

Platinated DNA Adducts Enhance Poisoning of DNA Topoisomerase I by Camptothecin*

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Camptothecins constitute a novel class of chemotherapeutics that selectively target DNA topoisomerase I (Top1) by reversibly stabilizing a covalent enzyme-DNA intermediate. This cytotoxic mechanism contrasts with that of platinum drugs, such as cisplatin, which induce inter- and intrastrand DNA adducts. *In vitro* combination studies using platinum drugs combined with Top1 poisons, such as topotecan, showed a schedule-dependent synergistic activity, with promising results in the clinic. However, whereas the molecular mechanism of these single agents may be relatively well understood, the mode of action of these chemotherapeutic agents in combination necessitates a more complete understanding. Indeed, we recently reported that a functional homologous recombination pathway is required for cisplatin and topotecan synergy yet represses the synergistic toxicity of 1- β -D-arabinofuranosyl cytidine in combination with topotecan (van Waardenburg, R. C., de Jong, L. A., van Delft, F., van Eijndhoven, M. A., Bohlander, M., Bjornsti, M. A., Brouwer, J., and Schellens, J. H. (2004) *Mol. Cancer Ther.* 3, 393–402). Here we provide direct evidence for Pt-1,3-d(GTG) poisoning of Top1 *in vitro* and demonstrate that persistent Pt-DNA adducts correlate with increased covalent Top1-DNA complexes *in vivo*. This contrasts with a lack of persistent lesions induced by the alkylating agent bis[chloroethyl]nitrosourea, which exhibits only additive activity with topotecan in a range of cell lines. In human IGROV-1 ovarian cancer cells, the synergistic activity of cisplatin with topotecan requires processive DNA polymerization, whereas overexpression of Top1 enhances yeast cell sensitivity to cisplatin. These results indicate that the cytotoxic activity of cisplatin is due, in part, to poisoning of Top1, which is exacerbated in the presence of topotecan.

Eukaryotic DNA topoisomerase I (Top1)¹, a ubiquitous nuclear enzyme, acts as a swivel during replication, transcription, and recombination to relieve overwinding of duplex DNA (reviewed in Refs. 1–3). Top1 catalyzes changes in the linkage of DNA strands via the formation of a covalent enzyme-DNA intermediate. A phosphotyrosyl linkage of Top1 to the 3'-end of the cleaved DNA strand allows the free 5' DNA end to rotate. The covalent Top1-DNA intermediate is the cellular target of camptothecin (CPT) and clinically useful analogs, such as topotecan (TPT) and SN-38, the active metabolite of irinotecan (CPT-11) (reviewed in Refs. 2 and 4–6). These Top1 poisons reversibly stabilize the covalent Top1-DNA intermediate by inhibiting DNA religation. During S-phase, these ternary Top1-DNA-drug intermediates are converted into potentially lethal lesions, which induce cell cycle arrest and cell death.

TPT and CPT-11 have been approved for first and second line treatment of advanced colorectal cancer and second line treatment for ovarian cancer (7–9). As with most chemotherapeutics, these CPT analogs are administered in combination with other agents (10–13). Although the molecular mechanisms of single agents may be well understood, the combination of two or more agents often induces surprising interactions, which necessitates a more complete understanding of the cytotoxic lesions induced by such combinations for optimal efficacy. For example, *in vitro* combination experiments using platinum agents, such as cisplatin (cDDP), carboplatin, or oxaliplatin, with a Top1 poison, TPT or CPT-11, demonstrated a sequence-dependent synergy in various human tumor cell lines (14–16). The most cytotoxic sequence was a platinum drug followed by a Top1 poison. Moreover, chemotherapeutic regimens that combine TPT/CPT-11 with platinum drug are showing some promise in the clinic (see Refs. 17–19 and references therein). However, the lack of insight into the mechanism of synergy hampers the optimal design of clinical studies based on the combination of platinum drugs and Top1 poisons.

We recently reported genetic evidence that homologous recombination (HR) is responsible for the synergistic cytotoxicity of certain drug/Top1 poison combinations (20). Indeed, a functional HR pathway was required for the synergistic activity of platinum or X-irradiation in combination with TPT yet acted to suppress the synergistic combination of 1- β -D-arabinofuranosyl cytidine

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¹ The abbreviations used are: Top1, DNA topoisomerase I; CPT, camptothecin; TPT, topotecan; cDDP, cisplatin; HR, homologous recombination; BCNU, bis[chloroethyl]nitrosourea; ara-C, 1- β -D-arabinofuranosyl cytidine; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; Bleo, bleomycin; CI, combination index; ICE, immunocomplex of enzyme.

(ara-C) with TPT. Recent structural and biochemical studies indicate that ara-C-substituted DNA acts as an endogenous Top1 poison to increase the stability of the covalent Top1-DNA intermediate (21, 22). These studies derive from earlier reports that endogenous DNA lesions, such as abasic sites, mismatches, and oxidative damage (7,8-dihydro-8-oxoguanine), enhance the stability of Top1 and/or Top2 covalent complexes (23–26). However, the contrary effects of HR on the synergistic activity of cDDP plus TPT *versus* ara-C plus TPT lead us to ask whether cDDP adducts would also act to poison Top1 or induce distinct DNA lesions that trigger aberrant repair by the HR pathways (20).

cDDP may interact with nucleophiles in many cellular components, including DNA, RNA, and proteins. However, it is generally accepted that the formation of intra- and interstrand platinum-DNA adducts is the underlying cause of the cytotoxic activity of platinum drugs, such as cDDP, oxaliplatin, and carboplatin (27, 28). Unfortunately, whereas much is known about the repair of these DNA adducts, the mechanisms by which Pt-DNA adducts induce cell death remain poorly understood.

Here we report that the synergistic activity of cDDP with TPT required processive DNA polymerization and that the formation of persistent Pt-DNA adducts in human cancer cell lines correlates with increased covalent Top1-DNA complexes. We further provide direct evidence for Pt-1,3-d(GTG) poisoning of Top1 *in vitro*. Taken together, these data suggest that the cytotoxic activity of cDDP derives, in part, from poisoning of Top1, which is exacerbated in the presence of TPT. Moreover, these findings suggest that the distinct effects of HR on the synergistic activity of cDDP/TPT *versus* ara-C/TPT may be a consequence of differences in the persistence of covalent complex stabilization rather than the ability of either agent to transiently poison Top1.

EXPERIMENTAL PROCEDURES

Plasmids, Cell Lines, and Drugs—IGROV-1 (ovarian adenocarcinoma), MCF-7 (breast cancer), and WiDr (colon cancer) human cell lines were cultured under standard conditions in RPMI 1640 medium with 10% bovine calf serum, 10 mM NaHCO₃, 2 mM glutamine. The nucleotide excision repair-defective yeast strain, W303rad4, was previously described (20). The yeast *TOP1* vector, YCpGAL1-*TOP1*-L, expresses yeast DNA topoisomerase I from the galactose-inducible *GAL1* promoter (29). Yeast transformation and culture conditions were as described (20).

