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Eukaryotic DNA Topoisomerases Mediated DNA Cleavage Induced by a New Inhibitor: NSC 665517

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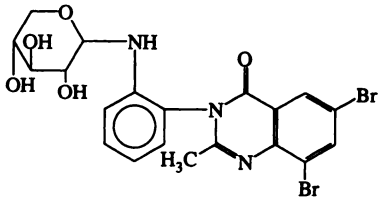
SUMMARY

A compound with a novel structure, NSC 665517, was tested in the National Cancer Institute Preclinical Drug Discovery Screen. With the COMPARE algorithm, the pattern of differential cytotoxicity for NSC 665517 most closely resembled those of known topoisomerase II (top2) inhibitors. *In vitro* data showed that NSC 665517 induced DNA cleavage in the presence of top2 and topoisomerase I (top1) (at a higher concentration). The minimum concentration required to induce top2 cleavage was 0.5 μM. A substantial decrease in top2-induced cleavage by NSC 665517 was seen when the reaction mixtures were shifted to elevated temperature (55°), suggesting that top2-induced cleavage occurs through the mechanism of stabilizing the reversible enzyme/DNA complex and inhibiting religation. The DNA cleavage pattern induced by NSC 665517 with top2 was different than that of other known top2 inhibitors, including etoposide, mitoxantrone, anthracyclines, amsacrine, and ellipticine. top2 cleavage sites induced by NSC 665517 showed strong preference for G located 3′ to the top2-mediated DNA cleavage (position +1). NSC 665517 produced limited DNA unwinding at high drug concentration. DNA damage analyzed in KB cells by alkaline elution showed that NSC 665517 induced strand break. Data from the cytotoxicity in KB-V1 overexpressing P-glycoprotein and COMPARE analysis with rhodamine efflux assay indicated that NSC 665517 is a substrate of P-glycoprotein. These results strongly suggest that NSC 665517 is a novel topoisomerase-targeted drug. Preclinical evaluation of NSC 665517 as an antitumor agent is under way.

Topoisomerases are nuclear enzymes that catalyze topological changes of DNA and are involved in DNA replication, transcription, and recombination (1). Furthermore, top2 is essential for the survival of eukaryotic cells because its activity is required for the segregation of mitotic (2) and meiotic (3) chromosomes and in the maintenance of chromosomal structure (4). top2 acts by making a transient double-strand break in both DNA strands, passing another duplex DNA through, and religating the double-strand break (5), thus changing the linking number by 2. The cleavage created by top2 during catalysis results in a four-base staggered double-stranded DNA break. The 5′ ends of the cleaved DNA strands are covalently linked to the subunits of the top2 homodimer via an O^4 phosphotyrosine bond (6). Normally, these DNA breaks are rapidly resealed by the enzyme. However, several classes of anticancer drugs, such as aminoacridines (7), anthracyclines (8), ellipticines (9), epipodophyllotoxins (10), and azatoxins (11), interfere with the religation step of the top2-mediated catalysis, resulting in the stabilization of the top2/DNA cleavable complex (1, 5). The cytotoxicity of these drugs is likely due to the chromosomal breakage. Although all of these agents inhibit top2, they have a different clinical potency and exhibit a wide activity spectra. This could in part be due to the differences in base sequence specificity among different drug families of top2 inhibitors (5).

To identify new anticancer agents, the National Cancer Institute uses a screening strategy in which the cytotoxicity of each potential drug is compared with standard agents of known mechanism of action with the use of 60 human tumor cell lines derived from seven cancer types (brain, colon, leukemia, lung, melanoma, ovarian, and renal). NSC 665517 (Fig. 1) was evaluated in this human tumor cell line screen. Based on the COMPARE analysis (12), it was suggested that NSC 665517 might be a potential top2 inhibitor. We have shown using *in vitro* data that NSC 665517 is a new top2 inhibitor with a novel structure. NSC 665517 inhibits top2 by stabilizing the cleavable complexes, thereby preventing DNA religation by the enzyme. Unlike other DNA binders, top2-mediated DNA breaks induced by NSC 665517 increased with drug concentration. The most preferred base in the NSC 665517-induced top2 cleavage sites was a guanine at +1 position of the DNA break. NSC 665517 was also found to

ABBREVIATIONS: top2, topoisomerase II; top1, topoisomerase I; VP-16, etoposide; TAE, Tris/acetate/EDTA; GI_{50}, 50% growth-inhibitory concentration; P-gp, P-glycoprotein; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; BSA, bovine serum albumin; MDR, multidrug resistance.



NSC 665517

Fig. 1. Chemical structure of NSC 665517.

induce top1 cleavage, suggesting that it might behave like a dual inhibitor of top1 and top2 at higher drug concentration.

Materials and Methods

Mitoxantrone, amsacrine, 5-iminodaunorubicin, 2-methyl-9-hydroxy-ellipticinium, 5-iminodaunorubicin, and camptothecin were obtained from the Drug Synthesis and Chemistry Branch (National Cancer Institute, Bethesda, MD). NSC 665517 was synthesized by Dr. M. F. Abdel-Megeed (Chemistry Department, Tanta University, Tanta, Egypt), and teniposide (VM-16) was provided by Dr. B. H. Long (Bristol-Myers Co., Wallingford, CT). Drug stock solutions were made in dimethylsulfoxide at 10 mM and aliquots were stored at −20°. Further dilutions were made in distilled water immediately before use. The final concentration of dimethylsulfoxide in the reactions did not exceed 1% (v/v).

