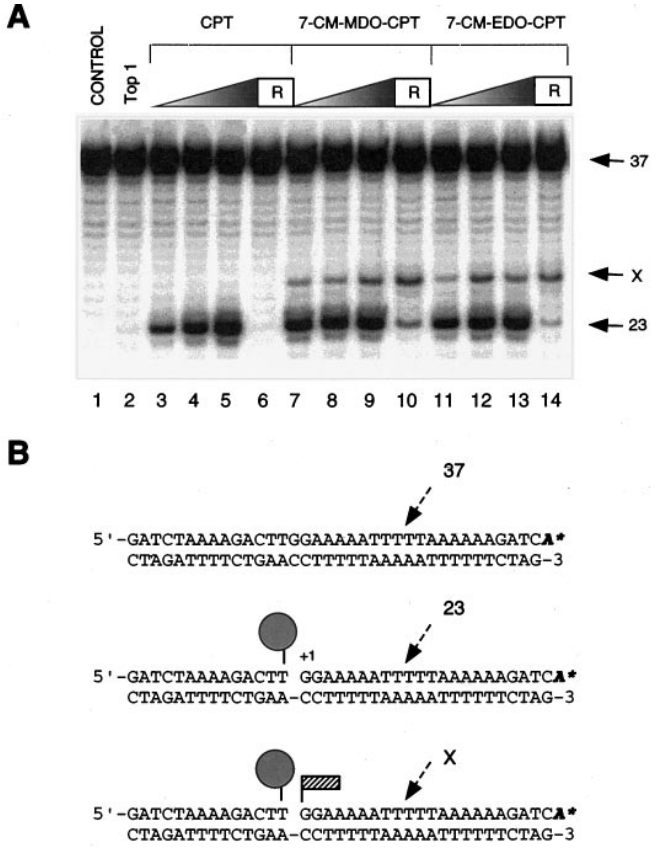

Fig. 2. Effects of CPT, 7-CM-MDO-CPT, and 7-CM-EDO-CPT on top1-mediated DNA cleavage. A, PhosphorImager results of a typical experiment. Reactions were performed for 15 min at 25° and stopped immediately with 0.5% SDS (*lanes 1–5*, *7–9*, and *11–13*) or after an additional hour and the addition of 0.5 M NaCl (*lanes 6*, *10*, and *14*) (*R* for salt-induced reversal). *Lane 1*, DNA alone; *lane 2*, + top1; *lanes 3–6*, *7–10*, and *11–14*, + top1 + 0.1, 1, and 10 μM CPT, 7-CM-MDO-CPT, or 7-CM-EDO-CPT, respectively. *Numbers to the right,* size of the detected oligonucleotides; *X*, drug alkylated oligonucleotide (see B). B, Sequence of the oligonucleotide used and size of the fragments generated by top1 cleavage (*shaded circle*). *A**, ^{32}P-Cordycepin used to label the 3′-end of the upper strand. ▨, drug alkylated to the 5′-terminal base of the cleaved oligonucleotide.

Cleavable Complexes in Drug-Treated Cells

Alkaline elution was used to assess cleavable complexes, which were detected as protein-linked DNA strand breaks (for reviews, see Refs. 11–13). Characteristically, each DNA SSB is associated with one DPC, and the SSBs are not detectable under nondeproteinizing conditions (2).

DNA SSBs. SSBs were analyzed using DNA-denaturing (pH 12.1) alkaline elution carried out under deproteinizing conditions. Briefly, [^{14}C]thymidine-labeled SW620 cells were layered onto polycarbonate filters (2 μm, Poretics, Livermore, CA) and were lysed with a solution containing 2% SDS, 0.1 M glycine, 0.025 M disodium EDTA, and 0.5 mg/ml proteinase K, pH 10 (SDS lysis). The lysis solution was washed from filters with 5 ml of 0.02 M EDTA, pH 10, and the DNA was eluted with tetrapropylammonium hydroxide-EDTA containing 0.1% SDS, pH 12.1, at a flow rate of 0.14 ml/min. Fractions were collected at 5-min intervals for 30 min. Fractions and filters were processed and counted. Computation of SSB was performed as de-

scribed previously (11, 12). Drug-induced SSB frequencies were expressed as the γ-radiation dose that produces an equivalent elution rate (rad-equivalents).

