Substituted Purine Analogues Define a Novel Structural Class of Catalytic Topoisomerase II Inhibitors

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Abstract

By screening 1,990 compounds from the National Cancer Institute diversity set library against human topoisomerase IIα, we identified a novel catalytic topoisomerase II inhibitor NSC35866, a 6-substituted analogue of thioguanine. In addition to inhibiting the DNA strand passage reaction of human topoisomerase IIα, NSC35866 also inhibited its ATPase reaction. NSC35866 primarily inhibited DNA-stimulated ATPase activity, whereas DNA-independent ATPase activity was less sensitive to inhibition. We compared the mode of topoisomerase II ATPase inhibition induced by NSC35866 with that of 12 other substituted purine analogues of different chemical classes. The ability of thiopurines with free SH functionalities to inhibit topoisomerase II ATPase activity was completely abolished by DTT, suggesting that these thiopurines inhibit topoisomerase II ATPase activity by covalently modifying free cysteine residues. In contrast, NSC35866 as well as two 6-substituted guanine analogues, 6-benzylguanine and NU2058, could inhibit topoisomerase II ATPase activity in the presence of DTT, indicating that they have a different mechanism of inhibition. NSC35866 did not increase the level of topoisomerase II covalent cleavable complexes with DNA, indicating that it is a catalytic inhibitor and not a poison. NSC35866 was also capable of inducing a salt-stable complex of topoisomerase II on closed circular DNA. In accordance with these biochemical data, NSC35866 could antagonize etoposide-induced cytotoxicity and DNA breaks in human and murine cancer cells, confirming that NSC35866 also functions as a catalytic topoisomerase II inhibitor in cells. (Cancer Res 2005; 65(16): 7470-7)

Introduction

Topoisomerase II is an essential nuclear enzyme found in all living cells. The basic activity of this enzyme is to transiently create a double-strand break in one DNA molecule through which a second dsDNA molecule is transported (1). During this gating process, topoisomerase II is covalently attached to DNA, and this configuration of topoisomerase II covalently attached to DNA is called the cleavable complex (2). Topoisomerase II participates in various DNA metabolic processes, such as transcription, DNA replication, chromosome condensation, and decondensation, and is essential at the time of chromosome segregation following cell division (3). Whereas lower eukaryotes have only one type II topoisomerase, higher vertebrates have α and β isoforms. Topoisomerase IIα is essential for cell proliferation and is expressed only in dividing cells (3, 4). The β isoform is not required for cell proliferation, but knockout mice lacking this isoform die shortly after birth due to defects in their central nervous system (CNS; ref. 5).

Next to compounds targeting the activity of the mitotic spindle apparatus, topoisomerase II–directed drugs are among the most successful clinically applied anticancer compounds encompassing such important classes as the epipodophyllotoxins (exemplified by etoposide), the aminocarboxylic acids (exemplified by ansamycin), and the anthracyclines (exemplified by doxorubicin, daunorubicin, and idarubicin; ref. 6). The success of topoisomerase II as an anticancer target relates to its essential role in cells, its selective expression in proliferating cells (the α isoform), and its lack of biological redundancy.

Most topoisomerase II–directed compounds currently in clinical use, like the ones mentioned above, work by a rather unusual mechanism. Instead of inhibiting the catalytic activity of topoisomerase II, these compounds increase the levels of covalent cleavable complexes in cells (2). The action of DNA metabolic processes then renders these complexes into permanent double-strand breaks, which are highly toxic to cells (7). Topoisomerase II poisons display some level of cancer selectivity because malignant cells tend to divide more rapidly than cells in normal tissues and that they have high levels of topoisomerase II expression. Despite these facts, all topoisomerase II poisons clinically used are toxic to several types of rapidly dividing cells in normal tissues, such as the bone marrow and the gut lining, causing these compounds to have unwanted side effects. One possible way of improving cancer selectivity is to modulate the activity of known topoisomerase II poisons by the use of topoisomerase II catalytic inhibitors (8).

