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Protein-Linked DNA Strand Breaks Induced by NSC 314622, a Novel Noncamptothecin Topoisomerase I Poison

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ABSTRACT

NSC 314622 was found to have a cytotoxicity profile comparable to the topoisomerase I (top1) inhibitors camptothecin (CPT) and saintopin in the National Cancer Institute *In Vitro* Anticancer Drug Discovery Screen using the COMPARE analysis. *In vitro* data showed that NSC 314622 induced DNA cleavage in the presence of top1 at micromolar concentrations. Cleavage specificity was different from CPT in that NSC 314622 did not cleave all sites induced by CPT whereas some sites were unique to the NSC 314622 treatment. Top1-induced DNA cleavage was also more stable than cleavage induced by CPT. NSC 314622 did not induce DNA cleavage in the presence of human topoisomerase II. High concentrations of NSC 314622 did not produce detectable DNA unwinding, which suggests that NSC 314622 is not a DNA intercalator. DNA damage analyzed in human breast carcinoma MCF7 cells by alkaline elution showed that NSC 314622 induced protein-linked DNA single-strand breaks that reversed more slowly than CPT-induced strand breaks. CEM/C2, a CPT-resistant cell line because of a top1 point mutation [*Cancer Res* **55:**1339–1346 (1995)], was cross-resistant to NSC 314622. These results demonstrate that NSC 314622 is a novel top1-targeted drug with a unique chemical structure.

CPT derivatives have recently been introduced in the clinic and are among the most promising novel anticancer drugs. The only known cellular target of CPTs is mammalian DNA top1 [for reviews, see Chen and Liu (1994) and Pommier (1996)]. Top1 plays an essential role in DNA metabolism and its catalytic intermediates are DNA cleavage complexes [for reviews see Champoux (1990), Gupta *et al.* (1995b), Wang (1996), and Pommier (1998)]. These cleavage complexes consist of DNA single-strand breaks that are generated by top1 as the enzyme cleaves the DNA and forms a covalent bond with the 3′-DNA termini. Under physiological conditions, the cleavage complexes are reversible and top1 religates the broken DNA. Camptothecin and its clinical derivatives trap top1 cleavage complexes by inhibiting their religation and as a consequence generate DNA damage.

The fact that camptothecins are active against solid tumors including ovarian and colon carcinomas and are the only class of top1 poisons used in the clinic to date prompted us to look for novel top1 inhibitors. Here we report a novel top1 poison, NSC 314622 (Fig. 1), with original structure and describe its molecular and cellular interactions with mammalian top1.

Materials and Methods

Synthesis of NSC 314622. This compound was synthesized in two steps from 4,5-dimethoxyhomphtalic anhydride and 3,4-methylenedioxy benzylidenemethylamine according to our previously published procedure (Cushman and Cheng, 1978). The melting point and IR- and ^{1}H-NMR spectra of the synthetic compound compared favorably with the published data and were identical with those produced by a reference sample on the same spectrometer at the same time. NSC 314622 is a stable substance that displayed no signs of decomposition when stored over long periods of time in the solid state.

Drugs, enzymes, and chemicals. Camptothecin was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD). Drug stock solutions were made in DMSO at 10 mM and aliquots were stored at −20°. Further dilutions were made in DMSO immediately before use. The final concentration of DMSO in the reactions did not exceed 10% (v/v).

Calf thymus top1, T4 polynucleotide kinase, terminal deoxynucleotidyl transferase, *Taq* DNA polymerase, DNA polymerase I (Klenow fragment), dNTP [where N is A (adenosine), C (cytidine), G (guanosine), or T (thymidine)], agarose, and polyacrylamide/bis were purchased from Perkin-Elmer Cetus (Norwalk, CT), GIBCO BRL (Gaithersburg, MD) or New England Biolabs (Beverly, MA). [γ-^{32}P]ATP, [α-^{32}P]deoxyCTP, and [α-^{32}P]cordycepin were purchased

ABBREVIATIONS: CPT, camptothecin; top, topoisomerase; bp, base pair(s); DMSO, dimethylsulfoxide; GI50, growth inhibition 50% concentrations; NSC 314622, 5,6-dihydro-5,11-diketo-2,3-dimethoxy-6-methyl-8,9-methylenedioxy-11*H*-indeno(1,2-*c*)isoquinoline; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; SV40, simian virus 40; TBE, Tris/borate/EDTA.

from DuPont-New England Nuclear (Boston, MA). SV40, c-myc human DNA inserted in pBR322, and oligonucleotides were purchased from GIBCO BRL, the American Type Culture Collection (Rockville, MD), and Midland Certified Reagent (Midland, TX), respectively.

Cell cultures. The MCF7 cells were obtained from Dr. Dominic Scudiero (Developmental Therapeutics Program, National Cancer Institute, Frederick, MD). The CEM/C2 cell line was established as described previously (Fujimori *et al.*, 1995, 1996). Cells were maintained in RPMI 1640 (GIBCO BRL, Gaithersburg MD) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA) and 2 mM L-glutamine in a 5% CO_2 incubator at 37°.

Top1-mediated DNA cleavage reactions using end-labeled *c-myc* DNA, 161-bp plasmid DNA, and the top1 oligonucleotides. The 401-bp DNA fragment from the human *c-myc* proto-oncogene includes the junction between the first intron and first exon. It was prepared by PCR using the sense primer (oligo 2671–2692) and the antisense primer (oligo 3052–3072); the numbers in parenthesis refer to the genomic position of the corresponding base in *c-myc* DNA (Leteurtre *et al.*, 1994). Single-end labeling was obtained by ^{32}P-5′-end labeling of the sense primer. Twenty picomoles of DNA were incubated for 30 min at 37° with 10 units of T4 polynucleotide kinase and 10 pmol of [γ-^{32}P]ATP (approximately 100 μCi) in kinase buffer (70 mM Tris·HCl, pH 7.6, 0.1 M KCl, 10 mM $MgCl_2$, 5 mM dithiothreitol, and 0.5 mg/ml bovine serum albumin). Reactions were stopped by heat denaturation at 70° for 10 min. Five picomoles of the labeled primer and 10 pmol of the antisense primer (unlabeled) were used for the PCR. Approximately 0.1 μg of the c-myc plasmid that had been cleaved with *Sma*I and *Sac*I was used as a template during the 22 temperature cycle reactions (each cycle: 95° for 1 min, 56° for 1.5 min, and 72° for 2 min). The last extension was for 7 min. DNA was purified using a G-50 quick spin column (Boehringer-Mannheim Biochemicals, Indianapolis, IN). The resulting 401-bp PCR fragment corresponded to the human *c-myc* DNA region between positions 2671 and 3072, according to the GenBank numbers. This fragment was used to assay top1-induced DNA cleavage by CPT or NSC 314622. Top1 reactions were performed at 30° for 30 min in the presence of CPT or NSC 314622. The same volume of DMSO used in the drug-treated samples was added to the reactions without drug. Reactions were terminated by adding 0.5% SDS followed by digestion with 0.5 mg/ml proteinase K for 60 min at 50°. Reversibility of cleavage complexes was tested by adding 0.5 M NaCl 15 min before terminating the reactions.