DPCs. DPCs were analyzed under nondeproteinizing, DNA-denaturing conditions using protein-adsorbing filters (polyvinylchloride-acrylic copolymer filters, 0.8-μm pore size; Gelman Science, Ann Harbor, MI), and LS10 lysis solution (2 M NaCl, 0.2% Sarkosyl, and 0.04 M disodium EDTA, pH 10). After scraping cells in drug-containing ice-cold nucleus buffer (13), all cell suspensions were irradiated with 30 Gy. The DNA was eluted from filters with tetrapropylammonium hydroxide-EDTA, pH 12.1, without SDS at a flow rate of 0.035 ml/min. Fractions were collected at 3-hr intervals for 15 hr. DPC frequencies were calculated according to the bound to one terminus model formula (12, 14).

Demonstration of the protein linkage of DNA breaks. DNA fragments not tightly bound to protein (frank breaks) were detected by alkaline elution carried out under the nondeproteinizing conditions described for the DPC assay except that cell samples were not irradiated before elution. Elution rates were compared with those from γ-irradiated control cells (14).

Results

Effects of the 7-alkyl CPT derivatives on purified mammalian DNA-top1. Inasmuch as previous work suggested that 10,11-ethylenedioxy CPT derivatives exhibit better anti-tumor activity than the more potent 10,11-methylenedioxy derivatives (9, 15), we sought to test whether 7-CM-EDO-CPT was able to alkylate the top1-DNA complex (6). The result of a typical experiment is shown in Fig. 2A and a schematic representation of the reaction products in Fig. 2B. Both 7-CM-MDO-CPT and 7-CM-EDO-CPT produced two bands in the presence of top1, whereas CPT produced only the faster migrating 23-mer band. The 23-mer band corresponds to the expected 3′-end-labeled DNA cleavage product generated by top1 (Fig. 2B) (6). The band labeled *X* corresponds to the 23-mer oligonucleotide with the drug covalently bound to guanine N3 at the 5′-end of the oligonucleotide (Fig. 2B) (6). These results demonstrate that 7-CM-EDO-CPT exhibits the same top1 cleavable complex alkylating activity as does 7-CM-MDO-CPT.

Fig. 2A also shows the lack of reversibility of the X products (*lane 4*, labeled *R* for each compound). As described in Materials and Methods, reaction mixtures were incubated in the presence of 0.5 M NaCl for 1 hr at room temperature and then stopped with SDS. Under these conditions, the normal top1 cleavage product (23-mer) reversed completely in the case of CPT (*lane 6*). As expected (6), the X band did not reverse for either the 7-CM-MDO-CPT- or 7-CM-EDO-CPT-treated samples. This is consistent with the formation of irreversible top1 complexes. It is also interesting to note that the 23-mer band did not reverse completely in the case of 7-CM-MDO-CPT, suggesting that this compound produces markedly more stable top1 cleavable complexes than CPT.

Kinetics analyses were performed to determine the stability of top1 cleavable complexes. Fig. 3 shows the results of a typical experiment demonstrating that the reversal of top1 cleavable complexes was markedly slower for 7-CM-MDO-CPT and its nonalkylating analog, 7-ethyl-MDO-CPT (6) (see Fig. 1 for structures) (half-time reversal around 25 min) than for CPT (<1 min). MDO-CPT also produced more stable cleavable complexes (half-time reversal around 6 min) than CPT, with kinetics comparable to SN-38, the active metabolite of CPT-11. However, the MDO-CPT-induced cleavable