Human c-*myc* DNA was purchased from the American Type Culture Collection (Rockville, MD). pUC-BENN plasmid containing the HIV-LTR was a gift from Dr. K.-T. Jeang (Laboratory of Molecular Microbiology, National Institute for Allergy and Infectious Diseases, Bethesda, MD). Restriction enzymes, T4 polynucleotide kinase, and *Taq* DNA polymerase were purchased from New England Biolabs (Beverly, MA) and Perkin Elmer (Norwalk, CT) respectively. [γ-^{32}P]ATP was purchased from DuPont-NEN (Boston, MA). top2 was purified from mouse leukemia L1210 cell nuclei as described previously (13). top2 was stored at −70° in 40% (v/v) glycerol, 0.35 M NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM KH_2PO_4, 0.2 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride, pH 6.6. top1 was purchased from GIBCO-BRL (Grand Island, NY).

Human carcinoma cell lines KB3–1 and KB-V1 were provided by Dr. Michael M Gottesman (National Cancer Institute, Bethesda, MD). The cells were grown in Dulbecco's modified Eagle's medium (ABI, Columbia, MD) supplemented with 10% fetal bovine serum, 2 mM L-glutamine (GIBCO, Gaithersburg, MD), penicillin (100 units/ml), and streptomycin (100 μg/ml). KB-V1 is an MDR-expressing clone isolated from KB3–1 by stepwise selection with vinblastin (14). The cell lines were maintained at 37° in a humidified atmosphere of 95% air/5% CO_2.

National Cancer Institute Preclinical Antitumor Drug Discovery Screening Assays. The protocols used by the National Cancer Institute drug discovery screening program have been discussed in detail previously (12). Briefly, the human tumor cell lines making up the screening panel were grown in RPMI-1640 medium containing 5% fetal bovine serum and 2 mM L-glutamine. Cells were inoculated into 96-well culture plates and incubated at 37° for 24 hr before treatment. Drugs were added in sequential 10-fold dilutions. After a 48-hr incubation at 37°, cell cultures were fixed *in situ* with trichloroacetic acid, washed, and then stained with sulforhodamine B. The solubilized stain was measured spectrophotometrically at 515 nm.

The pattern-recognition algorithm COMPARE has become an analytical tool for identifying new agents with a similar mechanism of action based on biological data obtained from the National Cancer Institute human tumor cell line screen (15). The analysis is designed to find mean graph pattern matches to compounds of interest. The compound of interest is selected as a seed for a probe of the database. The program then ranks the similarity patterns of differential cytotoxicity (GI_{50}) of a group of ~175 standard agents composed of approved anticancer agents. The analysis uses an SAS statistical program for calculating Pearson correlation coefficients.

Rhodamine 123, the fluorescent dye, is a substrate for P-gp (16). The National Cancer Institute Drug Screen Program has analyzed 58 human tumor cell lines for their ability to efflux rhodamine 123 as a functional assay for P-gp (17). Briefly, a profile was first generated with the efflux values, in the presence and absence of cyclosporin A. This value is referred to as the level of rhodamine efflux. A range of values for rhodamine efflux was obtained from all of the cell lines used in the screen. The SW620 cell line was chosen as a reference because it has detectable *mdr*-1 expression and P-gp activity and can be sensitized to vincristine 4–6-fold by verapamil (18). The level of rhodamine efflux observed in SW620 cells was 31. Thus, levels of rhodamine efflux of >30 were considered to represent significant P-gp activity. Twelve cell lines, including HOP-62, SW-620, DLD-1, HCT-15, CCRT-CEM, UO-31, A498, CAKI-1, RXF-393, RXF-631, ACHN, and SF-295, had rhodamine efflux values of >30 (17). With the rhodamine efflux data as a seed for COMPARE analysis with cytotoxicity data on >30,000 compounds in the National Cancer Institute drug screen database, hundreds of compounds with high correlation coefficients were identified.

DNA unwinding assay. The reactions were performed as described previously (19). A 20-μl final reaction volume containing 0.3 μg supercoiled SV40 DNA, 10 mM Tris·HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 μg/ml BSA, and drug or water was reacted with 10 units of top1. Reactions were performed at 37° for 30 min and stopped by the addition of 2 μl of 10% SDS. Five microliters of loading buffer (50 mM EDTA, 50% v/w sucrose, 0.1% bromophenol blue) were added to each reaction mixture before loading onto a 1% agarose gel made in 1× TAE buffer. After electrophoresis, DNA bands were stained in a 10 μg/ml solution of ethidium bromide and visualized by transillumination with UV light (300 nm).

Topoisomerase II-mediated DNA relaxation. Reactions mixture contained 0.3 μg negatively supercoiled SV40 DNA and 10 units human top2 (TopoGen, Columbus, OH) in a total of 10 μl of relaxation buffer (10 mM Tris·HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM ATP, and 15 μg/ml BSA). DNA relaxation was carried out at 37° for 1 hr, and reactions were stopped by the addition of 1 μl of stop solution (1% SDS and 1 mM EDTA, pH 8.0). Samples were mixed with 3 μl of loading buffer and subjected to electrophoresis in 1% agarose gel in 1× TAE buffer. Gels were stained with ethidium bromide (10 μg/ml) for 5 min and photographed under UV light.