The 161-bp fragment from pBluescript SK(−) phagemid DNA (Stratagene, La Jolla, CA) was cleaved with the restriction endonucleases *Pvu*II and *Hin*dIII (New England Biolabs, Beverly, MA) in supplied NE buffer 2 (50-μl reactions) for 1 hr at 37°, separated by electrophoresis in a 1% agarose gel made in 1 × TBE buffer. The 161-bp fragment was eluted from the gel slice (centrilutor by Amicon, Beverly, MA) and concentrated in a centricon 50 centrifugal concentrator (Amicon). Approximately 200 ng of the fragment was 3′-end labeled at the *Hin*dIII site by fill-in reaction with [α-^{32}P]dCTP and 0.5 mM dATP, dGTP, and dTTP, in REact 2 buffer (50 mM Tris·HCl, pH 8.0, 100 mM MgCl, and 50 mM NaCl; New England Biolabs) with 0.5 units of DNA polymerase I (Klenow fragment). Labeling reactions were followed by phenol/chloroform extraction and ethanol precipitation. The resulting 161-bp 3′-end-labeled DNA fragment was resuspended in water. Aliquots (approximately 50,000 dpm/reaction) were incubated with top1 at 30° for 15 min in the presence of NSC 314622 or CPT. Reactions were terminated by adding 0.5% SDS. After ethanol precipitation the samples were resuspended in loading buffer (80% formamide, 10 mM sodium hydroxide, 1 mM sodium EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue, pH 8.0), and separated in a denaturing gel (16% polyacrylamide and 7 M urea) run at 51°. The gel was dried and visualized by using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

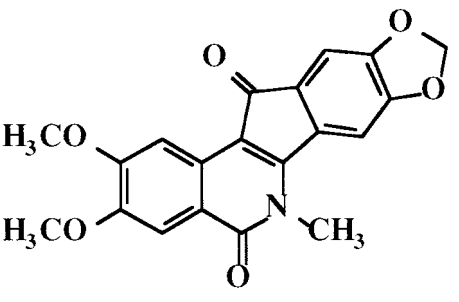


Fig. 1. Chemical structure of NSC 314622.

The DNA oligonucleotide with a single top1 cleavage site (Pommier *et al.*, 1995; synthesized by Midland Certified Reagent) was used to assay top1 sensitivity to CPT and NSC 314622. The 32-mer DNA was labeled at the 3′-terminus on the sense strand with [α-^{32}P]cordycepin and terminal transferase, as described previously (Pommier *et al.*, 1995). Aliquots of this oligonucleotide (20,000-50,000 dpm/reaction) were incubated with top1 at 37° for 15 min in the presence of CPT or NSC 314622. Reactions were terminated by adding 0.5% SDS. Samples were denatured by adding four volumes of loading buffer (80% formamide, 10 mM sodium hydroxide, 1 mM sodium EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue, pH 8.0), and separated in a denaturing gel (16% polyacrylamide and 7 M urea) run at 51°. Top1-mediated cleavage generates a fast migrating DNA band that corresponds to a cleaved (19-mer) product from the uncleaved substrate (33-mer) (see Fig. 5A). Radioactivity of the cleaved and uncleaved products was quantified using a PhosphorImager and ImageQuant software.

SV40 DNA unwinding assay. Reaction mixtures (10 μl final volume) contained 0.3 μg supercoiled SV40 DNA in reaction buffer (10 mM Tris·HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, and 15 μg/ml bovine serum albumin) and 10 units of purified calf thymus top1 (Pommier *et al.*, 1987). Reactions were performed at 37° for 30 min and terminated by the addition of 0.5% SDS. Next, 1.1 μl of 10× loading buffer (20% Ficol 400; 0.1 M Na_2EDTA, pH 8.0, 1.0% SDS, and 0.25% bromphenol blue) was added and reactions mixtures were loaded onto a 1% agarose gel made in 1 × TBE buffer. After electrophoresis, DNA bands were stained in 10 μg/ml of ethidium bromide and visualized by transillumination with ultraviolet light (300 nm).

Top1-mediated DNA relaxation assays. Reactions were performed in a 10-μl final volume at 37° in reaction buffer (see above) with 0.3 μg supercoiled SV40 DNA and 1 unit of top1 per reaction. Aliquots were taken at indicated times and stopped with 0.5% SDS (final concentration). Samples were digested with 0.5 mg/ml proteinase K for 15 min at 50°, mixed with 1.1 μl of 10 × loading buffer (see above), and subjected to electrophoresis in a 1% agarose gel made in 1 × TBE buffer. The ethidium bromide (0.5 μg/ml) stained supercoiled bands were visualized using a Molecular Dynamics FluorImager and quantified with ImageQuant software.

DNA single-strand breaks, measurements by alkaline elution. Human MCF7 breast carcinoma cells were treated with NSC 314622 or CPT for 1 hr. Aliquots were analyzed by alkaline elution (Covey *et al.*, 1989; Bertrand and Pommier, 1995).

Cytotoxicity assays in human leukemia CEM and CPT-resistant CEM/C2 cells. Exponentially growing CEM and CEM/C2 cells were treated with NSC 314622 or CPT for the times indicated in Table 1. The cells were counted with a Coulter Electronics (Miami Lake, FL) Counter model ZBI before and 48 hr after beginning of treatment. Growth fraction was calculated relative to control (untreated cells) with control being 1.

Results

COMPARE analysis of NSC 314622. NSC 314622 was selected as a potential top1 inhibitor by COMPARE analysis in the National Cancer Institute *In Vitro* Anticancer Drug

Discovery Screen. The COMPARE algorithm was developed to permit the rapid selection of compounds with similar or novel cytotoxicity relative to established anticancer agents with known mechanisms of action (Boyd and Paull, 1995; Paull *et al.*, 1995). If the data pattern of an agent of interest correlates well with the data patterns of one or more agents of known mechanism of action, then the hypothesis is that the agent of interest may have the same mechanism of action as those agents of known mechanism. It is always necessary to confirm this hypothesis in the laboratory. If the agent of interest has no good correlation with a set of agents comprising most known biochemical mechanisms of action, the hypothesis is that it has a novel mechanism. This hypothesis is difficult to prove so it remains tentative until the agent's mechanism is actually elucidated in the laboratory.

First, using saintopin, a non-CPT top1 inhibitor (Leteurtre *et al.*, 1994) as a seed in the COMPARE analysis, we found that NSC 314622 was ranked fifth after saintopin and three of its derivatives and that the compounds with highest ranking included 20 CPT derivatives as well as other top1 inhibitors including benzophenanthridines and indolocarbazoles (Pommier, 1998). This result strongly suggested that NSC 314622 was a potential top1 inhibitor.

Fig. 2 shows the cytotoxicity profile of NSC 314622 as a mean graph representation and the similarity between the cytotoxicity profiles of NSC 314622, CPT, and topotecan. A large number of CPT derivatives (33) showed a high Pearson correlation coefficient, 0.84–0.78, with NSC 314622 (Table 1). The correlation coefficient for NSC 314622 with topotecan was also high, 0.74, but with the unsubstituted CPT, NSC 94600, it was 0.63.