Topotecan (TPT) was from Smith Kline Beecham Pharmaceuticals (King of Prussia, PA), SN-38, the active metabolite of CPT-11, was obtained from Aventis (Alfortville, France), and cDDP was either from TEVA Pharma (Mijdrecht, the Netherlands) or the kind gift of J. Reedijk (Leiden University). Bis(chloroethyl)nitrosourea (BCNU) was from Bristol-Myers Squibb Co., whereas ara-C was from Amersham Biosciences. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was from Sigma, and bleomycin (Bleo) was obtained from the Netherlands Cancer Institute. A ¹³⁷Cs source was used with a dose rate of ~1 gray/min.

Drug Cytotoxicity Assays—IGROV-1, MCF-7, and WiDr cells, treated with the indicated agents, were assayed for cell viability using the sulforhodamine B assay as described (14). For each experiment, cells were plated with a density of 1500 cells in 200 μ l per well in 96-well plates. Cells were then exposed to increasing concentrations of drug or treated with increasing doses of X-rays to determine IC₅₀ values at day 3 or 5. On the basis of the IC₅₀ values of the single agents, a constant drug ratio of agent 1/agent 2 was used in combination according to the following schedule: exposure with the first agent for 2 days, followed by co-treatment with the second agent for an additional 3 days. Cell viability was determined using the sulforhodamine B assay. This prolonged treatment schedule was used to avoid potential complications of the sulforhodamine B assay associated with short term drug exposure.

Drug combination data were evaluated according to the median effect analysis, based on the multiple drug effect equation (30, 31). The combination index (CI) for each fraction killed was calculated using the equation, $CI = d_1/D_1 + d_2/D_2 + ((d_1d_2)/(D_1D_2))$, where D_1 and D_2 are the doses of drug 1 and 2, which by themselves result in a given fraction killed, and d_1 and d_2 are the doses resulting in the same fraction killed

in combination. The CI values were interpreted as defining an antagonistic effect if $CI > 1.2$; an additive effect if $1.2 > CI > 0.8$; and a synergistic effect if $CI < 0.8$.

³²P Postlabeling Assay—The quantification of platinum-DNA intrastand 1,2-d(GG) and 1,2-d(AG) adducts was performed with the ³²P postlabeling assay according to Ref. 32. Subconfluent IGROV-1 cells were incubated for 2 h with 55 μ M cDDP, with or without 25 nM SN-38, washed, and subsequently cultured in medium with or without 25 nM SN-38 until DNA isolation. Cells were harvested at 0, 2, 6, 24, and 48 h after removal of cDDP and processed as in Ref. 32.

Top1 DNA Binding Assays—Oligonucleotides containing a unique 1,3-d(GTG) site (5'-CTAAAAACATGTGCATATCTTC-3'), untreated or treated with cDDP as described (33), were annealed to a 5'-³²P-end-labeled complementary strand (5'-GAAGATATGCACATGTGTTTT-TAG-3'). This oligonucleotide lacks the canonical high affinity Top1 cleavage site. Approximately 32 fmol/reaction of damaged or undamaged double-stranded oligonucleotide was incubated with 0, 125, or 250 ng of purified human Top1 protein (Topogen, Columbus, OH) in the presence of 0.4 μ g/ μ l CPT, 5% Me₂SO, and 50 mM Tris, pH 7.5, in a final 20- μ l volume at room temperature. Following the addition of loading buffer (10 mM Tris, pH 7.5, 30% glycerol, and 0.1% bromophenol blue), protein-DNA complexes were resolved in a 4% polyacrylamide gel and visualized by autoradiography.

Top1 Catalytic Activity Assays—Nuclear extracts were isolated from subconfluent IGROV-1 cells. Cells were harvested by centrifugation and resuspended in NucA buffer (1 mM KH₂PO₄, pH 6.4, 150 mM NaCl, 5 mM MgCl₂, 1 mM EGTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride), diluted 1:10 with NucA buffer supplemented with 0.3% Triton X-100, and incubated for 10 min on ice. Following centrifugation at 4 °C, the nuclear pellet was washed with NucA buffer and resuspended in 0.2 volumes of NucA buffer and an equal volume of NucC buffer (1 mM KH₂PO₄, pH 6.4, 550 mM NaCl, 5 mM MgCl₂, 1 mM EGTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) and incubated on ice for 30 min. Following centrifugation, the clarified nuclear extract was adjusted to a final concentration of 43% glycerol and stored at -80 °C.

Top1 catalytic activity was assessed in a plasmid DNA relaxation assay, using either untreated or platinated pBR322 DNA as substrate. Pt-pBR322 DNA was prepared by incubating 25 μ g of DNA with 33.33 μ M cDDP overnight at room temperature, followed by ethanol precipitation and resuspension in 10 mM Tris, pH 7.5, 1 mM EDTA. 250 ng of supercoiled pBR322 or Pt-pBR322 DNA was incubated with increasing concentrations of IGROV-1 cell nuclear extracts in 50 mM Tris, pH 7.4, 85 mM KCl, 15 mM MgCl₂, 0.5 mM EDTA, 30 μ g/ml bovine serum albumin for 30 min at 37 °C. The reaction products were treated with proteinase K and resolved by agarose gel electrophoresis in the absence of ethidium bromide. However, to facilitate detection of nicked DNA (34), the gels were stained with ethidium bromide and subjected to an additional 3 h of electrophoresis. Intact DNA topoisomers and nicked circles were quantitated using the Eagle-eye II system (Stratagene, La Jolla, CA) and TINA software (Isotopenmessgeräte GmbH).

Oligonucleotide-based DNA Cleavage Assay—The same oligonucleotides described for the DNA binding assays above were used to assess DNA cleavage by human Top1. However, in this case, the scissile strand (with or without a platinum adduct) was 3'-end labeled with [³²P]cordycepin using terminal deoxynucleotidyltransferase (Stratagene, La Jolla, CA) according to the manufacturer's instructions using a CoCl₂-free buffer (20 mM MES, pH 6, 0.4 mM MgCl₂). Excess unincorporated ³²P-label was removed by centrifugation through a G25-Sephadex quick spin column. The labeled oligonucleotide was annealed to the complementary unlabeled strand in equimolar amounts by heating for 5 min at 95 °C followed by cooling down to room temperature. Cleavage reactions (10 μ l) were performed at room temperature by incubating 1 pmol of DNA (with or without platinum adduct) with or without 50 μ M CPT and equal concentrations of purified human Top1 (as described in Ref. 35) in 20 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 0.1 mM EDTA, 50 mM KCl, and 50 μ g/ml gelatin. Reactions were terminated after 10 s and 5, 10, and 20 min by the addition of 3.3 volumes of loading buffer (USB-stop buffer) followed by heating to 70 °C. Cleavage products were resolved in denaturing 20% polyacrylamide, 7 M urea gels and visualized with a PhosphorImager (Amersham Biosciences).