Topoisomerase II-mediated DNA cleavage. Human c-*myc* DNA fragments used for top2-mediated cleavage reactions were prepared by polymerase chain reaction as described previously (20). A 254-bp DNA fragment was prepared between positions 3035 and 3288, using sequence information obtained from the GeneBank sequence data. The DNA fragment was prepared using oligonucleotides 5-GTAATCCAGAACTGGATCGG-3′ as the sense primer and 5′-GGAACCTCATCCCTGGCG-3′ as the antisense primer. A 418-bp DNA fragment from the first exon was prepared between positions 2265 and 2745 using oligonucleotides 5′-GATCCTCTCTCGCTAATCTCCGC-3′ as the sense primer and 5′-TCCTTGCTCGGGTGTTGTAAGTTCC-3′ as the antisense primer. A 435-bp HIV-LTR DNA fragment was prepared using 5′-GGCTAATTCACTCCCAACGAAGAC-3′ as the sense primer and 5′GGGAGTCTAGGACGTATATTCGT-3′ as the antisense primer (21). Single-end labeling of these DNA fragments was obtained by 5′ end labeling one of the primer oligonucleotide by T4 polynucleotide kinase and [^{32}P-ATP].

DNA fragments were equilibrated with or without drug in 10 mM Tris·HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM ATP, and 15 μg/ml BSA for 5 min before the addition of purified top2 (40–70 ng) in 20 μl final reaction volume. Reactions were performed at 37° for 30 min and then stopped by the addition of SDS to a final concentration of 1% and proteinase K to 0.4 mg/ml, followed by

TABLE 1

COMPARE analysis of NSC 665517 using all the cell lines or excluding the multidrug-resistant cell lines of the National Cancer Institute Anticancer Drug Discovery Screen

The cytotoxicity profile of NSC 665517 was compared with those of selected anticancer agents with known mechanism of action. The 59 cell lines (middle) included human lines from brain, colon, leukemia, lung, melanoma, ovarian, and renal tumor. For rhodamine efflux analysis (right), MDR-positive cell lines HOP-62, SW-620, DLD-1, HCT-15, CCRT-CEM, UO-31, A498, CAKI-1, RXF-393, RXF-631, ACHN, and SF-295 were excluded. The COMPARE algorithm ranked all compounds in this database according to the similarity of their mean graph patterns to the mean graph pattern of NSC 665517.

Matches	Pearson correlation coefficient	
	All cell lines	Excluding Rhod 30 cell lines
Rhodamine	0.803	
Adriamycin	0.752	0.702
VM-26	0.707	0.712
Mitoxantrone	0.685	0.679
Amsacrine	0.616	0.672
Mitomycin C	0.579	0.558
Melphalan	0.577	0.704
Hydroxyurea	0.535	0.688
Aphidicolin glycinate	0.534	0.658
Cisplatin	0.521	0.583
Actinomycin D	0.471	0.413
Topotecan	0.465	0.572
Cytosine arabinoside	0.443	0.536
Vinblastine sulfate	0.390	0.341
5-Fluorouracil	0.353	0.542
Methotrexate	0.313	0.440
Bryostatin-1	0.160	0.087
5-Azacytidine	0.072	0.160

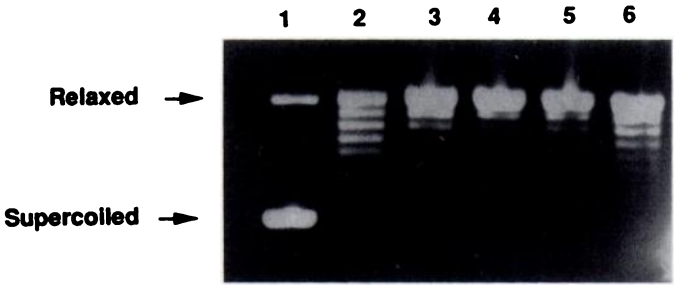


Fig. 2. DNA unwinding in the presence of NSC 665517. Native supercoiled SV40 DNA (*lane 1*) was reacted with excess top1 in the absence (*lane 2*) or the presence of NSC 665517 (*lanes 3–6*) for 30 min at 37°. Drug concentrations were 10 μM (*lane 3*), 100 μM (*lane 4*), 250 μM (*lane 5*), and 500 μM (*lane 6*). Reactions were stopped by adding SDS and analyzed in 1% agarose TAE buffer gel. DNA was visualized by staining the gel with ethidium bromide.

incubation for 30 min at 42°. Samples were ethanol precipitated and resuspended in 2.5 μl loading buffer (80% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue). Samples were heated to 90° and immediately loaded onto a 7% DNA sequencing gel containing 7 M urea in 1× Tris/Borate/EDTA buffer. Electrophoresis was carried out at 2500 V for 3 hr. Gels were dried on 3-mm paper sheets and autoradiographed with Kodak XAR-2 film.

Clonogenic assays. Exponentially growing KB3–1 or KB-V1 cells were seeded in 25-cm^2 flasks. Semiconfluent cells were treated with NSC 665517 for 8 hr. Cells were washed with PBS, trypsinized, and counted with a Coulter counter (Hialeah, FL), and 200 cells were plated in triplicate in 25-cm^2 flasks with 5 ml of fresh medium. Colonies were grown for 7–10 days, after which they were washed with PBS, fixed with methanol (95%), and stained with methylene blue (0.05%). Survival fractions were calculated as the ratio of the cloning efficiency of drug-treated cells to the cloning efficiency of control cells. Cloning efficiencies for the control cells ranged from 60% to 70%.