Several classes of structurally unrelated compounds, including the anthracycline derivative aclarubicin, the conjugated thiobarbituric acid derivate miberanone, the coumarin drugs novobiocin and coumermycin A1, the epipodophyllotoxin analogue F 11782, fostrexin, chloroquine, and the bisdioxopiperazines ICRF-187, ICRF-193, and ICRF-154, have been shown to act as catalytic inhibitors of eukaryotic topoisomerase II as extensively reviewed (9, 10). The bisdioxopiperazine compounds have been shown to antagonize DNA damage and cytotoxicity of topoisomerase II poisons (8, 11–14), an antagonism that can be extended to in vivo settings, where ICRF-187 antagonizes the effect of etoposide in the mouse (15), thereby allowing etoposide dose-escalation resulting in improved targeting of tumors in the CNS (16). In a similar fashion, aclarubicin has been shown to protect human cells from the action of topoisomerase II poisons (17), an antagonism that has also been
extended to a mouse model (18). Finally, chloroquine has been shown to protect human cancer cells from etoposide- and camptothecin-induced DNA breaks and cytotoxicity in a pH-dependent fashion (19, 20), serving as proof of principle that topoisomerase catalytic inhibitors can modulate the activity of topoisomerase poisons by targeting their cytotoxicity to acidic environments, such as solid tumors.

To identify novel structural entities targeting topoisomerase II as catalytic inhibitors, we have screened 1,990 compounds from the National Cancer Institute (NCI) diversity set library against human topoisomerase IIb by using a filter-based decatenation assay (21). Here, we characterize the biological activity of NSC35866, a 6'-substituted thioguanine analogue identified in this screen. Results presented show that NSC35866 is a catalytic inhibitor of topoisomerase II in vitro and in cells and represents a novel structural class of inhibitors of this enzyme. We also present data showing that NSC35866 inhibits phosphorylation of histone H1 in nocodazole-treated human cancer cells, suggesting that this compound is also a cyclin-dependent inhibitor (CDK) inhibitor. The potential uses of purine-based compounds as pharmacologic modulators of topoisomerase II are discussed.

Materials and Methods

Drugs and reagents. ICRF-187 (Cardoxane, Chiron Group, Emeryville, CA) was dissolved in sterile water. Etoposide was purchased from Bristol-Myers Squibb (Lyngh by, Denmark) and diluted further in sterile water. Methanesulfone-m-anisidine-4'-(9-acridinyl)amino] hydrochloride (m-AMS A; Amekrin, Pfizer, New York, NY) was diluted in DMSO. NSC35866 was supplied by the Drug Synthesis and Chemistry Branch, Development Therapeutics Program, Division of Cancer Treatment and Diagnosis, NCI (Bethesda, MD) and dissolved in DMSO. [3H]dATP, [3H]thymidine, and [14C]thymidine were purchased from Amersham Biosciences (Uppsala, Sweden). All other compounds were purchased from Sigma-Aldrich (Brondby, Denmark) and dissolved in DMSO. The structural formulas of substituted purine analogues are depicted in Fig. 1.

Purification of human topoisomerase IIb from overexpressing yeast cells. Wild-type and Y165S mutant human topoisomerase IIb was purified from overexpressing yeast cells as described in ref. 22 with modifications described in ref. 23 and was purified to >95% purity as judged by SDS-PAGE and Coomassie blue staining.

Inhibition of topoisomerase II DNA strand passage assay (decatenation assay). Topoisomerase II catalytic activity (DNA strand passage activity) was measured using a filter-based kinetoplast DNA (kDNA) decatenation assay as described (21).

Topoisomerase II ATPase assay. ATP hydrolysis by human topoisomerase IIb was linked to the oxidation of NADH as described (ref. 24 and references therein). The reaction was monitored spectrophotometrically at 340 nm by using a Bio-Tek EL808 Ultra Microplate Reader connected to a PC with KC4 Software installed (Bio-Tek Instruments, Winooski, VT). Reactions were done in 96-well plates (Microtest 96-well Clear Plate, BD Biosciences, San Jose, CA) at 37°C in a total volume of 400 L. The samples contained 17.65 nmol/L human topoisomerase IIb and 1 nmol/L ATP. When present, closed circular DNA was added to obtain a DNA base pair to enzyme-dimer ratio of 425.