Immunocomplex of Enzyme (ICE) Assay—To quantitate covalent Top1-DNA complexes formed in drug treated IGROV-1 cells, we used a modification of the ICE assay (36). Subconfluent cells were treated for 2 days at the IC₅₀ of the first agent followed by a 30-min co-incubation with 0.5 μ M TPT. In the reverse schedule, cells were incubated with the IC₅₀ of TPT followed by the IC₅₀ of cDDP for 30 min. For 1-h treatment schedules, cells were treated with 5 \times IC₅₀ of the first agent, followed

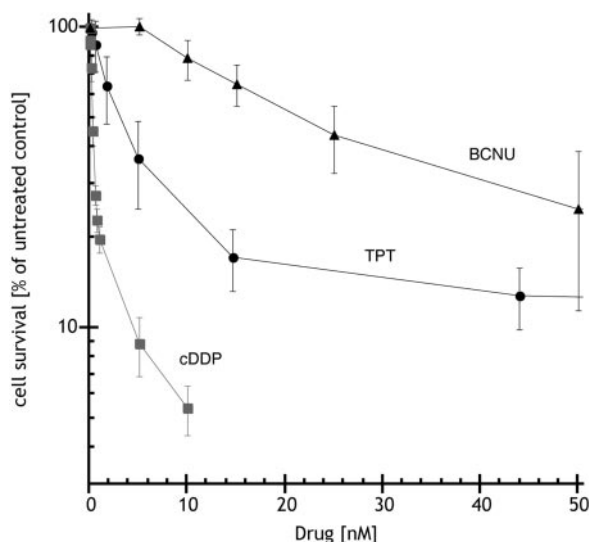


FIG. 1. IGROV-1 cell survival following exposure to increasing concentrations of TPT, cDDP, or BCNU. Subconfluent cultures of IGROV-1 cells were incubated in the presence of increasing concentrations of TPT (●), cDDP (■), or BCNU (▲). After 5 days, the number of viable cells was determined using the sulforhodamine B assay and plotted relative to the untreated control. The mean and S.D. of at least three independent experiments are shown.

TABLE I
IGROV-1 cell sensitivity to single agents

Therapeutic agent	IC ₅₀ value ^a
cDDP	0.29 nM
TPT	29.9 nM
BCNU	27.5 nM
Ara-C	41.1 nM
Bleo	149.5 microunits/ml
MNNG	1731.4 nM
X-ray	1.8 grays

^a IC₅₀ values were defined as drug concentration or x-ray dose required to yield 50% survival following 5 days of exposure (cDDP, BCNU, Ara-C, Bleo, MNNG, or x-ray) or 3 days of exposure (TPT). Values were the average of at least three independent experiments.

with 0.5 μM TPT for the last 30 min. For all schedules, cells were lysed with 1% sarkosyl preheated to 37 °C. Following the addition of an equal volume of 3 M NaSCN, the lysates were overlaid on a CsCl step gradient (1.82, 1.72, 1.50, and 1.35 g/ml) and centrifuged in a Beckman SW40 Ti rotor at 31,000 rpm, 20 °C for 16 h. The upper layer of CsCl (1.35 g/ml) also contained 3 M NaSCN. Fractions of 0.4 ml were collected, and 50-μl aliquots were slot-blotted onto polyvinylidene difluoride membranes. Each blot contained a 10-point calibration curve of nuclear extracts as an internal control for blot-to-blot variation. DNA topoisomerase I protein was detected using the polyclonal human anti-human Top1 scleroderma antibody (Topogen, Columbus, OH), visualized with autoradiography using ECL chemiluminescence (Amersham Biosciences), and quantified using the Eagle-eye II system (Stratagene). Relative levels of Top1-DNA covalent complexes obtained with various drug combinations were quantitated relative to the amount obtained with TPT alone.

Yeast Cell Viability Assays—W303rad4 cells, transformed with YCp-GAL1-TOP1-L or pRS415 vector control, were grown overnight at 28 °C in selective media (S.C. -leucine) supplemented with 2% galactose to A₆₀₀ = 1.0 and stored at -80 °C in 250-μl fractions. Thawed cells were grown overnight in S.C. -leucine + galactose to an A₆₀₀ value of 0.8–1.2. The cells were harvested by centrifugation and resuspended in water, and 1-ml fractions were incubated with various concentrations of cDDP for 2 h. Aliquots were 10-fold serially diluted and plated onto S.C. -leucine + galactose plates, and the number of viable cells forming colonies was determined after incubation at 28 °C.

RESULTS

Schedule-dependent Synergy of cDDP, Bleo, Ara-C, or X-ray with TPT in Human Cancer Cell Lines—TPT, a water-soluble analog of CPT, and SN-38, the active metabolite of the prodrug CPT-11, have demonstrated considerable activity against a

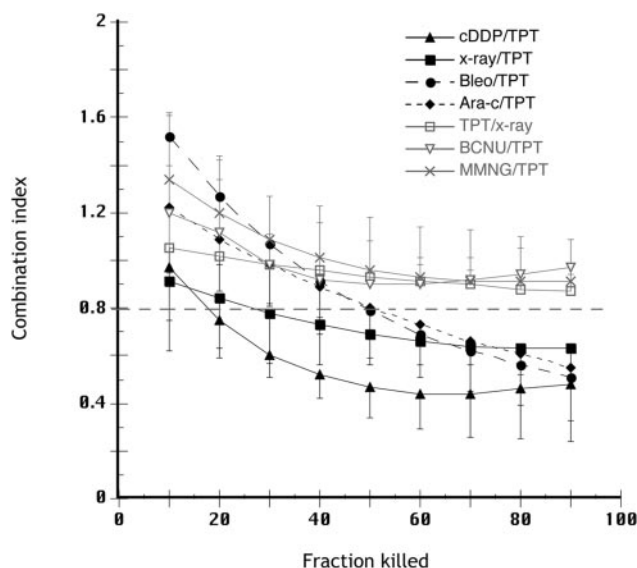


FIG. 2. cDDP, x-ray, Bleo, and ara-C exhibit schedule-dependent synergistic cytotoxicity in combination with TPT. Subconfluent IGROV-1 cells were treated with the following combination of agents according to the schedule (x-ray irradiation or continuous exposure with the indicated first agent for 2 days/the addition of either TPT for an additional 3 days or x-ray irradiation followed by 3 days of incubation). ▲, cDDP/TPT; ■, x-ray/TPT; ●, Bleo/TPT; ◆, ara-C/TPT; □, TPT/x-ray; ▽, BCNU/TPT; ×, MNNG/TPT. CI values were plotted relative to the fraction of cells killed. CI values at greater than 50% fraction killed were interpreted as antagonistic if CI was >1.2, additive if 1.2 > CI > 0.8, and synergistic if CI was <0.8. Data shown are the mean at least three independent experiments. S.D. values are as indicated.