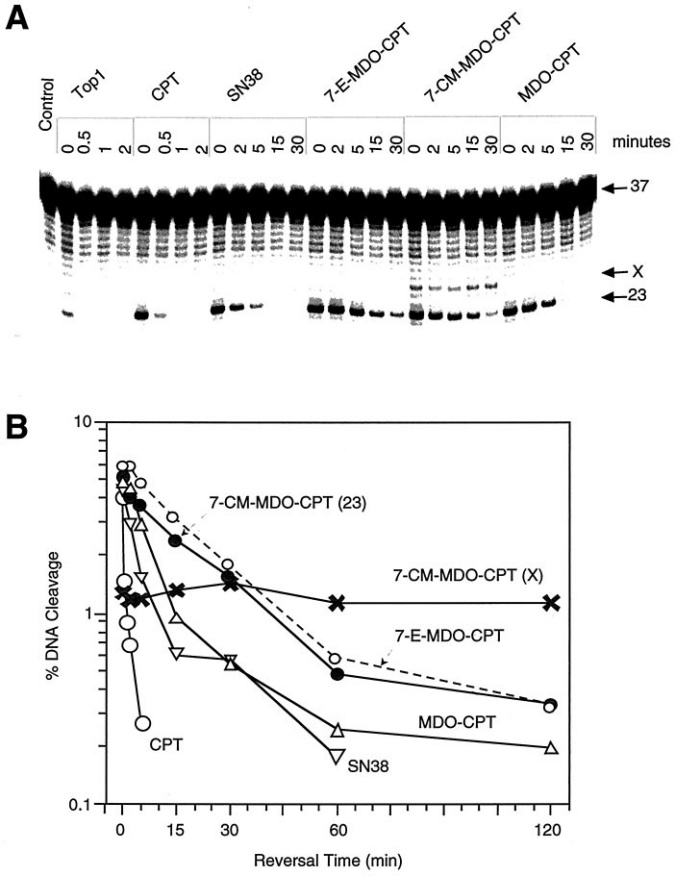

Fig. 3. Differential stability of top1 cleavable complexes induced by CPT derivatives. Reactions were performed at 25° for 30 min, after which an aliquot was taken (time 0) and 0.35 M NaCl was added. *Times above each lane,* aliquots were taken after NaCl addition and stopped by adding 0.5% SDS. A, PhosphorImager results of a typical gel. *Numbers to the right*, size in nucleotides of the DNA fragments (see Fig. 2B). B, Quantitation of the data shown in A. ●, 7-CM-MDO-CPT cleavable complexes; ✕, 7-CM-MDO-CPT irreversible complexes; ○ (*dashed line*), 7-E-MDO-CPT; ▽, SN-38; △, MDO-CPT; ○ (*solid line*), CPT.

complexes were noticeably less stable than those induced by 7-E-MDO-CPT and 7-CM-MDO-CPT. Thus, the slow reversal of the 7-E-MDO-CPT or 7-CM-MDO-CPT-induced cleavable complexes is due to both the 7-alkyl and 10,11-methylenedioxy substitutions. Together, these results indicate that 7-CM-MDO-CPT induces two types of persistent top1 complexes: (i) slowly reversible cleavable complexes (23-mer in our oligonucleotide experiments) and (ii) irreversible complexes (X product). 7-E-MDO-CPT induces only slowly reversible cleavable complexes, which is consistent with its nonalkylating activity.

DNA lesions induced by 7-alkyl CPT derivatives in SW620 Cells. The cellular pharmacology of 7-E-MDO-CPT, 7-CM-MDO-CPT, 7-CM-EDO-CPT, and CPT was evaluated in human colon carcinoma SW620 cells. This cell line was chosen because we previously characterized its relative CPT sensitivity among the colon carcinoma cell lines of the NCI Anticancer Drug Screen and its functional and genetic responses to CPT (16–18). SW620 cells also grow well in culture and can be used for clonogenic assays.

Cellular top1 cleavable complexes were assayed by alkaline elution as DPC and DNA SSB. Topoisomerase-induced

cleavable complexes typically produce 1) similar frequency of DPC and SSB, as a topoisomerase molecule is covalently linked to a DNA terminus at each break site, and 2) no break under nondeproteinizing conditions (11, 13, 14). The three analogs induced DPC (Fig. 4) and DNA SSBs (Fig. 5 and data not shown). 7-E-MDO-CPT was the most potent compound, followed by 7-CM-MDO-CPT and 7-CM-EDO-CPT, which in turn were more potent than CPT (Fig. 4). The DNA SSBs induced by 7-CM-MDO-CPT and 7-CM-EDO-CPT were protein-concealed because no detectable DNA elution was observed under nondeproteinizing conditions (14) (data not shown). These results demonstrate that 7-E-MDO-CPT, 7-CM-MDO-CPT, and 7-CM-EDO-CPT are more potent top1 poisons than CPT in cell culture.

Reversal of drug-induced cleavable complexes was studied after 30-min treatments by measuring DNA-SSBs (Fig. 5). For the three analogs, the DNA lesions were noticeably more persistent than for CPT. The DNA SSBs induced by 7-E-MDO-CPT were the most persistent and remained detectable up to 4 hr after drug removal (not shown).

Cytotoxicity of the 7-alkyl CPT derivatives in SW620 Cells. The cytototoxicity of CPT is known to be dependent upon duration of drug exposure (for review, see Ref. 2) and active DNA replication, as aphidicolin, a DNA polymerase inhibitor protects cells against CPT-induced cytotoxicity (19, 20). The results of Table 1 demonstrate that the compounds studied can be ranked by increasing cytotoxicity: CPT < 7-CM-EDO-CPT < 7-CM-MDO-CPT < 7-E-MDO-CPT. A difference between CPT and 7-E-MDO-CPT, 7-CM-MDO-CPT, and 7-CM-EDO-CPT is that CPT-induced cytototoxicity reached a plateau below one log cell kill for short drug exposures (19, 21), whereas cytotoxicity continued to increase with higher concentrations of 7-E-MDO-CPT or of the two alkylating derivatives.