Topoisomerase II DNA cleavage assay. A 950-bp linear 3H-labeled DNA was synthesized by performing PCR in the presence of [3H]dATP. The DNA template was derived from a cDNA sequence of human topoisomerase II. Primers used in the PCR reaction were forward 5'-ATGATACGAGA-CTGCTCGGC-3' and reverse 5'-TTAAAACCTATGGCTTATCAG-3'. The DNA fragment was isolated from unincorporated deoxynucleotide triphosphates by ethanol precipitation at 0.3 mol/L NaCl followed by washing in 70% ethanol. The specific activity of the fragment was typically 10,000 to 20,000 cpm/µg. Reaction mixtures containing 100 ng labeled DNA fragment, 300 ng human topoisomerase IIb, topoisomerase II cleavage buffer (10 mmol/L Tris-HCl (pH 7.9), 50 mmol/L NaCl, 50 mmol/L KCl, 5 mmol/L MgCl2, 1 mmol/L EDTA, 15 µg/mL bovine serum albumin, 1 mmol/L Na2ATP), and increasing concentrations of drug in 50 µL reaction volumes were next incubated for 30 minutes at 37°C. A "no topoisomerase II" sample was always included for normalization purposes. The cleavable complex was trapped by adding 5 µL of 10% SDS. After vigorous vortexing for 2 minutes, the samples were centrifuged at 20,000 × g for 2 minutes and the upper water phase (90 µL) was used for scintillation counting using 15 mL Ultima gold scintillation fluid (Packard Biosciences, Groningen, The Netherlands).
Topoisomerase II retention on DNA/streptavidin beads. An assay capable of measuring noncovalent complexes of topoisomerase II on closed circular DNA was done as described (ref. 25 and references therein) with the only modification that 60 μL M280 streptavidin-coated beads slurry corresponding to 600 μg beads and 30 μL biotinylated DNA were used for a total of six reactions.

Cell lines. Human small cell lung cancer (SCLC) OC-NYH (26), NCI-H69 cells (27), human ovary cancer A2780 cells (28), and murine leukemic L1210 cells (29) were grown in RPMI 1640 supplemented with 10% FCS and 100 units/mL penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO2 in the dark.

Clonogenic assay. Clonogenic assay was done as described previously (30).

Alkaline elution assay. Alkaline elution assay was done as described in ref. 31 with modifications as described in ref. 32.

Band depletion assay. Band depletion assay was done with SCLC OC-NYH cells as described (32). For detection of topoisomerase Ila, a polyclonal antibody (Bio Trend, Cologne, Germany) was used.

Determination of histone H1 phosphorylation in cells. Human SCLC OC-NYH and A2780 ovary cancer cells were grown to 50% confluence in 75 cm² tissue culture flasks. The cells were then incubated with drugs for 24 hours. Total histones were extracted as described previously (33). Total histones (10 μg) were used for Western blotting using a polyclonal primary antibody against phosphorylated histone H1 (Upstate Cell Signaling Solutions, Waltham, MA).

Results

NSC35866 inhibits the DNA strand passage reaction of topoisomerase II. Initial screening results had shown that NSC35866 (Fig. 1) inhibited the DNA strand passage activity of purified recombinant human topoisomerase Ila. To establish a dose-response relationship, we next carried out decatenation of Crithidia fasciculata kDNA as described previously (ref. 21; Fig. 2). NSC35866 inhibited the DNA strand passage activity of wild-type human topoisomerase Ila at concentrations above 250 μmol/L, but was clearly less potent in comparison with the reference compound ICRF-187 (Fig. 2A). NSC35866 could also inhibit the catalytic activity of bisdioxopiperazine-resistant (34) mutant Y165S human topoisomerase Ila, whereas ICRF-187 could not as expected (Fig. 2B). This result shows that NSC35866 and the bisdioxopiperazines do not have similar interactions with topoisomerase II.