NSC 314622 induces top1-mediated DNA cleavage complexes with a different pattern from CPT. Induction of DNA cleavage in the presence of top1 was tested in a fragment of the human c-myc gene (Fig. 3A) and in the *Pvu*II/*Hin*dIII fragment of pBluescript SK(−) phagemid DNA (Fig. 3B). A number of cleavage sites detected in the presence of NSC 314622 were also induced by CPT. However, NSC 314622 induced top1 sites that were not observed with CPT, but did not stabilize all the CPT sites, and the relative cleavage intensity of similar sites varied between NSC 314622 and CPT. Top1-induced cleavage in the presence of NSC 314622 was detectable at 0.3 μM and increased with increasing drug concentrations up to 100 μM. These results confirmed the COMPARE analysis that NSC 314622 is a top1 poison and suggested that the DNA cleavage patterns induced by NSC 314622 exhibited similarities and differences from those of CPT.

Top1 linkage and reversibility of the top1 cleavage complexes induced by NSC 314622. Top1 cleavage complexes are protein linked (Hsiang *et al.*, 1985; Covey *et al.*, 1989), with top1 covalently linked to the 3′-terminus of the DNA break that it generates. Thus, with the use of 5′-end-labeled DNA, the labeled strand is expected to become covalently linked to top1. Fig. 4A shows that treatment of 5′-end-labeled c-myc DNA with either CPT or NSC 314622 in the presence of top1 resulted in DNA retention at the top of the gel when the proteinase K digestion step was omitted. Treatment with proteinase K released the high molecular-weight DNA and generated the cleaved DNA fragments (Fig. 4B). This result demonstrates that the breaks induced by NSC 314622 are protein linked.

CPT enhances top1 cleavage by stabilizing the cleavable complexes and inhibiting DNA religation (Hsiang *et al.*, 1985). However, increasing salt concentration can reverse the CPT-induced cleavable complexes, and this method has been used to compare the molecular interactions between CPT derivatives and top1 cleavage complexes (Tanizawa *et al.*, 1995; Valenti *et al.*, 1997). As shown in Fig. 4B, cleavage sites induced by NSC 314622 and CPT were reversed with

TABLE 1
COMPARE analysis of NSC 314622 using GI50 data from the National Cancer Institute *In Vitro* Anticancer Drug Screen

Rank	NSC	LHICONC	Max X	CORR	N	Mechanism/class
1	314622	−4.00	5	1.000	60	top1
2	D	−4.00	3	0.845	60	top1
3–35				0.842–0.778		33 camptothecins
36	9706	−4.00	137	0.777	60	alkylating agent
37	D	−4.00	3	0.776	60	1 camptothecin
38	D	−4.00	2	0.775	59	analog NSC 314622
39	132313	−3.00	4	0.775	60	alkylating agent
40–52				0.774–0.765		12 camptothecins
53	D	−4.00	2	0.764	60	alkylating agent
54–61				0.763–0.760		8 camptothecins
62	167781	−4.00	4	0.760	60	alkylating agent
63–71				0.759–0.751		9 camptothecins
72	638436	−4.00	1	0.751	40	top1 and/or top2
73–74				0.751–0.750		2 camptothecins
75	3088	−3.12	127	0.749	60	alkylating agent
76	6396	−3.00	130	0.748	60	alkylating agent
77–79				0.747		3 camptothecins
80	620480	−4.00	3	0.747	58	alkylating agent
81	D	−4.00	2	0.744	60	bleomycin analog
82–91				0.744–0.740		10 camptothecins
92	48034	−4.00	2	0.739	59	alkylating agent
93	D	−6.00	2	0.739	58	alkylating agent
94	D	−4.12	2	0.739	59	1 camptothecin
95	275656	−4.00	3	0.738	57	alkylating agent
96–100				0.737–0.734		5 camptothecins

NSC, number key to the NCI database (D indicates discrete compound); LHICONC, decimal logarithm of the highest drug concentration tested (molar concentration); Max X, measure of the number of tests that were averaged for this compound; CORR, Pearson coefficient for the seed and the data base compound; and N, number of cell lines common to both the seed and the database compound, which therefore are used in the calculation of the CORR.