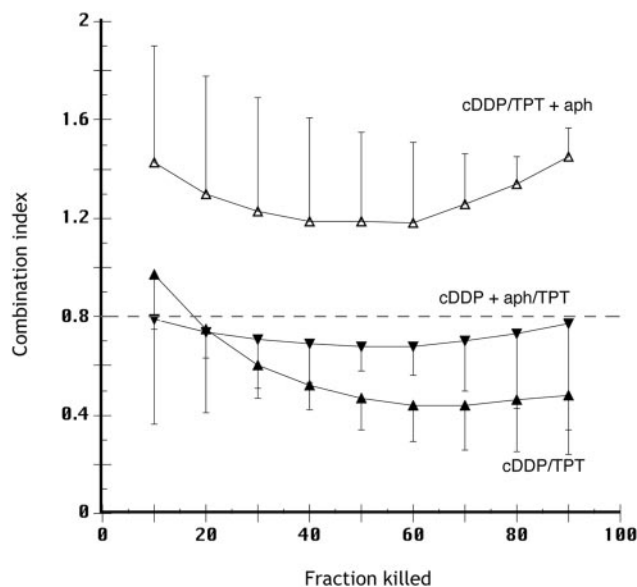


FIG. 3. The synergistic activity of cDDP/TPT requires processive DNA replication in the presence of TPT. As detailed in the legend to Fig. 2, subconfluent IGROV-1 cells were exposed to the combination of cDDP/TPT (▲; replotted from Fig. 2). In the cDDP + aph/TPT samples (▼), a sublethal dose of aphidicolin was added with cDDP for the entire duration of the experiment (5 days). In the cDDP/TPT + aph experiment (▲), cells were first exposed to cDDP for 2 days and then co-treated with TPT and aphidicolin for an additional 3 days. As for Fig. 2, the ratio of cDDP to TPT concentration was constant over the entire concentration range, whereas a fixed concentration of aphidicolin (<IC₁₀) was used. Data are the mean of at least three experiments. S.D. values are as indicated.

broad spectrum of adult and pediatric malignancies. These Top1 poisons also exhibit synergistic cytotoxic activity when used in combination with a wide range of DNA-damaging

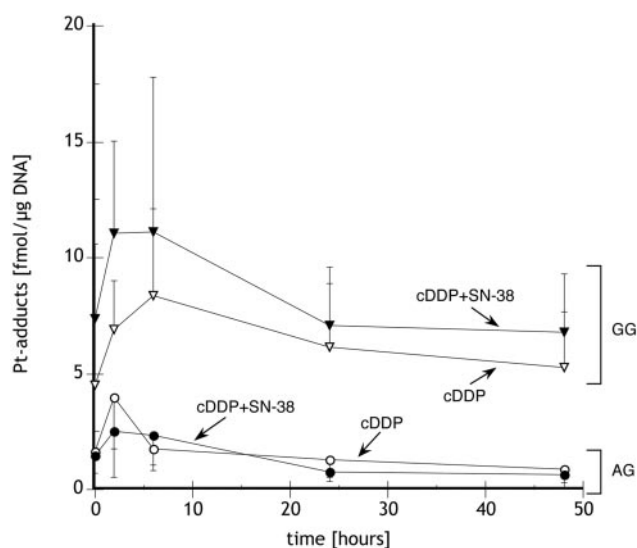


FIG. 4. The kinetics of Pt-DNA intrastrand adduct formation was unaffected by DNA topoisomerase I poisoning. As described under "Experimental Procedures," a ^{32}P -postlabeling assay was used to quantitate the formation of Pt-induced 1,2-d(GG) intrastrand adducts (\blacktriangledown and \triangledown) and 1,2-d(AG) intrastrand adducts (\bullet and \circ) in the absence (open symbols) or presence (closed symbols) of SN-38, the active metabolite of CPT-11. Subconfluent IGROV-1 cells, treated with $55\ \mu\text{M}$ cDDP for 2 h with or without 25 nM SN-38, were then cultured in medium with or without SN-38. Cells were harvested at 0, 2, 6, 24, and 48 h. The fmol of the indicated platinum adducts per μg of genomic DNA were plotted versus time. Data are the mean of at least three experiments. S.D. values are as indicated.

agents, including platinum drugs, Bleo, ara-C, and x-rays (14–16, 37–40). In the case of the nucleoside analogue ara-C, incorporation into DNA induces a structural change that increases the stability of Top1-DNA complexes, potentially acting as a Top1 poison (21, 22). However, there is considerably less understanding of the mechanisms underlying the interaction of these other agents with TPT. We recently reported that the synergistic activity of cDDP or x-ray with TPT in paired isogenic hamster cell lines was dependent on the presence of functional homologous recombination pathways. In contrast, a defect in homologous recombination enhanced the synergistic interactions of ara-C with TPT (20). Given the contrary effects of homologous recombination on cell sensitivity to these drug combinations, we asked whether poisoning of Top1 by platinated DNA adducts also contributed to the synergistic activity of these agents.

In addition to ara-C and platinum drugs, such as cisplatin (cDDP), we also included comparisons with other DNA-damaging agents, including X-rays, the alkylating agents BCNU and MNNG and Bleo. Based on the IC_{50} values of single agents obtained with a human ovarian carcinoma cell line IGROV-1 (shown for cDDP, TPT, and BCNU in Fig. 1 and summarized for all agents in Table I), the synergistic activity of these agents with TPT was examined. In drug combination experiments, the first agent (cDDP, x-ray, Bleo, ara-C, BCNU, or MNNG) was combined with TPT at a constant molar ratio, as described under "Experimental Procedures." The median effect analysis (30, 31) was then used to acquire a CI value for each drug combination, where $\text{CI} > 1.2$ was defined as antagonistic, $1.2 > \text{CI} > 0.8$ as additive, and a $\text{CI} < 0.8$ (indicated by a dashed line in Figs. 2 and 3) as synergistic. As reported for different schedules and cell lines (14, 31, 37, 38), the combinations of cDDP/TPT, x-ray/TPT, Bleo/TPT, and ara-C/TPT induced synergistic cell killing. Moreover, for cDDP/TPT (14), ara-C/TPT (data not shown), and x-ray/TPT (Fig. 2), this greater than additive cytotoxicity was schedule-dependent. Reversing the order in which the agents were adminis-

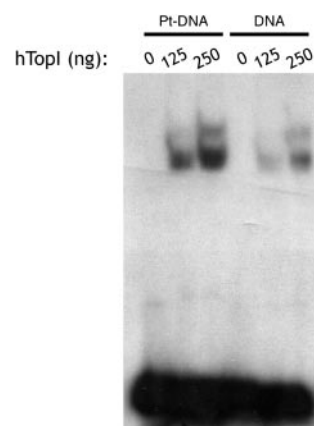


FIG. 5. DNA topoisomerase I exhibits enhanced covalent binding of platinated DNA. Purified human DNA topoisomerase I (125 or 250 ng) were incubated with ~ 32 fmol of duplex DNA (24-mer), in which a unique 1,3-d(GTG) site in one strand was undamaged (DNA) or platinated (Pt-DNA). The undamaged complementary oligonucleotide was $5'$ - ^{32}P -end-labeled. Following incubation with $0.4\ \mu\text{g}/\mu\text{l}$ CPT for 5 min at room temperature, the complexes were resolved in a 4% native polyacrylamide gel.

tered abolished the synergistic interaction of these agents when the fraction of cells killed exceeded 50%. Similar results were obtained for cDDP/TPT and x-ray/TPT in studies of the MCF-7 breast carcinoma cell line and for cDDP/TPT in the WiDr colon carcinoma cell line (data not shown). In all cases, the combinations of alkylating agents with Top1 poisons (BCNU/TPT and MNNG/TPT) exhibited additive cell kill. Thus, whereas the combinations of x-ray, cDDP, Bleo, or ara-C with TPT were synergistic, only the activity of TPT and Bleo was schedule-independent.