NSC35866 inhibits the ATPase reaction of topoisomerase II. We next assessed the effect of increasing concentrations of NSC35866 on the ATPase reaction of human topoisomerase Ila. Topoisomerase II is a DNA-stimulated ATPase (35, 36). In the presence of DNA, the rate of ATP hydrolysis by human topoisomerase Ila in the absence of drug was 35 nmol/L ATP hydrolyzed per second, whereas the rate was 7.5 nmol/L ATP hydrolyzed per second in the absence of DNA. To enable a clear comparison of the effects of NSC35866 in the absence and presence of DNA, the rate of ATP hydrolysis was arbitrary set to one in the absence of drug.

In the presence of DNA, NSC35866 inhibited topoisomerase II ATP hydrolysis with an IC50 of 50 μmol/L, whereas 300 μmol/L NSC35866 inhibited 75% of the total ATPase activity (Fig. 3A). NSC35866 could also inhibit the DNA-independent ATPase activity, but without DNA the IC50 value was increased to 300 μmol/L (Fig. 3A), suggesting that NSC35866 targets mainly the DNA-bound conformation of topoisomerase II.

Structure-activity ATPase studies with other substituted purines. To obtain information concerning the mechanism of action of NSC35866, we next did structure-activity ATPase studies, including 12 other substituted purine analogues of different chemical classes (Fig. 1). Two C9-substituted purine analogues, 9-benzylguanine and acyclovir (the latter being an inhibitor of viral DNA polymerase ref. 37), had no inhibitory effect on the ATPase reaction of human topoisomerase Ila at concentrations up to 300 μmol/L (data not shown). 6-Chloroguanine had also no inhibitory effect on the topoisomerase II ATPase reaction (data not shown).
Because NSC35866 is a $S^6$-substituted thioether of guanine, we also assessed the ability of two other $S^6$-substituted thioether purine analogues, 6-methylthioguanine and azathioprine (the latter being used as an antimetabolite prodrug in the clinic; ref. 38), to inhibit the topoisomerase II ATPase reaction (Fig. 3B). Both compounds were capable of inhibiting topoisomerase II ATPase activity but less efficiently than NSC35866.

To establish whether oxygen-based ether analogues may also work as topoisomerase II ATPase inhibitors, we tested the ability of a small series of $O^6$-substituted guanine analogues to inhibit topoisomerase II ATPase activity [i.e., $O^6$-methylguanine, $O^6$-benzylguanine (an inhibitor of the DNA repair protein $O^6$-alkylguanine-DNA alkyltransferase; ref. 39), and NU2058 (an inhibitor of CDK1 and CDK2; ref. 40)]. NU2058 can be regarded as an analogue of $O^6$-benzylguanine, where the benzyl group has been substituted by the more flexible cyclohexane group. Whereas $O^6$-methylguanine had no detectable inhibitory effect on topoisomerase II ATPase activity at concentrations up to 300 $\mu$mol/L (data not shown), $O^6$-benzylguanine and NU2058 were both active (Fig. 3C), having IC$_{50}$ values of 1,000 and 300 $\mu$mol/L, respectively.

We also tested the effect of four different thiopurines with free SH groups (i.e., 6-thioguanine, 6-thiopurine, 2-thiopurine, and 2,6-dithiopurine) as topoisomerase II ATPase inhibitors (see Fig. 3D; 6-thioguanine and 6-thiopurine are both used clinically as antimetabolites; ref. 38). 6-Thiopurine and 6-thioguanine both inhibited the ATPase activity of topoisomerase II, with 6-thioguanine having an IC$_{50}$ of $\sim$ 30 $\mu$mol/L and 6-thiopurine having an IC$_{50}$ of $\sim$ 100 $\mu$mol/L. 2-Thiopurine and 2,6-dithiopurine inhibited topoisomerase II ATPase activity having IC$_{50}$ values of $\sim$ 3 $\mu$mol/L.