The protective effect of aphidicolin (19, 20) was investigated at different concentrations of the four compounds (Table 1). As expected, CPT cytotoxicity was inhibited by aphidicolin. Interestingly, the cytotoxicity of 7-CM-MDO-CPT was not affected by aphidicolin, whereas the cytotoxicity of 7-E-MDO-CPT and 7-CM-EDO-CPT was only partially inhibited.

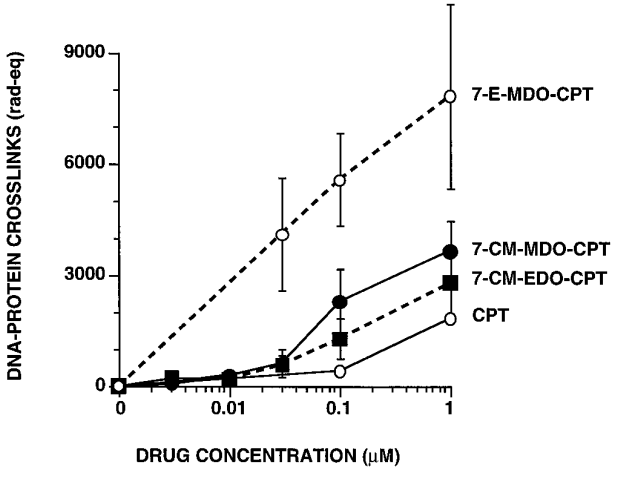

Fig. 4. Drug-induced cleavable complexes measured as DPCs calculated according to the bound-to-one terminus model and expressed in rad-eq (as described in Materials and Methods). SW620 cells were treated for 30 min with ○ (*solid line*), CPT; ●, 7-CM-MDO-CPT; ■, 7-CM-EDO-CPT; or ○ (*dashed line*), 7-E-MDO-CPT.

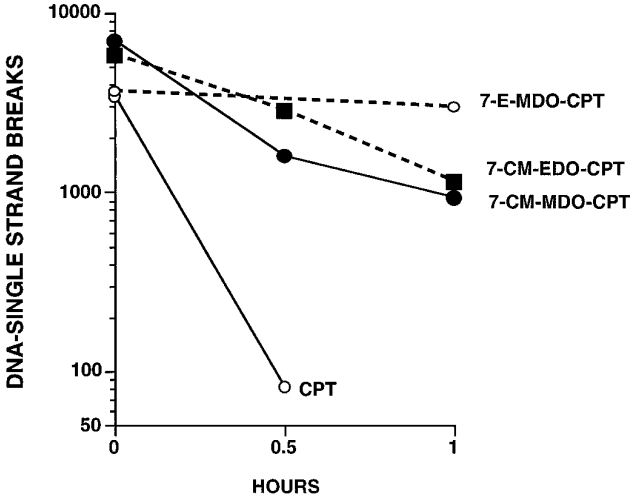

Fig. 5. Kinetics of the reversion of cleavable complexes measured as DNA-single strand breaks. SW620 cells were treated for 30 min with 1 μM CPT (○) (*solid line*), 7-CM-MDO-CPT (●), 7-CM-EDO-CPT (■), or 7-E-MDO-CPT (○) (*dashed line*). Drugs were removed at time 0, and alkaline elutions were performed at the indicated times.

TABLE 1

Cytotoxicity of CPTs and effects of APD in human colon carcinoma SW620 cells

Cells were treated with CPTs for 30 min in the absence or presence of 10 μM APD. APD treatments were started 5 min before CPT treatments (35-min total APD exposure). Under these conditions, APD had no noticeable cytotoxicity by itself. Cytotoxicity was determined by clonogenic assays.

CPT	Survival	
	− APD	+ APD (10 μM)
μM	%	
CPT		
0.01	95.1	ND[a]
0.3	38 ± 9	99 ± 1.4
1	34.4 ± 9	100
7-CM-MDO CPT		
0.01	68.3	ND
0.3	23.7 ± 11.3	37.3 ± 11.6
1	9.25 ± 3	13.7 ± 3.2
7-CM-EDO-CPT		
0.01	48.3	ND
0.3	26.7 ± 7.12	65 ± 1
1	5.35 ± 5.1	5 ± 1
7-E-MDO-CPT		
0.01	24.7 ± 5.2	48.0 ± 22.6
0.3	19.0 ± 9.5	39.2 ± 10.1
1	3.7	1.3

[a] ND, not determined.

These results demonstrate that the 7-alkyl CPT derivatives reported here are cytotoxic to cells with arrested DNA replication at the time of treatment with the CPT.