Determination of DNA single-strand breaks. KB3–1 and KB-V1 cells were labeled with ^{14}C-thymidine (0.04 μCi/ml) for 24 hr. CEM cells were labeled with ^{3}H-thymidine (0.2 μCi/ml) for one doubling time (24 hr) as internal control. All cells were chased in nonradioactive medium for 20–24 hr before drug treatment. To measure SSB, ^{14}C-labeled calibrator cells (KB3–1 and KB-V1) and ^{3}H-labeled internal standard cells (CEM) were irradiated on ice with 20 Gy. Cells were then loaded onto polycarbonate filters (2-μm pore, 25-mm diameter; Poretics Corporation, Livermore, CA). Cells were lysed in 5 ml of 0.1 M glycine, 0.025 M Na_2EDTA, 2% w/v SDS, and 0.5 mg/ml proteinase K (Boehringer Mannheim, Indianapolis, IL) at pH 10. DNA was eluted with tetrapropylamminoum hydroxide (RSA, Ardsley, NY)/EDTA, pH 12.1, containing 0.1% w/v SDS, at a flow rate of 0.15 ml/min. Fractions were collected at 5-min interval for 30 min, and single-strand break frequency was expressed in rad-equivalents and was calculated from the following formula: Single-strand break = $[\log(r_1/r_o)/\log(R_o/r_o)] \times 2000$, where R_o, r_o, and r_1 are the fractions of [^{14}C]DNA retained on the filter for 20-Gy irradiated control cells, unirradiated control cells, and drug-treated cells, respectively (22).

Results

COMPARE analysis of NSC 665517 in the National Cancer Institute Anticancer Drug Discovery Screen. The COMPARE algorithm was developed to permit the rapid selection of compounds with similar or novel patterns of cytotoxicity relative to establish anticancer agents with known mechanism of action. Differential cytotoxicity (mean graph) was obtained from the screening results based on $\log_{10}$ molar concentrations of NSC 665517 that caused a 50% growth inhibition. The patterns of differential cytotoxicity were compared with those for 175 standard agents. The agents that provided the closest matches (Table 1) were predominantly top2 inhibitors. Correlation coefficients were all >0.67. The rhodamine efflux data (Table 1) is discussed later.

DNA intercalation. To determine whether NSC 665517 intercalates into DNA, unwinding assays (19) were performed with increasing concentrations of the drug (Fig. 2). Supercoiled DNA was used as the starting substrate. Relaxation of supercoiled DNA by top1 produces closed circular duplex. NSC 665517 at 250 and 500 μM produced a shift in the pattern of DNA topoisomers. These results suggest that NSC 665517 is a weak DNA intercalator at high drug con-

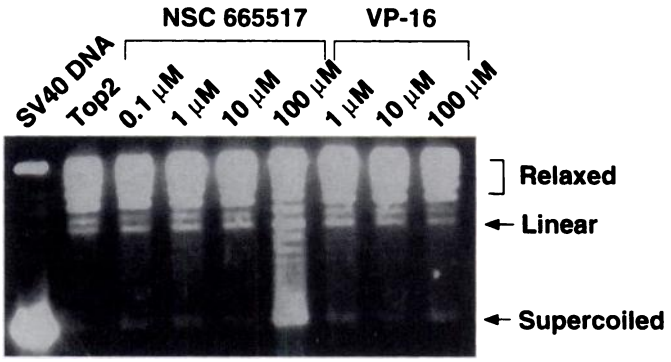


Fig. 3. Inhibition of top2 relaxation activity by NSC 665517. Supercoiled SV40 DNA was incubated with top2 in the presence of VP-16 or NSC 665517 at 37° for 1 hr. The reaction mixtures were analyzed on a 1% agarose gel. The supercoiled and relaxed product were visualized by staining the gel with ethidium bromide.