Recombinantly expressed human topoisomerase IIa purified by a protocol similar to the one used here has been shown to contain free cysteine residues (41). Furthermore, thiopurines having free SH functionalities have been shown to covalently modify proteins at free cysteine residues (42). We therefore tested the ability of all active compounds to inhibit topoisomerase II ATPase activity in the presence of DTT (data not shown). Because DTT inhibits the formation of thiopurine-topoisomerase II covalent interactions, this treatment is expected to abolish the effects attributed to covalent modification. Although NSC35866, $O^6$-benzylguanine, and NU2058 could inhibit topoisomerase II ATPase activity in the presence of DTT, this was not the case with four thiopurines having free SH functionalities. This result suggests that the latter inhibit topoisomerase II ATPase activity by covalent modification, whereas NSC35866, $O^6$-benzylguanine, and NU2058 inhibit topoisomerase II by noncovalent interactions. Covalent modification of free cysteines by thiol-reactive compounds has been associated previously with catalytic inhibition of topoisomerase II (21, 41).

Inhibition of topoisomerase DNA strand passage by selected thiopurines. Because some of the thiopurines used in the ATPase structure-activity studies above are used as antimetabolites in the clinic (6-thioguanine, 6-thiopurine, and azathioprine; ref. 38), we assessed their effect on the DNA strand passage reaction of human topoisomerase II (Fig. 4).
topoisomerase IIα. The results of these experiments are depicted in Fig. 2C. 6-Thioguanine inhibited the catalytic activity of topoisomerase II. Although this compound did not reach a maximal level of inhibition similar to that of the reference compound ICRF-187, it displayed a rapid onset and half-maximal inhibition was achieved ~50 μmol/L. 6-Thiopurine was much less potent, and maximal inhibition was apparently not reached at 1,000 μmol/L, suggesting that the NH2 group present only in 6-thioguanine plays a role for topoisomerase II inhibition. 2 Thiopurine and 2,6-dithiopurine were both less potent in inhibiting topoisomerase II DNA strand passage activity than 6-thioguanine, although these compounds were more potent than 6-thioguanine in their inhibition of topoisomerase II ATPase activity. This result indicates that specific types of cysteine modifications may have differential effects on the ATPase and DNA strand passage reactions of human topoisomerase II.

**NSC35866 is a catalytic topoisomerase II inhibitor in vitro and in cells.** To establish whether NSC35866 inhibits the DNA strand passage reaction of topoisomerase II by stabilizing a covalent reaction intermediate, we developed a new and highly sensitive assay for the detection of topoisomerase II-DNA covalent complexes in vitro. This assay is based on the fact that, after extraction with phenol/chloroform, protein-bound DNA is removed from the water phase, whereas naked DNA stays in the water phase. The covalent topoisomerase II-DNA complex is a DNA-protein complex. Therefore, in reactions containing topoisomerase II and linear DNA, the ability of compounds to remove DNA from the water phase after phenol/chloroform extraction should reflect their potency as topoisomerase II poisons. We first validated this assay by incubating 100 ng of a linear 3H-labeled 950-bp DNA fragment with 300 ng of purified human topoisomerase II in the presence of increasing concentrations of etoposide and m-AMSA.

Within each experiment, a “no topoisomerase II” sample served as internal control for normalization. That is, the amount of radioactivity retained in the water phase of this sample was set to 100%. Figure 4A and B depicts the percentage of DNA removed from the water phase as the function of increasing concentrations of etoposide and m-AMSA. Both compounds increased the percentage of DNA removed from the water phase in a dose-dependent manner. Omitting ATP from the reaction greatly reduced the effect of etoposide in accordance with this compound requiring ATP for efficient DNA cleavage (ref. 43; data not shown).

We next tested the ability of NSC35866 to increase the level of topoisomerase II-DNA covalent complexes using etoposide as a positive control (Fig. 4C). Whereas etoposide was highly effective in removing DNA from the water phase, NSC35866 had no effect at concentrations up to 1,000 μmol/L, showing that it is not a topoisomerase II poison. The ability of NSC35866 to inhibit the DNA strand passage reaction of topoisomerase II without increasing the level of covalent complex establishes this compound as a catalytic topoisomerase II inhibitor.