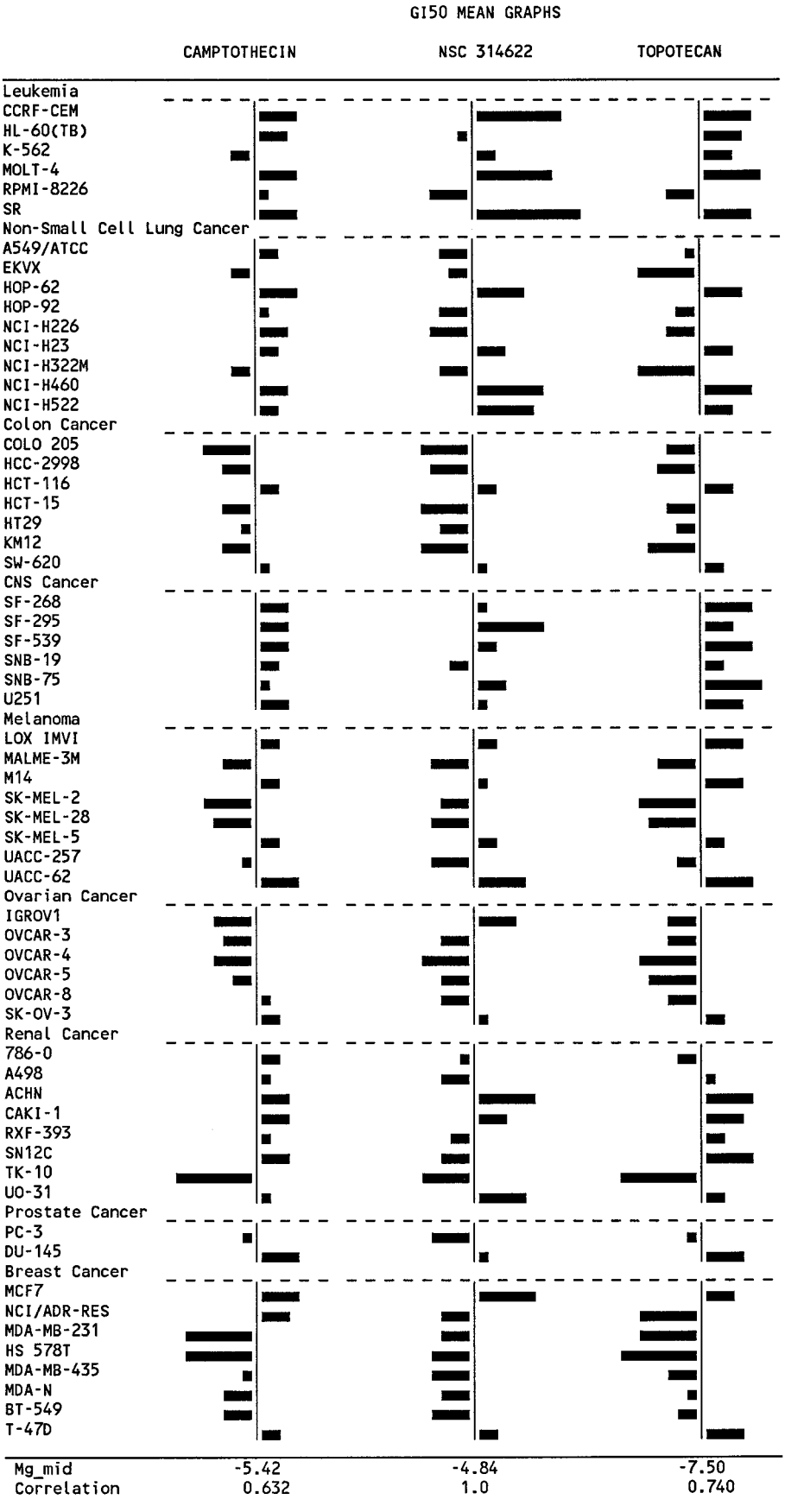


Fig. 2. Mean graph representation of the cytotoxicity profile of NSC 314622 in the 60 cell lines of the National Cancer Institute Anticancer Drug Screen. GI50s were used to generate the graph. The profiles of CPT and topotecan are shown for comparison. The mean GI50 (*Mg_mid*) and the Pearson correlation coefficient (*correlation*) of the COMPARE analysis of topotecan and CPT are indicated below each profile.

salt treatment. This reversibility is consistent with the reversible trapping of top1 cleavable complexes by NSC 314622.

Stability of the top1 cleavage complexes induced by NSC 314622. We also tested the induction of top1 cleavage complexes using an oligonucleotide containing a single top1 cleavage site (Fig. 5A) (Pommier *et al.*, 1995). In this system, CPT-induced DNA cleavage was seen as an increase of the 19-mer product, and after 1 hr CPT-induced DNA cleavage decreased. This decrease could be due to inactivation of top1 or CPT lability during the time course of the reaction. NSC 314622 also enhanced top1 cleavage in this oligonucleotide at the same site as CPT. The cleavage complexes induced by NSC 314622 were more persistent than the cleavable complexes induced by CPT (Fig. 5, B and C).

NSC 314622 inhibits top1-mediated DNA relaxation. Top1 is very efficient at relaxing DNA supercoiling (Champoux, 1990; Wang, 1996). Relaxation of SV40 DNA by top1 was reduced at every time point by NSC 314622 when compared with the top1-mediated SV40 DNA reactions in the absence of drug (Fig. 6). Thus, NSC 314622 inhibits top1 catalytic activity.

NSC 314622 does not unwind DNA. Unwinding assay using supercoiled DNA in the presence of top1 is a simple procedure to detect DNA intercalation (Pommier *et al.*, 1987). In this assay, excess top1 (10 units instead of the 1 unit per reaction shown in Fig. 6) is used to compensate for an eventual inhibitory effect of the drug tested in the assay (Pommier *et al.*, 1987). Fig. 7 shows that NSC 314622 had no detectable effect on top1-mediated relaxation of SV40 DNA. These results suggest that NSC 314622 is not a DNA intercalator up to a concentration of 100 μM.

Protein-linked DNA single-strand breaks induced by NSC 314622 in human breast carcinoma MCF7 cells. Drug-induced top1 cleavage complexes were assayed in MCF7 cells by alkaline elution. Typically, in the case of CPT (Covey *et al.*, 1989), top1 cleavage complexes can be found as DNA single-strand breaks that are detectable only after proteinase digestion of the cell lysates. Fig. 8 shows that 1 μM NSC 314622 produced approximately 300 rad-equivalent DNA single-strand breaks and that, as in the case of CPT, these breaks were protein associated (top 1 linked).

Reversal kinetics of DNA single-strand breaks induced by NSC 314622 in MCF7 cells. As shown in Fig. 9,

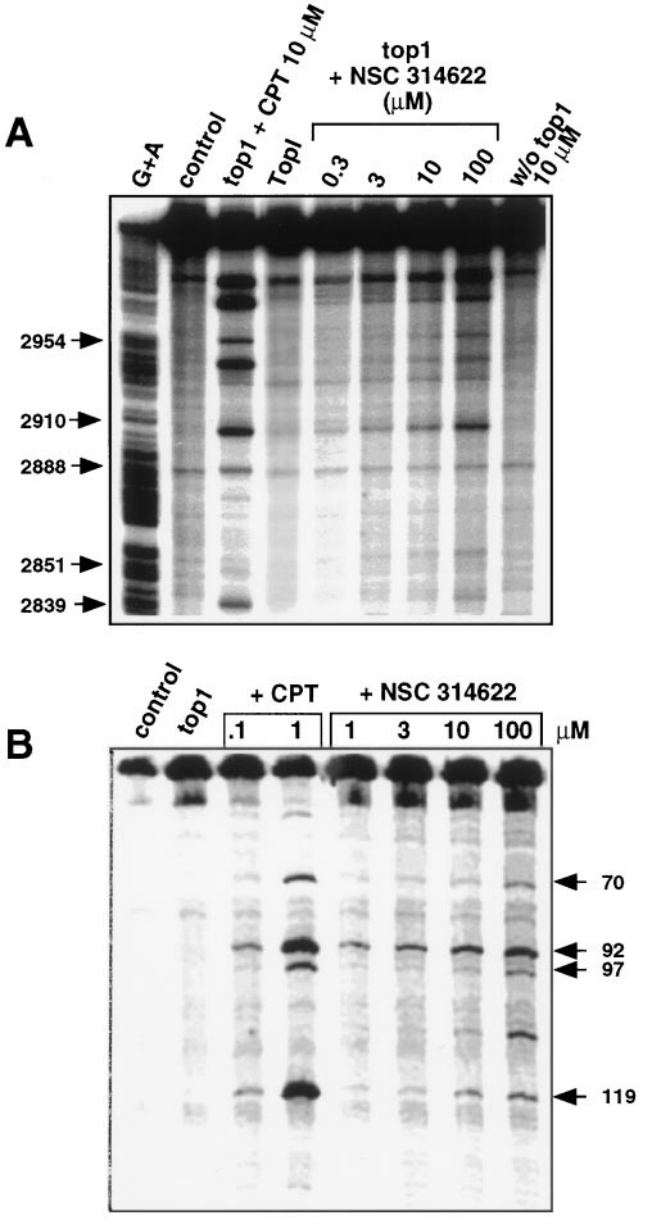


Fig. 3. Top1-mediated cleavage induced by NSC 314622. A, The DNA fragment used corresponds to the 5′-end-labeled sense strand of the human c-myc protooncogene encompassing the junction of the first intron and the first exon. B, The DNA corresponds to the 3′-end-labeled *Pvu*II/*Hin*dIII fragment of pBluescript SK(−) phagemid DNA. DNA fragments were reacted with top1 in the presence of the indicated concentrations of NSC 314622 or CPT. Reactions were at 30° for 30 min and stopped by adding 0.5% SDS followed by proteinase K digestion. DNA fragments were separated on 7% denaturing polyacrylamide gels. A, *Lane G + A*: purine ladder; *right lane*: DNA reacted with 10 μM NSC 314622 in the absence of top1. *Numbers to the side of the gels*, migration position of DNA fragments cleaved at this position in the DNA fragment analyzed.