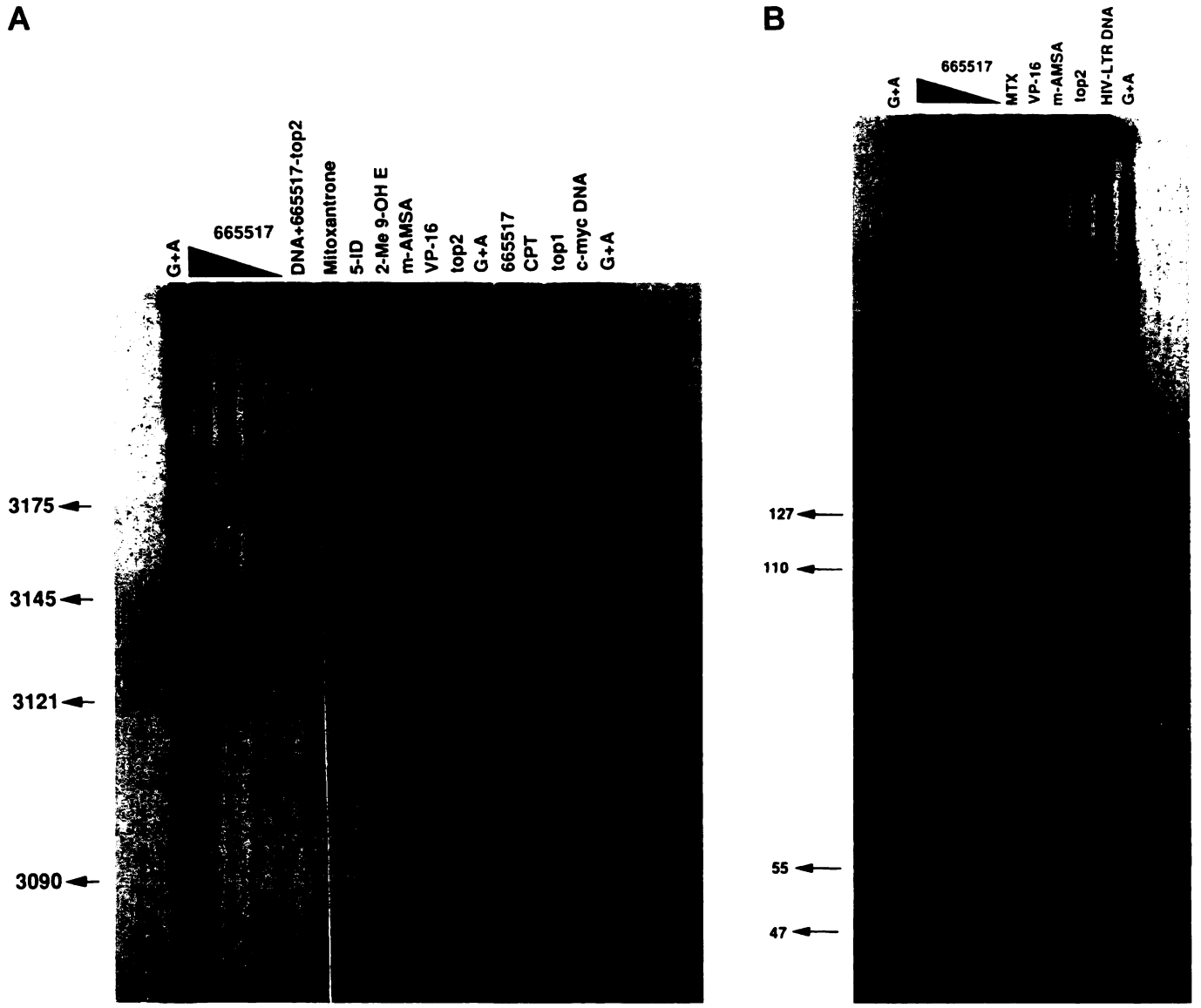


Fig. 4. Stimulation of top2- and top1-mediated DNA cleavage by NSC 665517. The DNA fragments were prepared by polymerase chain reaction with 5′ ^{32}P-labeled sense primers (20). Cleavage reactions were performed with purified top2 at 37° for 30 min, stopped with SDS, and digested with proteinase K. The reaction mixtures were analyzed on 7% denaturing acrylamide gels. A, Sense strand of the first intron of the human c-*myc* proto-oncogene. From left to right, *G+A*, purine ladder obtained after formic acid reaction; [665517 (from right to left), top2 + 10 μM, + 100 μM, + 250 μM, and + 500 μM NSC 665517] *DNA* + (100 μM) *665517-top2*, in the absence of enzyme; *mitoxantrone*, top2 + 0.5 μM mitoxantrone; *5-ID*, top2 + 2 μM 5-iminodaunorubicin; *2-Me 9-OH E*, top2 + 10 μM 2-methyl-9-hydroxyl-ellipticinium; *m-AMSA*, top2 + 10 μM amsacrine; *VP-16*, top2 + 100 μM etoposide; *top2*, +top2 without drug; *665517*, top1 + 100 μM NSC 665517; *CPT*, top1 + 100 μM camptothecin; *top1*, +top1 without drug; *c-myc DNA*, control DNA. B, Sense strand of the HIV-LTR DNA; same concentrations of amsacrine, VP-16, and mitoxantrone were used as with c-*myc* DNA. For 665517, the concentrations used were 1, 10, 100, and 250 μM in the presence of top2. *Numbers on left*, positions of cleavage sites.

centrations. top1 cleavage is probably minimal in SV40 DNA, as unwinding is observed.

Inhibition of catalytic activity of DNA topoisomerase II. The effect of NSC 665517 was examined on the relaxation activity of mammalian top2 and compared with a known top2 inhibitor, etoposide (VP-16). As shown in Fig. 3, in the absence of the drug (*control*), top2 relaxed supercoiled SV40 DNA, While NSC 665517 inhibited the relaxation of supercoiled DNA at 100 μM by ~50%. At 100 μM, VP-16 inhibited the relaxation activity by ~10–15%. Thus, NSC 665517 inhibits the catalytic activity of top2 with a higher potency than VP-16.

Induction of top2-mediated cleavable complex. Induction of cleavage was seen in several fragments of the human c-*myc* gene, (Fig. 4A). NSC 665517 induced top2 cleavage sites with a specific pattern which differed from those induced by other top2 inhibitors such as VP-16, amsacrine, 2-methyl-9-hydroxy-ellipticinium, 5-iminodaunorubicin, and mitoxantrone. Higher concentrations of NSC 665517 resulted in an enhancement of DNA cleavage consistent with the unwinding data suggesting minimal DNA distortion by NSC 665517 at pharmacological concentrations. NSC 665517 was also found to induce top1-mediated cleavage at a higher

concentration (100 μM). The cleavage pattern of NSC 665517-induced top1 cleavage differed from that of camptothecin.

NSC 665517-induced top2 cleavage was also mapped in the HIV-LTR DNA, which had previously been shown to contain hotspots for topoisomerases cleavage (21) (Fig. 4B). Some of the cleavage sites, such as those at nucleotide positions 47, 55, and 127, were enhanced by NSC 665517, amsacrine, and VP-16. top2-induced DNA cleavage in the presence of NSC 665517 was detectable at ~0.5 μM (data not shown), and the number of sites increased with higher drug concentrations. The presence of strong top2 sites in the promoter (+1 position) and in the TATAA box are consistent with our previous report (21).

Reversibility of NSC 665517-induced DNA cleavage. The cleavable complex induced by top2 inhibitors can be reversed by salt, EDTA, and heat treatment (23, 24). The salt-reversibility of the top2-mediated DNA cleavage suggests that the enzyme behaves in a distributive manner, i.e., the enzyme dissociates from the substrate after cleavage and religation. Addition of salt to the cleavable complexes formed at low salt results in religation of the cleavage complexes (23). Biochemical studies have demonstrated that antitumor drugs such as VP-16 stimulate top2-mediated cleavage by stabilizing the cleavable complex (23) and thus preventing DNA religation. To test whether NSC 665517-induced DNA cleavage with top2 was reversible, reaction mixtures were heated at 55° before termination with SDS and proteinase K, and an aliquot was withdrawn at various times during the heat treatment. The reversibility of top2-mediated DNA cleavage induced by NSC 665517 and VP-16 (included for comparison) was analyzed. As shown in Fig. 5, cleavage site 2308 of the human c-*myc* gene was induced by NSC 665517, whereas it was not by VP-16 and did not rapidly reverse. Site 2313 was enhanced by both NSC 665517 and VP-16 and was reversed (within 2 min) to background level (produced in the absence of the drug). Cleavage site 2338 was stable and did not reverse completely up to ~45 min, whereas site 2339 was only stimulated by VP-16 and not by NSC 665517. These results suggest that the mechanism of DNA cleavage induced by NSC 665517 is through stabilization of the reversible enzyme/DNA complex and thus inhibition of DNA religation.