The alkaline elution assay represents a direct and highly sensitive way of measuring DNA breaks in cells (31). Figure 4D depicts the result of an alkaline elution assay. It is evident that 3 μmol/L etoposide results in extensive fragmentation of DNA. Whereas 100 μmol/L NSC35866 had no detectable effect on the level of etoposide-induced DNA breaks, 500 μmol/L NSC35866 partly antagonized the effect of etoposide and 1,000 μmol/L NSC35866 completely antagonized etoposide-induced DNA breaks. From Fig. 4D, it is also evident that NSC35866 does not induce detectable levels of DNA breaks by itself at concentrations up to 1,000 μmol/L in accordance with our DNA cleavage results (Fig. 4C). The ability of NSC35866 to antagonize etoposide-induced DNA breaks in cells is consistent with this compound being a catalytic topoisomerase II inhibitor.
NSC35866 induces a salt-stable complex of topoisomerase II on closed circular DNA in vitro and in cells. We next assessed the ability of NSC35866 to induce a salt-stable complex of human topoisomerase IIα around circular DNA. We used an assay measuring the retention of topoisomerase II on circular plasmid DNA attached to magnetic beads via biotin-streptavidin linkage as described (44). Figure 5A depicts the result of a typical experiment preformed in the presence of 1 mM ATP. In the absence of any drug, very little protein was retained on the beads after washing with 2 mM KCl (Fig. 5A, lane 1). Addition of 200 mM ICRF-187 to the reaction mixture strongly induced the retention of topoisomerase II to the beads (Fig. 5A, lane 2). Figure 5A (lanes 3-6) depicts protein retention in the presence of increasing concentrations of NSC35866 (30, 100, 300, and 1,000 μM). It is evident that NSC35866 traps human topoisomerase IIα as a salt-stable complex on circular closed DNA in a dose-dependent manner. NSC35866 was also capable of trapping the protein as a salt-stable complex on DNA in the absence of ATP but only at 300 and 1,000 μM (Fig. 5B). Protein retention induced by ICRF-187 depended strongly on ATP as expected (compare Fig. 5A, lane 2, with Fig. 5B, lane 2).

The band depletion assay can be used to assess the binding of proteins to DNA in cells under various conditions (45). Figure 5C depicts the result of a band depletion assay measuring the extractable amount of human topoisomerase IIα protein from SCLC OC-NYH cells as determined by Western blot. ICRF-187 (200 μM/L) strongly reduced the amount of extractable topoisomerase IIα compared with the “no drug” sample (compare Fig. 5C, lanes 1 and 2) as expected. Exposure of cells to 500 μM/L NSC35866 (Fig. 5C, lane 4) and 1,000 μM/L NSC35866 (Fig. 5C, lane 5) clearly reduced the amount of extractable topoisomerase II, showing that NSC35866 induces a complex of topoisomerase IIα on DNA in cells.

NSC35866 abrogates hyperphosphorylation of histone H1, a phosphorylation target for cyclin-dependent kinase 1 and 2 in nucodazole-treated cells. NU2058 and NSC35866 are both analogues of guanine substituted at C6. Because NU2058 is a CDK1/CDK2 inhibitor, this prompted us ask whether NSC35866 might also inhibit CDK activity in cells. Phosphorylation of histone H1 in human SCLC OC-NYH and A2780 ovary cancer cells, respectively. Cells were treated with the drugs indicated for 24 hours and total histones were isolated. Lane 1, no drug; lane 2, 100 ng/mL nucodazole; lane 3, 100 ng/mL nucodazole + 125 μM/L NU2058; lane 4, 100 ng/mL nucodazole + 40 μM/L roscovitine; lane 5, 100 ng/mL nucodazole + 500 μM/L NSC35866.

NSC35866 protects cancer cells from etoposide-induced cytotoxicity. Catalytic topoisomerase II inhibitors have the potential to protect cultured cells from topoisomerase II poison-induced cytotoxicity (8, 11–13, 17, 20). Preexposure of human SCLC OC-NYH cells to increasing concentrations of NSC35866 for 20 minutes followed by coexposure to etoposide for 60 minutes antagonized etoposide-induced cytotoxicity in a dose-dependent manner. A typical experiment of three is depicted in Fig. 6. NSC35866 could reduce cytotoxicity derived from a 1-hour incubation with 20 μM/L etoposide in a dose-dependent manner and conferred up to 50-fold protection. NSC35866 was likewise capable of protecting human SCLC NCI-H69 cells, A2780 ovary cancer cells, and murine leukemic L1210 cells from etoposide-induced cytotoxicity (data not shown). Together, these data show that NSC35866 functions as a catalytic inhibitor of topoisomerase II in cells.