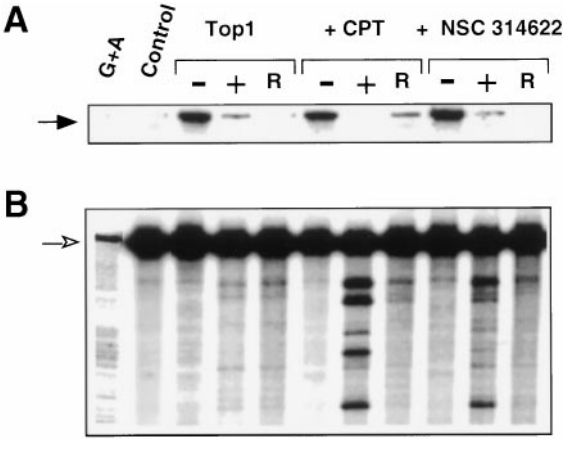


Fig. 4. Protein linkage and reversibility of the top1 cleavage complexes (DNA breaks and top1-DNA complexes) induced by NSC 314622. The 5′-end-labeled c-myc DNA fragment used was the same as the one shown in Fig. 3a. Reactions were performed with top1 in the absence of drug (top1) or in the presence of 10 μM CPT (+ CPT) or 100 μM NSC 314622 (+ NSC 314622). After incubation at 30° for 30 min, reactions were stopped with 0.5% SDS with (+) or without (−) subsequent digestion with proteinase K (0.5 mg/ml for 60 min at 50°). Reactions (R) were reversed in 0.5 M NaCl for 15 min before addition of 0.5% SDS and proteinase K digestion. The gel picture was cut to show the top of gel (A) with the top 1-linked DNA fragments (*black arrow*) and the bottom part of the gel (B) with the full size DNA (*white arrow*) and the top1-mediated DNA cleavage products. *G + A*, purine ladder after formic acid sequencing of the control DNA.

CPT-induced DNA single-strand breaks were completely reversed 30 min after the drug was removed from the cell cultures (Covey *et al.*, 1989; Valenti *et al.*, 1997), whereas it took more than 1 hr for the NSC 314622-induced DNA single-strand breaks to reverse. Thus, the DNA single-strand breaks induced by NSC 314622 reversed more slowly than the breaks induced by CPT.

Cytotoxicity of NSC 314622 in a CPT-resistant top1-mutant cell line. CEM/C2 cells are highly resistant to CPT as a result of a mutation of one of the top1 alleles, Asn722Ser (Fujimori *et al.*, 1995), and inactivation of the other top1 allele(s) (Fujimori *et al.*, 1996). Table 2 shows that CEM/C2 cells were cross-resistant to NSC 314622, consistent with the possibility that top1 is a cytotoxic target of NSC 314622.

Discussion

DNA topoisomerases are important targets for cancer chemotherapy. Two of the cellular topoisomerases, top2 and top1, are targeted specifically by potent anticancer drugs, although no inhibitor has been reported for the more recently discovered third class of cellular topoisomerases, top3 (Wang, 1996). Top2 is the target of several of the most effective anticancer drugs to date: the anthracyclines (doxorubicin, daunorubicin, epirubicin, and idarubicin), the epipodophyllotoxins etoposide (VP-16) and teniposide (VM-26), the anthracenediones (mitoxantrone and derivatives), the ellipticines, the acridine amsacrine and the bis-dioxopiperazines, ICRF 187 and 193 [for review, see Pommier (1997)]. Each of these top2 inhibitors has preferential activity for different cancers. Hence, it is likely that non-CPT top1 inhibitors will exhibit different anticancer activity from camptothecins.

Several methods have been applied to the discovery of novel top1 inhibitors. Biochemical assays with top1 and purified DNA have led to the recent discovery of several novel classes of top1 inhibitors [for recent review see Pommier (1998)], including actinomycin D (Trask and Muller, 1988; Wassermann *et al.*, 1990); morpholinodoxorubicin (Wassermann *et al.*, 1990); saintopin (Yamashita *et al.*, 1991; Leteurtre *et al.* 1994); and other benzoanthracenes, nitidine, and other benzophenanthridines (Fang *et al.*, 1993; Larsen *et al.*, 1993); indoloquinolinediones (Riou *et al.*, 1991); intoplicine (Poddevin *et al.*, 1993); indolocarbazoles (Yamashita *et al.*, 1992; Yoshinari *et al.*, 1995); and benzimidazoles (Chen *et al.*, 1993; Kim *et al.*, 1996). Yeast strains with deletion of the top1 gene can also be a useful screening tool because these mutants are selectively resistant to CPT and other top1

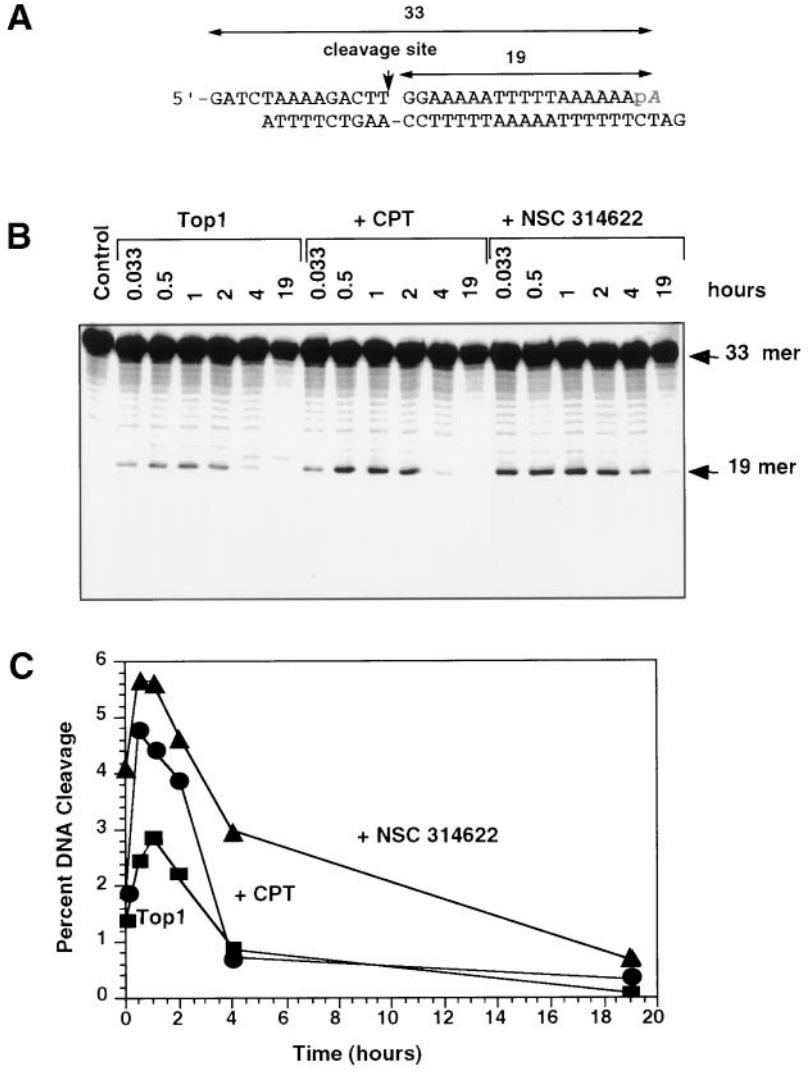
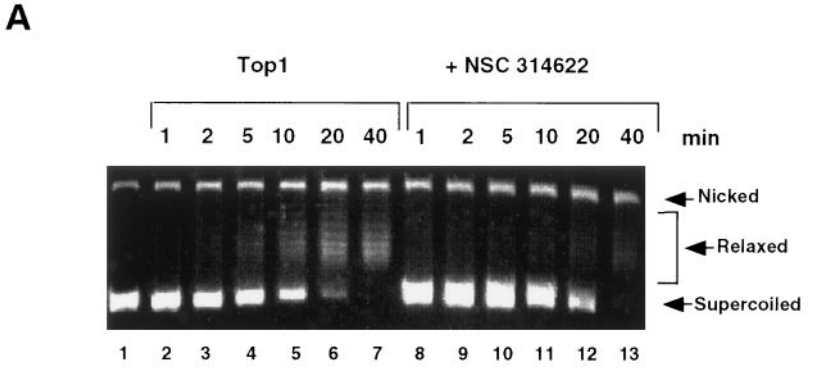