Discussion

We have extended our previous observation with 7-CM-MDO-CPT (6) to 7-CM-EDO-CPT and 7-E-MDO-CPT. The alkylating CPT derivatives (7-CM-MDO-CPT and 7-CM-EDO-CPT) produce irreversible top1 cleavage detected as a new cleavage product that we refer to as the "X-band" in DNA denaturing acrylamide gels. We previously showed that the X-band probably corresponds to alkylation of the CPT 7-alkyl group to N3 of the +1 purine 3′ to the top1 cleavage site (Fig.

2B). This finding provided the most direct evidence that CPTs inhibit the religation of top1-cleavable complexes by binding at the enzyme-DNA interface (6). Previous evidence for this hypothesis was that top1 cleavage sites trapped by CPT exhibited a strong preference for guanines at the +1 position, whereas no preference was observed in the absence of CPT (for review, see Ref. 2). The present results obtained with both 7-CM-MDO-CPT and 7-CM-EDO-CPT are consistent with CPT binding at the enzyme-DNA interface. The CPT binding site (22) seems to accommodate either the 10,11-methylene- or the 10,11-ethylene-dioxy substitutions. The fact that 7-CM-MDO-CPT was more potent and produced more stable top1-cleavable complexes than 7-CM-EDO-CPT suggests that the 10,11-methylenedioxy derivative binds better than the 10,11-ethylenedioxy derivative to the top1 cleavable complex.

Both 7-CM-MDO-CPT and 7-E-MDO-CPT (its nonalkylating derivative) produced the typical reversible cleavable complexes (6, 23). Interestingly, both compounds were more potent than was CPT, and their cleavable complexes were markedly more stable to salt reversal than those induced by CPT and even SN-38. Therefore, both the 7-alkyl and the 10,11-methylene- (or 10,11-ethylene-) dioxy substitutions probably increase the affinity of the CPT derivative for top1 cleavable complexes, which is consistent with previous data showing that, in the case of SN-38, both the 10-hydroxy and 7-ethyl substitutions are responsible for its greater affinity for top1-cleavable complexes and that the 10,11-methylenedioxy substitution markedly enhances the stabilization of top1-cleavable complexes (8).

The cellular data demonstrate that both 7-E-MDO-CPT and 7-CM-MDO-CPT are more potent than CPT, especially at low doses. This enhanced potency is probably due to the persistence of the top1-mediated DNA damage, as detected by alkaline elution. It is indeed well established that the cytotoxicity of CPTs increases with drug exposure time (2), probably because of the conversion of top1-cleavable complexes into irreversible top1 complexes by DNA replication fork collision (for review, see Ref. 2). One of the observations leading to this model is that aphidicolin has been shown to protect against CPT-induced cytotoxicity (Table 1) (19, 24). More recently, the cytotoxicity of CPT has been shown to depend also on an aphidicolin-independent mechanism in the colon carcinoma cell line, SW620 treated with a wide range of CPT concentrations (17), and more generally in most cell lines at high CPT concentrations (17, 19). A DNA replication-independent cytotoxicity has also just been reported in neuronal cells (25). The activity of 7-CM-MDO-CPT and 7-E-MDO-CPT in aphidicolin-treated cells and may be related to the persistence of the cleavable complexes induced by these derivatives.

The rationales for developing non-CPT top1 poisons are that 1) the cleavable complexes induced by CPT are rapidly reversible upon drug removal; 2) DNA replication plays a role in converting cleavable complexes into DNA damage, which imposes long CPT exposures; 3) CPTs contain a labile lactone moiety that is critical for activity; opening of the lactone E-ring of CPTs at physiological pH inactivates CPTs to their carboxylate forms (2); and 4) other inhibitors may have a different cytotoxic and clinical profile of activity, because this is observed in the case of top2 poisons (26). Non-CPT top1 poisons have been discovered, but these top1 inhibitors are less potent and probably less specific than CPTs. Most of them are DNA intercalators: saintopins (27, 28), actinomycin D (29, 30), morpholinyldoxorubicin (29, 30), intoplicine (31), indolocarbazoles (32, 33), benzo[*c*]phenanthridines, protoberberines (34, 36), or DNA minor groove binders in the case of bi- and *tert*-benzimidazoles (37, 39). A number are also topoisomerase II inhibitors: saintopins, actinomycin D, intoplicine, and nitidine. 7-CM-MDO-CPT and 7-CM-EDO-CPT are in preclinical evaluation. 7-E-MDO-CPT and 7-CM-MDO-CPT are promising compounds as they may alleviate the first two limitations of CPTs (rapid reversibility of cleavable complexes upon drug removal; DNA replication-dependent cytotoxicity) and represent leads for second generation CPTs.

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