DNA Replication and Platinum Adduct Formation in Drug Combinations—To begin defining potential mechanisms of cDDP/TPT synergy, we first asked whether the cytotoxic activity of the single agents was altered in cells treated with both agents. As shown in Fig. 3, the synergistic activity of the cDDP/TPT schedule was abolished when aphidicolin was added with TPT, which followed treatment with cDDP. The concentration of aphidicolin used ($< \text{IC}_{10}$) has been empirically determined to be the most effective in reducing the cytotoxic activity of TPT in IGROV-1 cells, with the least effect on cell viability in the absence of TPT (data not shown). When aphidicolin was co-administered with cDDP, 2 days prior to TPT, there was significantly less effect on the synergistic activity of cDDP/TPT (Fig. 3). Since aphidicolin inhibits processive DNA polymerization, these findings demonstrate that on-going DNA replication was required for the synergistic activity of cDDP/TPT. Moreover, since this effect was only evidenced in the presence of TPT, these results are consistent with previous models in which the collision of advancing replication forks with TPT-stabilized Top1-DNA complexes is necessary for the cytotoxic activity of TPT and may also contribute to the synergistic activity of cDDP/TPT (6, 41).

The cytotoxic activity of platinum drugs, such as cDDP, is thought to result from the formation of inter- and intrastrand cross-links. Previous studies have assessed the kinetics of interstrand cross-link reversal in cells treated with cDDP/TPT or related platinum drug/TPT analogs (14–16, 40). Here we show that the kinetics of intrastrand cross-link formation and reversal were largely unaffected in IGROV-1 cells treated with cDDP and the active metabolite of CPT-11, SN-38 (Fig. 4). The levels of the two major intrastrand Pt-DNA adducts induced by cDDP, 1,2-d(AG) and 1,2-d(GG) cross-links, were measured following treatment of IGROV-1 cells with cDDP alone or cDDP/SN-38. Although the levels of cDDP-induced 1,2-d(AG) cross-links were unaffected by SN-38, there was a slight in-

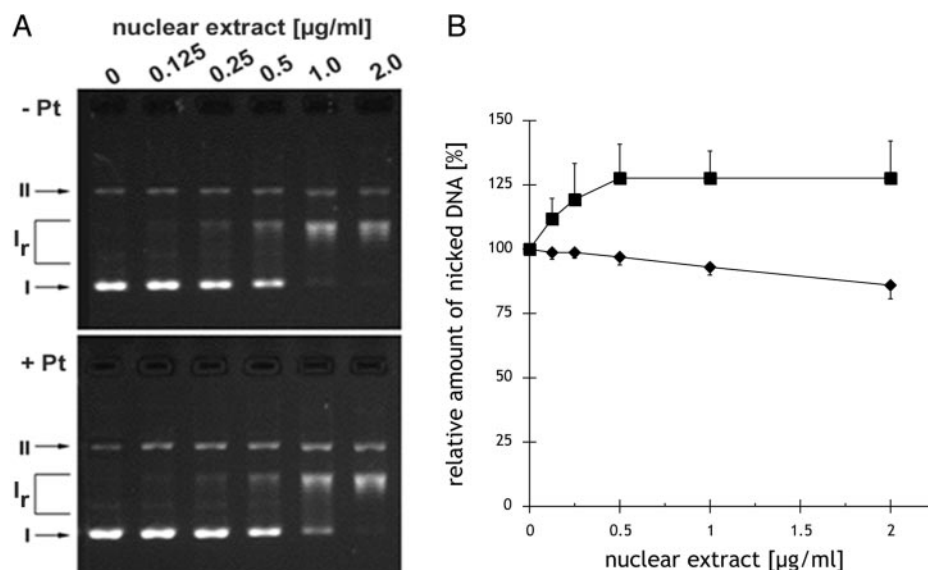


FIG. 6. DNA topoisomerase I exhibited increased nicking of platinated DNA. *A*, increasing concentrations of nuclear extracts prepared from subconfluent IGROV-1 cells were incubated with 250 ng of platinated (+Pt) or undamaged (-Pt) supercoiled pBR322 plasmid DNA in a relaxation assay as described under "Experimental Procedures." Following treatment with proteinase K, the reaction products were resolved by agarose gel electrophoresis in the absence of EtBr, followed by an additional 3-h electrophoresis in the presence of EtBr to effectively resolved supercoiled DNA (*I*), relaxed DNA topoisomers (*I_r*), and nicked DNA molecules (*II*). *B*, the intensity of the nicked DNA bands was determined for platinated (■) and undamaged (◆) pBR322 DNA substrates and plotted as a percentage of the values obtained in the no extract controls. The mean of at least three independent experiments is shown. S.D. values are as indicated.

crease in Pt-1,2-d(GG) adducts in the presence of SN-38. A similar increment in platinum-interstrand cross-links was also reported in the presence of Top1 poisons (14–16, 40); however, the biological significance of these findings remains unclear. Nevertheless, these data suggest that the underlying cytotoxic mechanism of each single agent was evident in cells treated with cDDP/TPT.

Pt-DNA Acts as a DNA Topoisomerase I Poison—To address the potential contribution of cDDP-induced DNA adducts, acting as Top1 poisons, to the schedule-dependent synergy of cDDP/TPT, we next asked whether DNA topoisomerase I activity was affected by platinated DNA.

First, the interaction between purified Top1 and Pt-DNA was assessed in a gel mobility retardation assay. A double-stranded oligonucleotide (24-mer) was used that contains a unique 1,3-d(GTG) site but lacks the canonical Top1 high affinity cleavage site. As diagrammed in Fig. 7C, a Pt-1,3-d(GTG) intrastrand adduct was introduced in one strand, which was subsequently annealed to a 5'-³²P-labeled complementary strand. This Pt-DNA and the corresponding undamaged DNA substrates were then incubated with human Top1 in the presence of CPT, and the reaction products were resolved in a native polyacrylamide gel. As shown in Fig. 5, a >4-fold increase in Top1-Pt-DNA complexes were detected with CPT, relative to those observed with undamaged DNA. In contrast, Top1-DNA or Top1-Pt-DNA complexes were barely detectable in the absence of CPT (data not shown). These results suggest the increased stabilization of Top1-DNA-CPT complexes in the presence of Pt-DNA adducts.

More direct evidence of platinated DNA acting as an endogenous Top1 poison in the absence of CPT was obtained in DNA nicking assays. As shown in Fig. 6A, there was a slight decrease in Top1 catalytic activity when IGROV-1 nuclear extracts were incubated with Pt-supercoiled plasmid DNA, relative to that observed in relaxation assays with unmodified DNA. However, as graphed in Fig. 6B, there was a significant increase in Pt-DNA nicking. These data show that Pt-DNA adducts act to enhance the stability of Top1-DNA covalent complexes. However, since these assays were based on Top1

activity in crude nuclear extracts, we investigated the stabilization of Top1-Pt-DNA complexes in the presence or absence of CPT in a well defined DNA cleavage assay.