Fig. 5. Kinetics of top1 cleavage complexes induced by NSC 314622 in an oligonucleotide containing a single top1 cleavage site. A, Sequence of the 3′-end-labeled oligonucleotide containing a single top1 cleavage site. The ^{32}P-radiolabeled cordycepin is shown as p*A*. B, Gel picture showing top1-mediated cleavage reactions for the indicated times at 37° in the absence of drug (*left*) or in the presence of 0.05 μM CPT (*middle*) or 5 μM NSC 314622 (*right*). Reactions were stopped with 0.5% SDS. Oligonucleotides were separated in a 16% denaturing polyacrylamide gel. C, Quantification of cleavage shown in B (performed with a PhosphorImager analyzer from Molecular Dynamics) plotted as a function of time: Top1 without drug (■); + CPT (●); + NSC 314622 (▲). A typical experiment is shown.

poisons (Nitiss and Wang, 1988; Gatto *et al.*, 1996; Nitiss *et al.*, 1997). A third approach is provided by the National Cancer Institute Anticancer Drug Screen and the COMPARE analysis (Paull *et al.*, 1989; Boyd and Paull, 1995; Paull *et al.*, 1995).

Table 1 provides a summary COMPARE listing for NSC



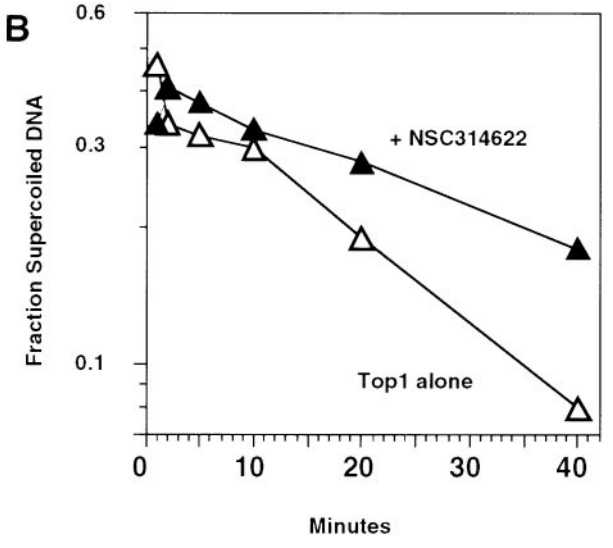


Fig. 6. NSC 314622 inhibits top1 catalytic activity. A, Picture of an agarose gel stained with ethidium bromide. Native supercoiled SV40 DNA (*lane 1*) was incubated with top1 in the absence of drug (*lanes 2–7*) or in the presence of 10 μM NSC 314622 (*lanes 8–13*) for the indicated times at 37°. Reactions were stopped with 0.5% SDS followed by proteinase K digestion. B, Quantification of the supercoiled bands shown in A with FluorImager using ImageQuant software.

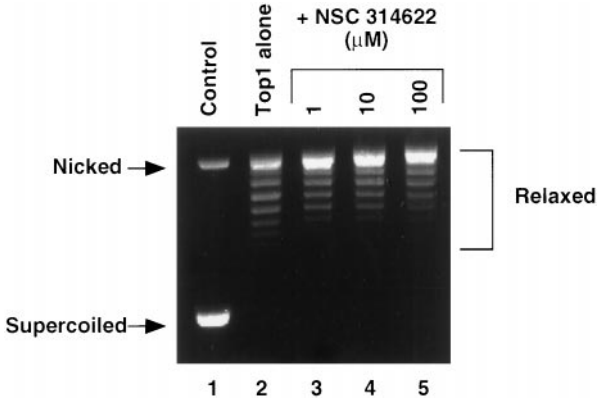


Fig. 7. NSC 314622 does not unwind DNA. Native supercoiled SV40 DNA (*lane 1*) was reacted with an excess of top1 in the absence of drug (*lane 2*) or in the presence of the indicated concentrations of NSC 314622 (*lanes 3–5*) for 30 min at 37°. Reactions were stopped with 0.5% SDS followed by proteinase K digestion and run in 1% agarose gel in TBE buffer. DNA was visualized after staining the gel with ethidium bromide.

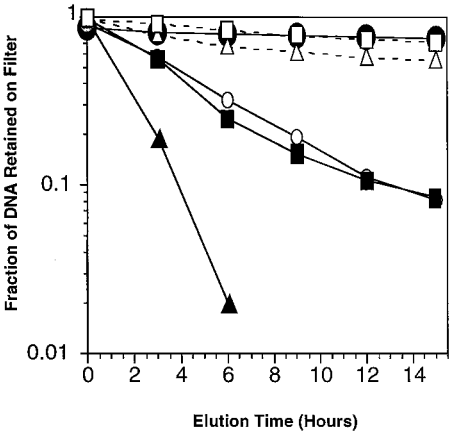


Fig. 8. Protein-linked DNA single-strand breaks induced by NSC 314622 in human breast carcinoma MCF7 cells. Cells were prelabeled with [^{14}C]thymidine as described in Materials and Methods and were treated with 0.1 μM CPT or 1 μM NSC 314622 for 1 hr at 37°. DNA single-strand breaks were assayed by alkaline elution (Covey *et al.*, 1989) with 300 rads (○); 0.1 μM CPT (▲); or 1 μM NSC 314622 (■). Protein-associated (top1-associated) strand breaks were assayed under nondeproteinizing conditions after treatment with 0.1 μM CPT (△) or 1 μM NSC 314622 (□).