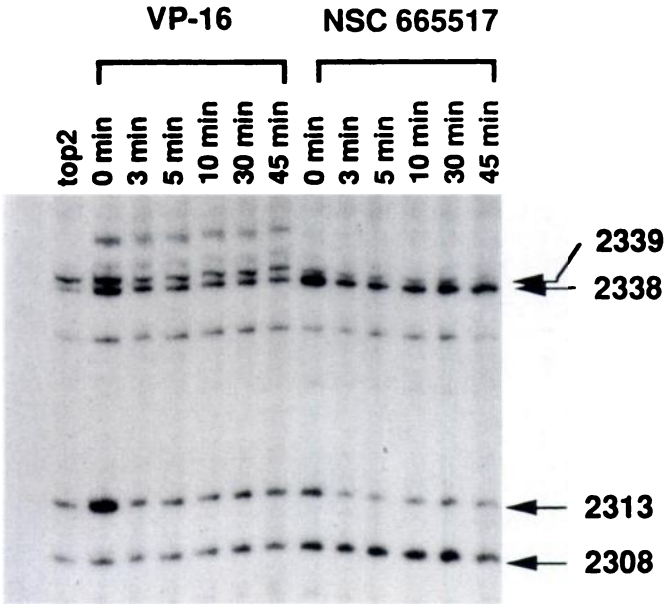


Fig. 5. Reversibility of NSC 665517-induced DNA cleavable complexes following heat treatment. The 5′-labeled sense strand of the human c-*myc* DNA was incubated with either 100 μM VP-16 or 100 μM NSC 665517 in the presence of top2 for 30 min at 37°. The reaction mixture was then shifted to 55°, and aliquots (20 μl) were withdrawn at various times after the heat treatment as indicated. The reactions were treated with SDS/proteinase K and analyzed on a 7% acrylamide gel.

Base preferences at the top2 cleavage site. Analysis of the base preferences around top2 cleavage sites in the presence of NSC 665517 was performed by aligning top2-induced DNA cleavage sites relative to the cleaved DNA phosphodiester bond (25). By convention, the nucleotide at the 3′ DNA termini of the DNA cleavage are numbered negatively with the nucleotide at the 3′ DNA terminus being −1. Nucleotides at the 5′ DNA termini are numbered positively with the nucleotide covalently linked to the top2 at the 5′ terminus of the DNA cleavage being +1. Having established the optimal NSC 665517 concentration required for observing top2 induced cleavage of DNA, we performed cleavage reactions on end-labeled DNA fragments with top2 in the presence of NSC 665517 (100 μM) as shown in Fig. 4. A total of 30 sites were analyzed, and the probabilities of base occurrence were determined at each position (Fig. 6). Position +1 exhibited the most striking differences, with a guanine preference at position +1.

Cytotoxicity of NSC 665517 in multidrug resistant cells. Most of the anticancer top2 inhibitors are substrates for P-gp/MDR, which is responsible for the multidrug-resistance phenotype (5, 26). To determine whether NSC 665517 was a substrate of MDR, cytotoxicity was performed by colony formation assay in KB-V1 and KB3–1 cells. KB-V1 is a multidrug resistant cell line that was established from human nasopharyngeal carcinoma KB cell line as an MDR-expressing clone by stepwise selection with vinblastin (14). Fig. 7 shows that in the parental KB3–1 cells, ~20 μM of NSC 665517 inhibited colony formation by almost 99%, whereas in the KB-V1 cells, even at 100 μM of NSC 665517, ~70–80% of the cell survived.

With the COMPARE analysis, the National Cancer Institute Drug Discovery database was screened for compounds with a cytotoxicity profile similar to the rhodamine efflux profile. Correlation coefficients were calculated for the relationship of the profile of NSC 665517 to the rhodamine efflux profile (Table 1). The high correlation coefficient (0.803) is consistent with NSC 665517 being a substrate of P-gp.

DNA strand breaks are induced by NSC 665517 in cells. The production of DNA single-strand break by NSC 665517 was measured by alkaline elution and was compared with that induced by VP-16 in KB3–1 and KB-V1 cells (Fig. 8). NSC 665517 induced DNA strand breaks that increased with concentration of the drug in KB3–1 cells but not in KB-V1 cells. The potency of NSC 665517 to induce DNA damage was comparable to that of VP-16.