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Figure 5. NSC35866 stabilizes a salt-stable complex of human topoisomerase IIα on DNA in vitro and in cells. A, levels of salt-stable topoisomerase II complexes formed in the presence of 1 mM ATP. Retention of salt-stable (to 2 mM KCl) complexes of human topoisomerase IIα on circular DNA attached to magnetic beads was determined by eluting retained protein by adding running buffer containing 4% SDS followed by heating to 100°C for 10 minutes followed by SDS-PAGE and staining with GelCode Blue Stain Reagent (Pierce, Rockford, IL). Lane 1, no drug; lane 2, 200 μM/L ICRF-187; lane 3, 30 μM/L NSC35866; lane 4, 100 μM/L NSC35866; lane 5, 300 μM/L NSC35866; lane 6, 1,000 μM/L NSC35866; lane K, 2 μg human topoisomerase IIα. Representative of four independent experiments. B, levels of salt-stable topoisomerase II complexes formed in the absence of ATP. Drug treatments were the same as described in A, with the exception that ATP was omitted from the reactions. Representative of three independent experiments. C, ability of NSC35866 to stabilize topoisomerase IIα as a nonextractable complex on DNA in human SCLC OC-NYH cells was assessed using the band depletion assay. The amounts of topoisomerase IIα were visualized by Western blotting using a topoisomerase IIα–specific primary antibody. Lane 1, no drug; lane 2, 200 μM/L ICRF-187; lane 3, 300 μM/L NSC35866; lane 4, 500 μM/L NSC35866; lane 5, 1,000 μM/L NSC35866. We detected band depletion of topoisomerase IIα in two independent experiments. D and E, phosphorylation of histone H1 in human SCLC OC-NYH and A2780 ovary cancer cells, respectively. Cells were treated with the drugs indicated for 24 hours and total histones were isolated. Lane 1, no drug; lane 2, 100 ng/mL nucodazole; lane 3, 100 ng/mL nucodazole + 125 μM/L NU2058; lane 4, 100 ng/mL nucodazole + 40 μM/L roscovitine; lane 5, 100 ng/mL nucodazole + 500 μM/L NSC35866.
We also tested the ability of relevant concentrations of 6-thiopurine, 6-thioguanine, azathioprine, 6-methylthioguanine, 2-thiopurine, and 2,6-dithiopurine to antagonize etoposide-induced cytotoxicity with OC-NYH cells and observed no effect (data not shown).

Although NSC35866 is nontoxic to cells at concentrations up to 500 μmol/L when applied for 20 plus 60 minutes as described above, continuous exposure is cytotoxic to cells as evidenced from NCI-60 cell line screening data available at http://dtp.nci.nih.gov/dtpstandard/servlet/dwindex?searchtype=NSC&chemnameboolea- n=and&outputformat=htmL&searchlist=35866&Submit=Submit. In these assays, the IC₅₀ of NSC35866 was in the range of 20 to 100 μmol/L. This mode of cytotoxicity is reminiscent of that of the bisdioxopiperazines where continuous exposure is also cytotoxic, whereas short time exposure is not.

**Discussion**

Data presented here establish that NSC35866 functions as a catalytic inhibitor of topoisomerase II *in vitro* and in cancer cells and that this compound stabilizes a noncovalent salt-stable complex of topoisomerase II on DNA. Although this mechanism of action is reminiscent of that of the bisdioxopiperazines (44, 48, 49), bisdioxopiperazine-resistant mutant topoisomerase IIα responds at least as well to inhibition by NSC35866 as wild-type protein. This result indicates that NSC35866 and the bisdioxopiperazines inhibit topoisomerase II by different mechanisms although similarities exist, which is not surprising given the lack of structural similarity between bisdioxopiperazines and NSC35866. The bisdioxopiperazine-binding pocket (ICRF-187) on yeast topoisomerase II has recently been resolved by X-ray crystallography (50), and the drug-binding site described does not suggest that NSC35866 interacts at this interaction site in agreement with our biochemical data.