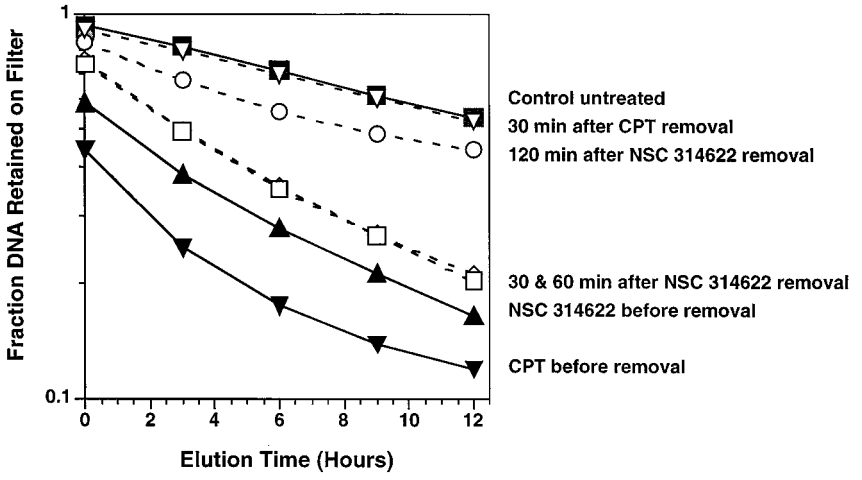


Fig. 9. Reversal kinetics of DNA single-strand breaks induced by NSC 314622 in MCF7 cells. MCF7 cells were treated with 10 μM NSC 314622 or 0.1 μM CPT for 1 hr at 37°. Cells were rinsed with fresh medium and incubated with drug-free medium for an additional 30, 60, or 120 min at 37°, after which cells were lysed and assayed by alkaline elution.

314622. Briefly, the 60 cell-line growth-inhibitory data obtained for NSC 314622 in the screen was correlated in pairs with the growth-inhibitory data for more than 60,000 compounds. The resulting list of NSC numbers and correlation coefficients was sorted by decreasing correlation coefficient. As shown in Table 1, portions of a list of the top 100 correlations were condensed to a single line if two or more camptothecins came together. Fig. 2 shows the GI50 mean graph (Boyd and Paull, 1995; Paull, 1995) for NSC 314622. These COMPARE data suggested that NSC 314622 was a top1 inhibitor. We previously used COMPARE to identify a novel top2 inhibitor, NSC 665517 (Gupta *et al.*, 1995a) and to discover that azatoxins, a novel class of top2 inhibitors, exhibited antitubulin activity (Solary *et al.*, 1993). COMPARE has also been used to identify p-glycoprotein^{MDR} substrates (Lee *et al.*, 1994; Alverez *et al.*, 1995) and new antitubulin agents (Paull *et al.*, 1992). The recent inclusion of a large number of CPT derivatives in the National Cancer Institute Database and the identification of other tested agents as top1 inhibitors have provided the means to identify novel top1 inhibitors. NSC 314622 is the first top1 inhibitor reported using COMPARE.

Our biochemical assays with purified top1 demonstrated that NSC 314622 is a top1 inhibitor. The cellular data with the top1 mutant and CPT-resistant CEM/C2 cell line (Fujimori *et al.*, 1995, 1996) also are consistent with the hypothesis that NSC 314622 targets top1 in cells. Top1 inhibition by NSC 314622 exhibits some differences from CPT. First, some of the drug-induced cleavage sites seemed different. Some sites were more intense with NSC 314622, whereas others were more intense with CPT. Such differences may be important in cells because some genes may be more selectively targeted by one compound than the other. Second, the top1 cleavage complexes trapped by NSC 314622 seemed more persistent than those induced by CPT in MCF7-treated cells (Fig. 9) and with purified enzyme (Fig. 5). This might offer an advantage for cancer chemotherapy because the rapid reversibility of the CPT-induced cleavage complexes imposes long infusions of CPTs to achieve maximum activity (Pommier, 1996). Third, NSC 314622 differs from CPTs in that NSC 314622 is chemically more stable than CPT. At physiological, and even more readily at alkaline pH, the lactone E ring of CPTs is hydrolyzed to the carboxylate form, which has no detectable activity against purified top1 (Jaxel *et al.*, 1989). Thus, it seems that NSC 314622 is a lead compound for developing novel non-CPT top1 inhibitors.

Most of the other non-CPT top1 inhibitors reported to date [see above and, for review, see Pommier (1998)] are DNA intercalators or minor groove binders. Our unwinding assay data suggested that NSC 314622 did not intercalate into DNA. The fact that the top1 cleavage complexes were not inhibited at high drug concentrations is also consistent with lack of strong DNA intercalation for NSC 314622.

TABLE 2
Cytotoxicity of NSC 314622 in human leukemia CEM and camptothecin-resistant CEM/C2 cells

NSC 314622 concentration	Exposure	Growth fraction[a]		
		CEM	CEM/C2	
6 μM	1 hr	0.23	0.53	Exp #1
10 μM	1 hr	0.03	0.29	Exp #2
0.1μM	48 hr	0.29	0.50	Exp #2
0.1μM	48 hr	0.33	0.60	Exp #3

[a] Cells were counted 48 hr after the beginning of drug treatment.

Acknowledgments

This article is dedicated to the memory of Dr. Ken Paull.

References

Alverez M, Paull K, Monks A, Hose C, Lee JS, Weinstein J, Grever M, Bates S, and Fojo T (1995) Generation of a drug resistance profile by quantitation of mdr-1/P-glycoprotein expression in cell lines of the NCI Anticancer Screen. *J Clin Invest* **95:**2205–2214.

Bertrand R and Pommier Y (1995) Assessment of DNA damage in mammalian cells by DNA filtration methods, in *Cell Growth and Apoptosis: A Practical Approach* (G Studzinski, ed) pp 96–117, IRL Press, Oxford University Press, Oxford.

Boyd MR and Paull KD (1995) Some practical considerations and applications of the National Cancer Institute *in vitro* anticancer drug discovery screen. *Drug Develop Res* **34:**91–109.

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.

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

Chen AY, Yu C, Bodley A, Peng LF, and Liu LF (1993) A new mammalian DNA topoisomerase I poison Hoechst 33342: cytotoxicity and drug resistance in human cell cultures. *Cancer Res* **53:**1332–1337.

Covey JM, Jaxel C, Kohn KW, and Pommier Y (1989) Protein-linked DNA strand breaks induced in mammalian cells by camptothecin, an inhibitor of topoisomerase I. *Cancer Res* **49:**5016–5022.

Cushman M and Cheng L (1978) Stereoselective oxidation by thionyl chloride leading to the indeno[1,2-c]isoquinoline system. *J Org Chem* **43:**3781–3783.

Fang S-D, Wang L-K, and Hecht SM (1993) Inhibitors of DNA topoisomerase I isolated from the roots of *Zanthoxylum nitidum*. *J Org Chem* **58:**5025–5027.