Discussion

Topoisomerases are important targets for the development of new cancer chemotherapeutic drugs. In an effort to screen new topoisomerases inhibitors, several different approaches have been used. Cultures of actinomycetes, fungi, and plant extracts have been screened for their ability to induce top2-mediated cleavage *in vitro*. As a result of these efforts, fla-

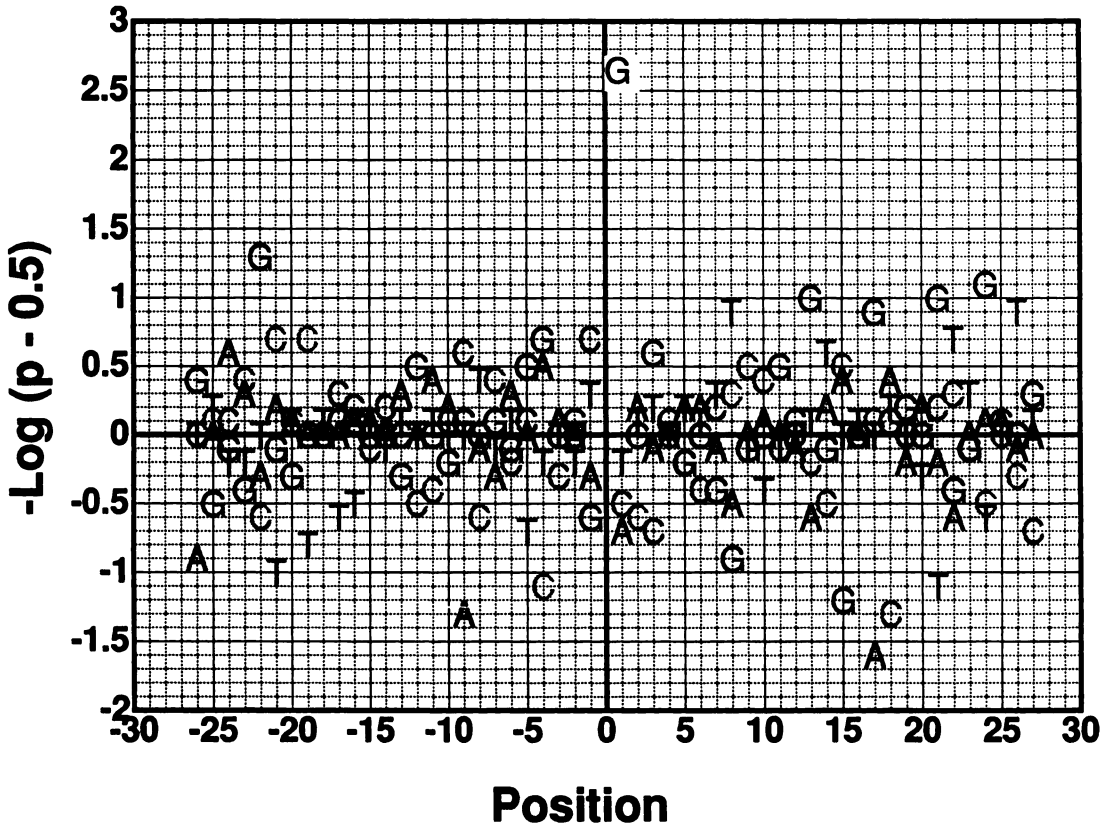


Fig. 6. Probabilities of the observed base frequency deviations at top2 cleavage sites in the presence of NSC 665517. Cleavage sites induced by 100 μM NSC 665517 were analyzed as described previously (20). Values above and below the baselines indicate preference and deficiency relative to the expected frequency, respectively.

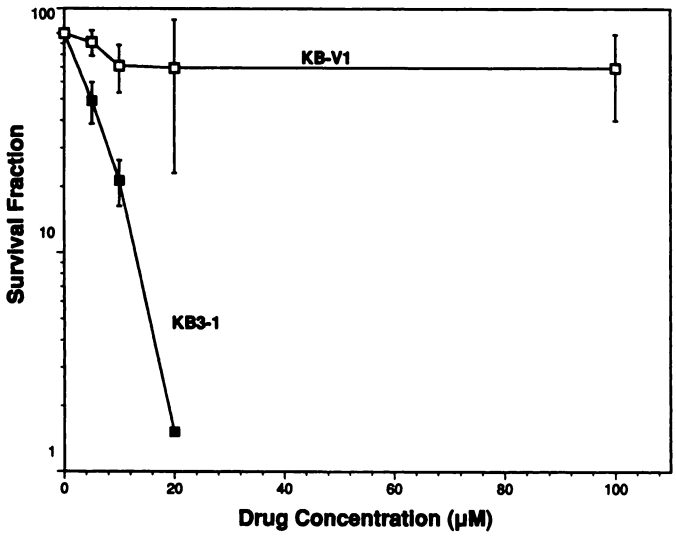


Fig. 7. Cytotoxicity of NSC 665517 in KB3–1 and KB-V1 cells. Cells were treated for 8 hr. After drug treatment, 200 cells were plated in triplicate and were counted after 10 days for colony formation. Percent survival was plotted as a function of drug concentration using a log scale.

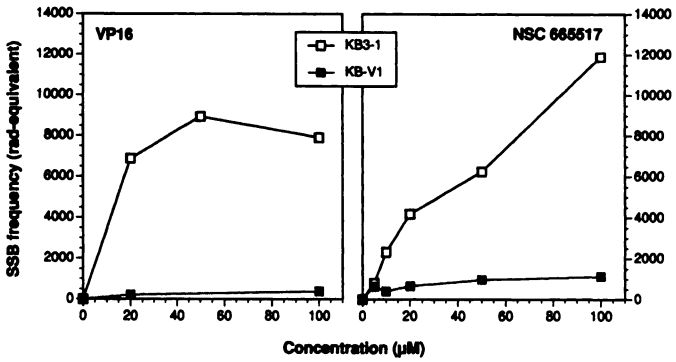


Fig. 8. Dependence of DNA single-strand breaks frequency on drug concentration in KB3–1 and P-gp expressing KB-V1 cells. Cells were exposed at 37° to VP-16 or NSC 665517 for 24 hr. DNA single-strand breaks (*SSB*) were assayed by alkaline elution.