We found the ATPase IC₅₀ value for NSC35866 to be 50 μmol/L in the presence of DNA, although the IC₅₀ value for this compound was considerably higher in the DNA strand passage assay. We observed the same pattern with the bisdioxopiperazine class of catalytic inhibitors.⁶ This apparent discrepancy is likely related to the mode of ATP usage by topoisomerase II. The ATPase and DNA strand passage reactions of topoisomerase II are not tightly coupled. At low ATP concentrations, ATP utilization is highly efficient, but at saturating ATP levels (a situation similar to our assay conditions) the number of ATP molecules hydrolyzed per DNA strand passage event is much higher (51). In addition, we have shown recently that mutations in yeast topoisomerase II and human topoisomerase IIα that greatly reduce the rate of ATPase hydrolysis do not result in impaired DNA strand passage activity at saturating ATP levels (52).

Taken together, these findings suggest that, at 1 mmol/L ATP, a significant fraction of topoisomerase II ATP hydrolysis must be inhibited before an effect on DNA strand passage is seen, which may explain the observed differences in NSC35866 IC₅₀ values observed in ATPase and DNA strand passage assays.

We did structure-activity studies, including 12 other substituted purine analogues. In this analysis, NSC35866 was capable of inhibiting topoisomerase II ATPase activity in the presence of DTT, whereas this was not the case for thiopurines with free SH groups. This result suggests that the latter inhibit topoisomerase II ATPase activity through covalent modification of free cysteine residues, a mechanism of protein interaction suggested previously for thiopurines having free SH functionalities (42). NSC35866 was highly efficient in protecting cancer cells from etoposide-induced cytotoxicity, whereas this was not the case for various thiopurines having free SH functionalities (data not shown), suggesting that clinically applied thiopurines, such as 6-thioguanine and 6-thiopurine, do not interfere with the function of topoisomerase II *in vivo*.

In the present work, NSC35866 is established as a catalytic inhibitor of topoisomerase II *in vitro* and in cells, having the potential to modulate the effects of topoisomerase II poisons. However, this compound must be regarded as a starting point for further drug development due to its low potency. This raises the question as to how more potent analogues may be developed. Our ATPase structure-activity data obtained with O⁶-methylguanine, O⁶-benzylguanine, and NU2058 suggest that modulating the size or flexibility of the side chain at C6 could result in analogues with higher potency. Furthermore, our DNA strand passage structure-activity data suggest that the presence of a substituent on C2 is also important for potency. Modifying the substituent attached to C2 in substituted purine analogues has pronounced effects on their potency as CDK inhibitors (53). Our finding that NU2058 and NSC35866 may target both topoisomerase II and CDK activity is interesting and suggests that dual inhibitors of topoisomerase II and CDK activity may be developed. Such agents should be useful as anticancer agents.

In summary, results presented here for the first time establish substituted purines as a novel class of catalytic topoisomerase II inhibitors. This modality adds topoisomerase II activity to a growing list of biological reactions/targets affected by these compounds now, including DNA polymerases (37), CDKs (40), DNA repair (39), and incorporation into DNA (38). Our structure-activity studies show that substitution at the C6 position is sufficient for topoisomerase II inhibition, leaving chemical space open for functional modifications. The identification of purine [Figure 6.](#) NSC35866 antagonizes etoposide-induced cytotoxicity with human cancer cells. SCLC OC-NYH cells were preincubated for 20 minutes with increasing concentrations of NSC35866. Etoposide (20 μmol/L) was then added and the cells were incubated for 1 hour. Next, the drugs were washed out and the cells were plated and counted after 3 weeks. The relative survival of cells receiving the various treatments compared with cells receiving no treatment is plotted against the NSC35866 concentration.

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⁶ Unpublished data.
analyses as catalytic inhibitors of topoisomerase II may therefore pave the way for the development of compounds capable of pH-dependent modulation of the activity of topoisomerase II, which may be useful in directing the cytotoxicity of established topoisomerase II poisons toward solid tumors.

References
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