In the representative experiments shown in Fig. 7, the same Pt-1,3-d(GTG) oligonucleotide used in the gel mobility shift assays was 3'-³²P-labeled with cordycepin (Fig. 7C) and annealed to an unlabeled complementary oligonucleotide. Equal concentrations of the labeled Pt-DNA and undamaged DNA control were then incubated in a DNA cleavage reaction with purified human Top1 in the absence or presence of CPT. As described under "Experimental Procedures," the reaction products were resolved in a denaturing gel, and the cleavage products were quantitated by PhosphorImager analysis. As seen in Fig. 7, *A* and *B*, there is an ~5-fold increase in Top1-mediated DNA cleavage at the C+1 position (*gray arrowhead* in Fig. 7C) of the Pt-DNA, relative to that obtained with undamaged DNA. Cleavage by Top1 at this C+1 site in the DNA control was enhanced ~12–18-fold in the presence of CPT; however, a novel platinum-specific cleavage site at the G0 site (*black arrowhead* in Fig. 7C) was enhanced ~3-fold in the presence of CPT. Cleavage at this site was not detected in the undamaged DNA control either with or without CPT. Taken together, these data indicate that a Pt-1,3-d(GTG) adduct increases the stability of a CPT-sensitive Top1p cleavage site at the C+1 position in the absence of CPT, whereas cleavage of the DNA backbone immediately 3' to the Pt-1,3-d(GTG) cross-link was selectively enhanced in the presence of CPT.

cDDP-induced Adducts Act as Persistent Top1 Poisons *In Vivo*—Since Pt-1,3-d(GTG) adducts comprise a relatively minor fraction of DNA lesions induced by platinum drugs, we next asked whether the ability of Pt-1,3-d(GTG) adducts to act as Top1 poisons *in vitro* correlated with increased Top1-DNA covalent complexes in treated cells. To address this, a modified ICE assay (36) was used to detect covalent Top1-DNA complexes in extracts of IGROV-1 cells treated with a synergistic schedule of cDDP/TPT. As controls, a reverse schedule of TPT/cDDP or BCNU/TPT was included, since these combinations failed to show any greater than additive activity. In these experiments, covalent Top1-DNA complexes, purified from Sar-

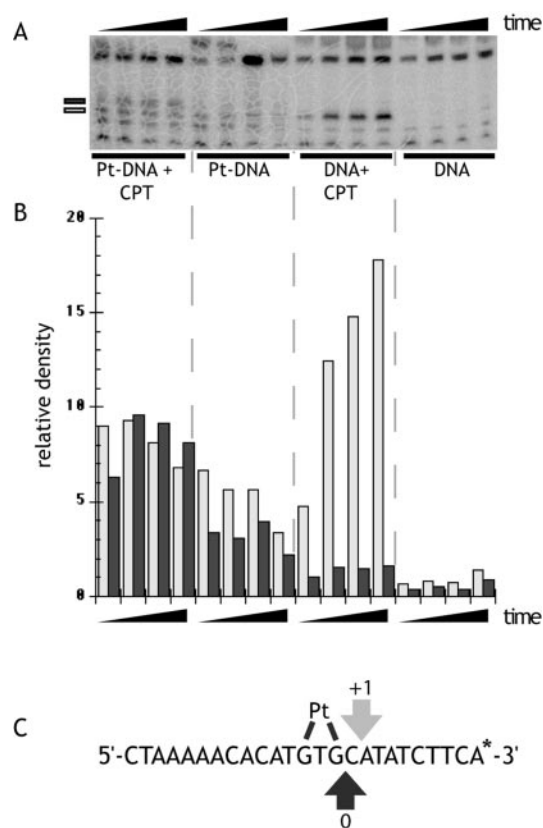


FIG. 7. Platinated DNA adducts act as endogenous DNA topoisomerase I poisons. A, purified human DNA topoisomerase I was incubated with a double-stranded DNA oligonucleotide (24-mer) in the presence of 50 μM CPT in a final 5% Me_2SO or Me_2SO alone. As shown in C, the scissile strand, labeled at the 3'-end with [^{32}P]cordycepin, contained a unique 1,3-d(GTG) site that was platinated (Pt-DNA) or undamaged (DNA). Following incubation for 10 s, 5 min, 10 min, and 20 min, the reaction products were denatured, resolved in a 20% polyacrylamide, 7 M urea gel, and visualized by PhosphorImager analysis. Cleavage sites were mapped using oligonucleotides of increasing length, containing the same sequences shown in C. In B, the relative density of the CPT-stabilized cleavage site (\square) and the unique cleavage site induced with platinated DNA (\blacksquare) are plotted. The same cleavage sites are indicated by the arrows in C.

kosyl-lysed cells by CsCl gradient centrifugation, were quantitated by slot blot analysis as detailed under "Experimental Procedures." As shown in Fig. 8, the relative amount of Top1-DNA covalent complexes for each of the drug combination were plotted relative to that obtained with TPT alone. Values obtained with cDDP as a single agent were slightly higher than those observed with TPT alone (data not shown). The short term (1-h) exposure of cells to cDDP/TPT or BCNU/TPT produced a 3.5- or 4.5-fold increase, respectively, in Top1-DNA covalent complexes. This was not observed with the reverse schedule of TPT/cDDP, consistent with the idea that preformed Pt-DNA adducts potentiate the activity of the Top1 poison, TPT. However, when cells were exposed to either cDDP or BCNU for 2 days, followed by TPT, only the combination of cDDP/TPT induced elevated levels of Top1-DNA covalent complexes (~ 6 -fold over TPT control). A similar rapid decay in Top1-DNA covalent complexes induced by MNNG/TPT has been reported (42). These findings suggest that the synergistic activity of cDDP/TPT results, in part, from the persistent formation of Pt-DNA adducts that act as Top1 poisons in concert with TPT. This contrasts with the rapid decay of Top1-DNA covalent complexes obtained with BCNU/TPT (Fig. 8) or MNNG/TPT (42) and the no greater than additive activity observed with either combination.