vonoids such as genistein and orobol (27), streptonigrin (28), terpentecin, clerocidin (29), saintopin (30), and naphthoquinones (31) have been identified as top2 inhibitors. Mutant Chinese hamster ovary cell lines xrs-6, which is deficient in DNA double-strand break repair, and a repair competent Chinese hamster ovary line BR1 were used to screen top2 inhibitors from natural marine products (32). The deficiency of the xrs-6 cells makes them more sensitive to top2 inhibitors that produce double-strand breaks. Wakayin, a marine pyrroloiminoquinone, was identified with this screen (32). The National Cancer Institute Preclinical Drug Discovery Screen uses the COMPARE algorithm to evaluate potential mechanism of action of novel drugs and natural product by comparing their cytotoxicity profiles with those of known agents in 60 different human tumor cell lines (12). In the present study, we have shown that NSC 665517 is a new inhibitor of top2. It was identified as a potential top2 inhibitor in the National Cancer Institute Preclinical Anticancer Drug Discovery Screen. The COMPARE analysis showed

that the cytotoxicity profile of NSC 665517 correlated best with those of known top2 inhibitors (Table 1). The COMPARE analysis has previously identified several novel tubulin-binding agents (33) and confirmed the activity of azatoxins on top2 and tubulin (34). Therefore, the COMPARE analysis of the National Cancer Institute anticancer screen can identify new topoisomerase inhibitors.

NSC 665517 belongs to a new class of drug with a novel structure that induces both top2- and top1- (at high concentration) mediated DNA cleavage. Unwinding data showed that NSC 665517 is only a weak DNA intercalator at high concentration, consistent with top2-mediated DNA cleavage not being suppressed at high drug concentrations. Previous studies with anthracyclines or ellipticine derivatives have shown that these DNA intercalators suppress top2-mediated DNA cleavage at high concentration (5 μM) (5). In the case of NSC 665517, however, top2-mediated DNA cleavage increased in a dose-dependent manner (Fig. 4B), and no suppression of DNA cleavage was observed at concentrations up to 500 μM. Thus, NSC 665517 can be categorized with the class of drugs with low DNA binding affinity, such as amsacrine, 5-iminodaunorubicin (8), epipodophyllotoxins (VP-16, VM-26), and azatoxins (11), that induce DNA cleavage without suppression at relatively high drug concentration. As for other classic top2 inhibitors, NSC 665517-induced top2-mediated cleavage was reversed efficiently by heat treatment (Fig. 5) or by the addition of high salt concentration (0.5 M NaCl) (data not shown).

Preferences for one of the bases flanking topoisomerase cleavage sites have been observed for all of the known top2 inhibitors. Drug-specific base preferences observed for top2 inhibitors are A−1 with anthracyclines (8); A+1 with amsacrine (35); T−1 with ellipticine (36); C−1 with epipodophyllotoxins, mitoxantrone, and anthrapyrazoles (37–39); T−1 or T+1 with genistein (37); and C-1 and A+1 with amonafide(40). The drug-specific preferences are either at the 3′ (position −1) or the 5′ (position +1) terminus of the DNA cleavage. Only one inhibitor, streptonigrin, is an exception, with strongly preferred bases in the middle of top2 DNA cleavage site, thymine at position +2, and adenine at position +3 (25, 41). This unusual base preference suggests that streptonigrin interacts with top2-cleavable complexes through a different mechanism compared with other top2 inhibitors. For top1 inhibitors, G+1 is preferred for camptothecin (5) and for the dual inhibitor of top1 and top2, saintopin (20). NSC 665517 induced a distinct DNA cleavage pattern with a strong preference for G at +1 position, showing that it belongs to a different drug class. The inhibitor-specific preferences for the base pairs adjacent to the cleaved phosphodiester bonds suggest that the DNA forms a part of the inhibitor binding site. The strong base preferences among top2 inhibitors led to a model suggesting that the drug interacts with the bases flanking at the enzyme/DNA break (5). Recently, direct evidence supporting this model has been obtained with 3-azido-amsacrine, a photoactivatible analogue of the inhibitor amsacrine with bacteriophage T4 top2 (42), and 7-chloromethylenedioxycamptothecin, an alkylating camptothecin derivative in the case of top1 (43).

Cytotoxicity of NSC 665517 was analyzed by colony formation and single-strand break formation in KB3–1 and KB-V1 cell lines. The cytotoxicity seen in the KB3–1 cells was likely due to the damage of DNA produced by NSC 665517. The cross-resistance exhibited against NSC 665517 in KB-V1, overexpressing the MDR phenotype, suggests that NSC 665517 is recognized and carried out of the cell by P-gp. The P-gp has been detected in several types of human cancers, including ovarian and renal cancer, soft tissue sarcoma, and occasional cases of leukemia and lymphoma (26). Rhodamine efflux assays are also consistent with NSC 665517 (Table 1) being a substrate of P-gp/MDR.

In conclusion, NSC 665517 is a new inhibitor of topoisomerases. Relaxation activity of top2 was inhibited in the presences of NSC 665517. top2- and top1-mediated cleavable complexes were induced by NSC 665517. NSC 665517 has a novel structure; there are no topoisomerase inhibitors of this structural type current in use in cancer treatment or clinical trails. It is novel mechanistically; it is the only known top2 agent that cleaves specifically at G+1 position. Importantly, it is quite likely that the clinical differences observed for the several top2 agents in current use for cancer chemotherapy (actinomycin D, amsacrine, doxorubicin, daunorubicin, etoposide, and mitoxantrone) may be attributable, at least in part, to their cleavage-site specificity. Therefore, the antitumor activity and thus the outcome of a clinical trial with NSC 665517 may not be even remotely predictable on the basis of clinical trials with previous top2 agents.

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