Fujimori A, Harker WG, Kohlhagen G, Hoki Y, and Pommier Y (1995) Mutation at the catalytic site of topoisomerase I in CEM/C2, a human leukemia cell resistant to camptothecin. *Cancer Res* **55:**1339–1346.

Fujimori A, Hoki Y, Popescu N, and Pommier Y (1996) Silencing and selective

methylation of the normal topoisomerase I gene in camptothecin-resistant CEM/C2 human leukemia cells. *Oncol Res* **8:**295–301
Gatto B, Sanders MM, Yu C, Wu HY, Makhey D, LaVoie EJ, and Liu LF (1996) Identification of topoisomerase I as the cytotoxic target of the protoberberine alkaloid coralyne. *Cancer Res* **56:**2795–2800.
Gupta M, Abdel-Megeed M, Hoki Y, Kohlhagen G, Paull K, and Pommier Y (1995a) Eukaryotic DNA topoisomerases mediated DNA cleavage induced by a new inhibitor: NSC 665517. *Mol Pharmacol* **48:**658–665.
Gupta M, Fujimori A, and Pommier Y (1995b) Eukaryotic DNA topoisomerases I. *Biochem Biophys Acta* **1262:**1–14.
Hsiang YH, 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.
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.
Kim JS, Gatto B, Yu C, Liu A, Liu LF, and LaVoie EJ (1996) Substituted 2,5′-Bi-1H-benzimidazoles: topoisomerase I inhibition and cytotoxicity. *J Med Chem* **39:**992–998.
Larsen AK, Grondard L, Couprie J, Desoizé B, Comof L, Jardillier J-C, and Riou J-F (1993) The antileukemic alkaloid fagaronine is an inhibitor of DNA topoisomerases I and II. *Biochem Pharmacol* **19:**1403–1412.
Lee J-S, Paull K, Alvarez M, Hose C, Monks A, Grever M, Fojo AT, and Bates SE (1994) Rhodamine efflux patterns predict P-glycoprotein substrates in the National Cancer Institute Drug Screen. *Mol Pharmacol* **46:**627–638.
Leteurtre F, Fujimori A, Tanizawa A, Chhabra A, Mazumder A, Kohlhagen G, Nakano H, and Pommier Y (1994) Saintopin, a dual inhibitor of DNA topoisomerases I and II, as a probe for drug-enzyme interactions. *J Biol Chem* **269:**28702–28707.
Nitiss JL, Pourquier P, and Pommier Y (1997) Aclarubicin stabilizes topoisomerase I covalent complexes. *Cancer Res* **57:**4564–4569.
Nitiss J and Wang JC (1988) DNA topoisomerase-targeting antitumor drugs can be studied in yeast. *Proc Natl Acad Sci USA* **85:**7501–7505.
Paull KD, Hamel E, and Malspeis L (1995) Prediction of biochemical mechanism of action from the *in vitro* antitumor screen of the National Cancer Institute, in *Cancer Chemotherapeutic Agents* (Foye WO, ed) pp 8–45, American Chemical Society, Washington, DC.
Paull KD, Lin CM, Malspeis L, and Hamel E (1992) Identification of novel antimitotic agents acting at the tubulin level by computer-assisted evaluation of differential cytotoxicity data. *Cancer Res* **52:**3892–3900.
Paull KD, Shoemaker RH, Hodes L, Monks A, Scudiero DA, Rubinstein L, Plowman J, and Boyd M (1989) Display and analysis of patterns of differential activity of drugs against human tumor cell lines: development of a mean graph and COMPARE algorithm. *J Natl Cancer Inst* **81:**1088–1092.
Poddevin B, Riou J-F, Lavelle F, and Pommier Y (1993) Dual topoisomerase I and II inhibition by intoplicine (RP-60475), a new antitumor agent in early clinical trials. *Mol Pharmacol* **44:**767–774.
Pommier Y (1996) Eukaryotic DNA topoisomerase I: genome gate keeper and its intruders, camptothecins. *Semin Oncol* **23:**1–10.
Pommier Y (1997) DNA topoisomerase II inhibitors, in *Cancer Therapeutics: Experimental and Clinical Agents* (Teicher BA, ed) pp 153–174, Humana, Totowa, NJ.
Pommier Y (1998) Diversity of DNA topoisomerases I and inhibitors. *Biochimie,* in press.
Pommier Y, Covey JM, Kerrigan D, Markovits J, and Pham R (1987) DNA unwinding and inhibition of mouse leukemia L1210 DNA topoisomerase I by intercalators. *Nucl Acids Res* **15:**6713–6731.
Pommier Y, Kohlhagen G, Kohn F, 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.
Riou J-F, Helissey P, Grondard L, and Giorgi-Renault S (1991) Inhibition of eukaryotic DNA topoisomerase I and II activities by indoloquinolinedione derivatives. *Mol Pharmacol* **40:**699–706.
Solary E, Leteurtre F, Paull KD, Scudiero D, Hamel E, and Pommier Y (1993) Dual inhibition of topoisomerase II and tubulin polymerization by azatoxin, a novel cytotoxic agent. *Biochem Pharmacol* **45:**2449–2456.
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* **43:**7200–7206.
Trask DK and Muller MT (1988) Stabilization of type I topoisomerase-DNA covalent complexes by actinomycin D. *Proc Natl Acad Sci USA* **85:**1417–1421.
Valenti M, Nieves-Neira W, Kohlhagen G, Kohn KW, Wall ME, Wani MC, and Pommier Y (1997) Novel 7-alkyl methylenedioxycamptothecin 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.
Wang JC (1996) DNA topoisomerases. *Annu Rev Biochem* **65:**635–692.
Wassermann K, Markovits J, Jaxel C, Capranico G, Kohn KW, and Pommier Y (1990) Effects of morpholinyl doxorubicins, doxorubicin and actinomycin D on mammalian DNA topoisomerases I and II. *Mol Pharmacol* **38:**38–45.
Yamashita Y, Fujii N, Murakata C, Ashizawa T, Okabe M, and Nakano H (1992) Induction of mammalian DNA topoisomerase I-mediated DNA cleavage by antitumor indolocarbazole derivatives. *Biochemistry* **31:**12069–12075.
Yamashita Y, Kawada S-Z, Fujii N, and Nakano H (1991) Induction of mammalian topoisomerase I and II mediated DNA cleavage by saintopin, a new antitumor agent from fungus. *Biochemistry* **30:**5838–5845.
Yoshinari T, Matsumoto M, Arakawa H, Okada H, Noguchi K, Suda H, Okura A, and Nishimura S (1995) Novel antitumor indolocarbazole compound 6-*N*-formylamino-12,13-dihydro-1,11-dihydroxy-13-(β-D-glucopyranosyl)-5H-indolo[2, 3-a] pyrrolo[3,4-c] carbazole-5,7(6H)-dione (NB-506): induction of topoisomerase I-mediated DNA cleavage and mechanisms of cell line-selective cytotoxicity. *Cancer Res* **55:**1310–1315.

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