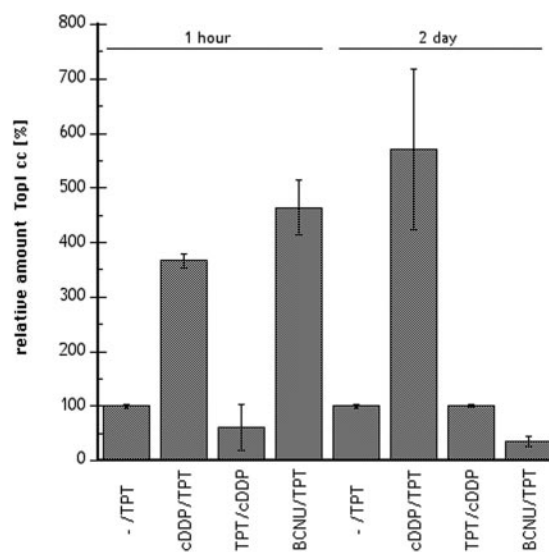


FIG. 8. The formation of Top1-DNA covalent complexes in IGROV-1 cells treated with cDDP/TPT is schedule-dependent. Subconfluent IGROV-1 cells were treated with the indicated drug combinations on a 1-h or 2-day schedule. For the 1-h samples, cells were treated with 5 times the IC_{50} concentration of the first drug, followed by either 0.5 μM TPT or 5 times the IC_{50} cDDP at 30 min. For the 2-day samples, cells were treated with the IC_{50} concentration of the first drug for 2 days, followed by a 30-min exposure to either 0.5 μM TPT or IC_{50} cDDP prior to cell lysis. As detailed under "Experimental Procedures," Top1-DNA covalent complexes were isolated from cell extracts by CsCl gradient centrifugation and immunoblotting of gradient fractions, based on the immunocomplex of enzyme assay. The Top1-DNA complexes were visualized by chemiluminescence and quantitated with the Eagle-eye II system. The relative percentage of Top1-DNA complexes was plotted relative to those obtained with TPT treatment alone. The mean and S.D. of three or more independent experiments are shown.

Increased Expression of DNA Topoisomerase I Enhances Cell Sensitivity to cDDP—The results obtained *in vitro* and with IGROV-1 cells indicate that Pt-DNA adducts may act as Top1 poisons. These data further suggest that the cytotoxic mechanism of Pt-DNA adducts might also derive from poisoning of Top1. However, the sensitivity of the ICE assay was insufficient to detect a dramatic increase in Top1-DNA covalent complexes isolated from cells treated with cDDP alone. Since DNA topoisomerase I overexpression is readily tolerated in yeast cells, we asked whether galactose-induced overexpression of DNA topoisomerase I from a YCpGAL-Top1 vector in a nucleotide excision repair-deficient W303*rad4* strain would increase cell sensitivity to cDDP in the absence of TPT or any other Top1 poison. Indeed, as shown in Fig. 9, W303*rad4* cells expressing elevated levels of yeast Top1 exhibited increased cell killing in response to cDDP relative to the same cells expressing wild-type levels of Top1.

DISCUSSION

As has been reported for ara-C (21, 22), we report here that Pt-DNA adducts also act as endogenous DNA topoisomerase I poisons to enhance the stability of covalent Top1-DNA complexes. These findings were consistent with the synergistic activity of platinum drugs in combination with the Top1 poisons, TPT or CPT-11. This cytotoxic mechanism was also evident in yeast cells overexpressing wild-type Top1 (Fig. 9). Pt-DNA adducts appear to selectively alter the DNA cleavage-religation equilibrium of Top1, since the same schedule of cDDP in combination with the DNA topoisomerase II poison, VP-16, failed to elicit synergistic activity or enhance TopII α or β cleavable complex formation (data not shown).

The ability of camptothecin to increase Pt-DNA adduct poisoning of Top1 (as shown in Figs. 5 and 7), provides further

insights into the schedule dependence of the synergistic toxicity of these agents, where formation of Pt-DNA adducts precedes exposure to TPT. Indeed, exposure of IGROV-1 cells to

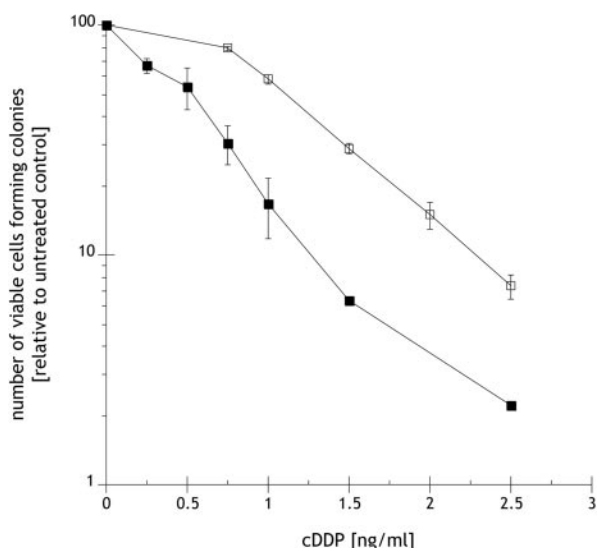


FIG. 9. Overexpression of DNA topoisomerase I increased yeast cell sensitivity to cDDP. Yeast W303*rad4* cells, transformed with expression vector YCpGAL1-*TOP1-L* (■) or vector control (□), were grown in selective media supplemented with galactose to induce *TOP1* expression. Exponential cultures were treated with increasing concentrations of cDDP for 2 h, and cell viability was assessed in a colony-forming assay. The percentage of viable cells was plotted relative to the untreated controls. The mean and S.D. of at least three independent experiments are shown.

cDDP for either 1 h or 2 days prior to a short exposure to TPT produced elevated levels of covalent Top1-DNA complexes, relative to either drug alone (Fig. 8 and data not shown). On the other hand, the additive activity of the alkylating agent BCNU in combination with TPT was independent of the order of drug addition (Fig. 2 and data not shown). However, previous studies demonstrated more effective cytotoxicity when cells were treated with BCNU during the first hour of a 24-h exposure to TPT (43). These findings are consistent with the results shown in Fig. 8, where the relatively high levels of covalent Top1-DNA complexes detected in the 1-hour BCNU/TPT schedule were not observed with the 2-day schedule. Taken together, these data suggest the persistence of Pt-DNA adducts, which potentiate TPT poisoning of Top1, is necessary for the synergistic activity of these agents. This contrasts with the relatively short lived BCNU-induced DNA lesions capable of poisoning Top1. Although the O6-G modifications induced by BCNU and the alkylating agent MNNG, O6-chloroethyl, and O6-methyl adducts, respectively, are both repaired by O6-alkylguanine-DNA alkyltransferase, only O6-methyl guanine has been shown to trap Top1 on DNA (42). Nevertheless, a similar pattern of short lived covalent Top1-DNA complexes was induced by MNNG (42), as shown here for BCNU/TPT. As the combinations of BCNU or MNNG with TPT were only additive at best, it appears that the persistence of DNA adducts capable of trapping Top1 is necessary to elicit synergistic toxicity in combination with TPT.

In the case of ara-C, incorporation of the modified nucleoside immediately opposite to the site of Top1-mediated cleavage in the complementary DNA strand has been shown to trap Top1 (21). Although ara-C incorporation has been shown to correlate with toxicity, it also acts to inhibit DNA synthesis. In contrast,

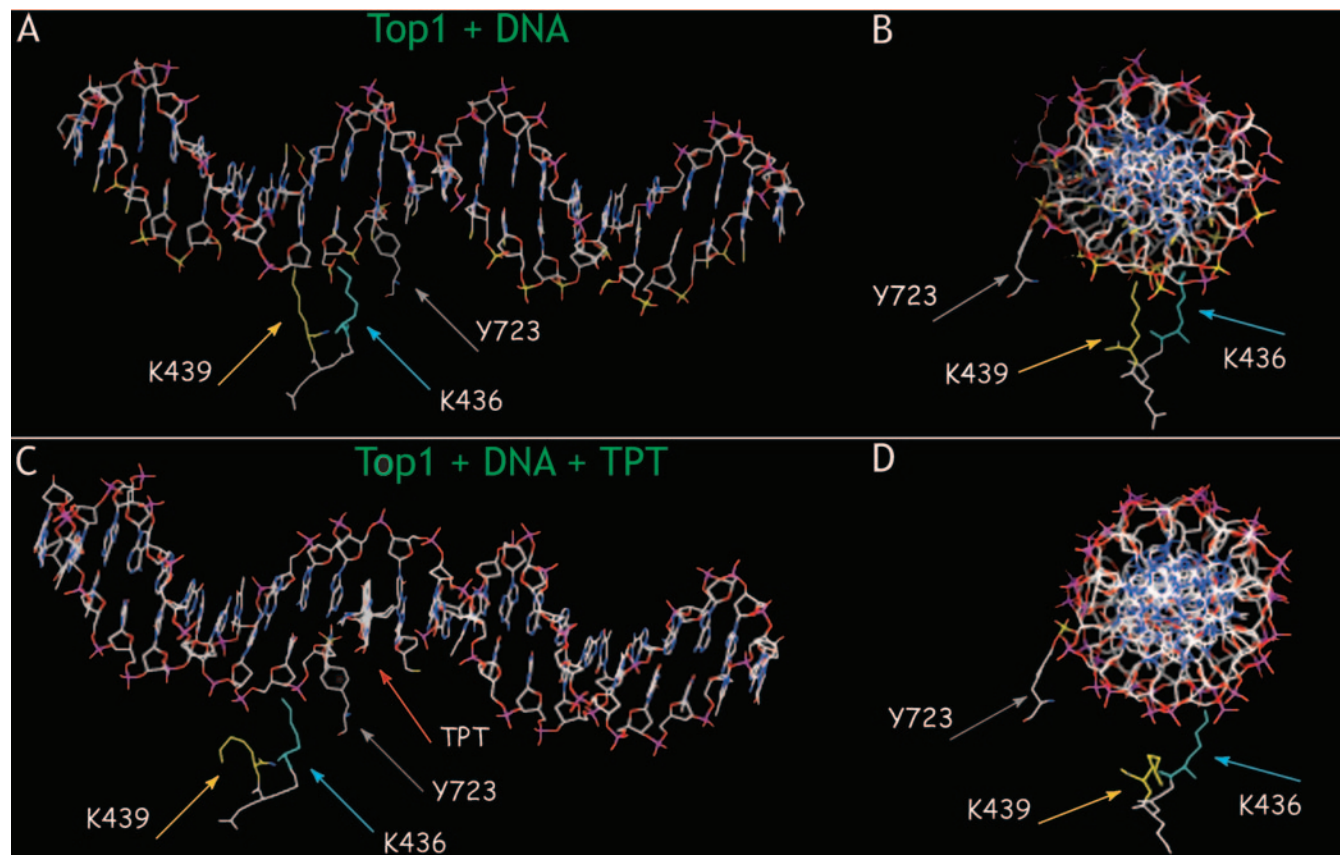


FIG. 10. Alterations in DNA minor groove interactions with Lys⁴³⁹ induced by TPT binding to the covalent Top1-DNA complex. Co-crystal structures of human Topo-70 with DNA (A and B) or with DNA and TPT (C and D), where only the active site tyrosine (Tyr⁷²³; Y723) and residues Lys⁴³⁶-Lys⁴³⁹ (K436-K439) of the flexible hinge connecting the CAP and CAT domains of the Top1 protein clamp are shown. A and C were rotated 90° to generate B and D, respectively. WebMol was used to generate these images from Protein Data Bank files 1K4S (human Topo-70 with 22-bp duplex DNA) and 1K4T (human Topo-70 with 22-bp duplex DNA and TPT), originally reported by Staker *et al.* (49).

Pt-DNA adducts occur independent of DNA synthesis. Although some Pt-DNA adducts are rapidly repaired, constant levels of Pt-1,2-d(GG) and Pt-1,2-d(AG) adducts are detected over time in IGROV-1 cells (Fig. 4). Both ara-C and Pt-DNA adducts act as Top1 poisons; however, the synergistic activity of ara-C/TPT is exacerbated by defects in HR repair pathways, whereas the synergy induced by cDDP/TPT is suppressed by the same deficiencies in HR (20). Whether these distinct mechanisms of drug synergy reflect the different effects of ara-C and Pt-DNA adducts on replication fork progression and/or fork stability have yet to be determined.

With cisplatin, the major cytotoxic DNA adduct (65%) is the 1,2-d(GG) intrastrand cross-link, consistent with the data in Fig. 4. However, with carboplatin, the major adducts are 1,3-d(GXG) (36%) and 1,2-d(GG) (30%) (44). Despite these variations in the spectrum of cytotoxic DNA adducts formed with these different cisplatin analogs, both demonstrate synergistic activity with TPT. Moreover, NMR and crystallographic analyses of duplex DNA containing 1,2-d(GG) or 1,3-d(GTG) platinum adducts indicate common structural aberrations (see Refs. 45–48 and references therein). In both cases, a significant bending of the helical axis of the DNA is detected, although the Pt-1,3-d(GTG)-adducted duplex is more distorted than DNA containing a Pt-1,2-d(GG) adduct. Watson-Crick base pairing of the 5' GT bases is completely disrupted as a consequence of 1,3-d(GTG) platination. The increased propeller twist and rotation of the 5' G, coupled with the extrusion of the T, further induces a flattening of the minor groove and a bulge in the phosphodiester backbone of the Pt-DNA strand. In contrast, there is much less distortion of the 3' G base pairing to C in the complementary strand for both Pt-1,3-d(GTG) and Pt-1,2-d(GG) adducts.

A slight distortion of the scissile DNA strand, 5' to the site of Top1 cleavage, is also induced by TPT binding to the covalent enzyme-DNA complex (49). As shown in Fig. 10, this distortion causes the phosphodiester backbone to clash with the side chain of Lys⁴³⁹, which lies in a flexible hinge that connects the CAP and CAT domains of Top1. In the context of Pt-1,3-d(GTG) or 1,2-d(GG) DNA, it is tempting to speculate that distortion of the minor groove in the 5' DNA strand also clashes with Lys⁴³⁹, inducing a 1-bp shift in DNA cleavage to the 3' G in the Pt-1,3-d(GTG) sequence, as seen in Fig. 7. Once formed, the structural distortion and unwinding of base pairs immediately 5' to the site of cleavage, evident in the NMR structures of Pt-1,3-d(GTG) and 1,2-d(GG) DNA, might preclude the alignment of the 5'-OH nucleophile of the DNA necessary for efficient DNA religation. This model of Pt-DNA adducts acting as endogenous Top1 poisons is also consistent with the results of the COMPARE program analysis of the activity of cisplatin against the NCI cell line panel, which defined a similar pattern of activity between cisplatin and camptothecin analogs (50).

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