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Topoisomerase I inactivation by a novel thiol reactive naphthoquinone

Steven Kennedy [a], John C. DiCesare [b], Robert J. Sheaff [a,*]

[a] Department of Chemistry and Biochemistry, The University of Tulsa, Tulsa, OK, United States
[b] Department of Chemistry, Georgia Southern University, Statesboro, Georgia

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ABSTRACT

The naphthoquinone adduct 12,13-dihydro-*N*-methyl-6,11,13-trioxo-5H-benzo[4,5]cyclohepta[1,2-b] naphthalen-5,12-imine (hereafter called TU100) contains structural features of both the anthracycline and isoquinone chemotherapeutics. An initial characterization showed TU100 is cytotoxic to mammalian cells and can inhibit topoisomerase I and II. Analysis using topoisomerase I now reveals TU100 is a slow acting inhibitor targeting the enzyme in the absence of DNA. Diluting pre-incubated TU100 and topoisomerase I failed to alleviate inhibition, suggesting the enzyme is being covalently modified. Critical cysteine thiols were identified as the possible target based on the ability of reducing agents to reverse TU100 inhibition. Consistent with this idea, TU100 protected topoisomerase I from inactivation by the sulfhydryl modifying agent *N*-ethylmaleimide (NEM). Unlike agents nonspecifically reacting with thiols, however, TU100 is specific for topoisomerase because it failed to inhibit a cysteine dependent protease. These results indicate TU100 is a novel naphthoquinone that inactivates free topoisomerase I via alkylation of cysteine residues.

1. Introduction

Cancer cells exhibit inappropriate cell division [1], making antiproliferative agents a major focus of drug development [2]. Topoisomerases are a well known target because they alleviate topological problems associated with DNA structure such as supercoiling, knotting and catenation [3]. Topoisomerase I and III are type I enzymes that cleave a single strand of the DNA duplex, whereas topoisomerase II-type enzymes cleave both strands [3,4]. Both types bind double stranded DNA and utilize a transesterification reaction in which a tyrosine hydroxyl forms a covalent but transient intermediate linking enzyme to the DNA backbone [3].

Small molecule inhibitors targeting both types of topoisomerase are an important component in the chemotherapeutic arsenal used to treat cancer [5–10]. Unfortunately, due to serious side-effects and/or development of resistant cancer cells, new improved versions are needed. Topoisomerase inhibitors can be divided into three categories based on specificity for the class of enzyme targeted (topoisomerase I, II, or the more rare dual I/II inhibitors). Mechanistically topoisomerase inhibitors also fall into three classes: (1) Topoisomerase poisons, so called because they stabilize enzyme:DNA strand cleavage intermediates that are then converted into lethal DNA lesions (e.g. double strand breaks) by DNA replication and/or transcriptional machinery [11–13]. (2) Catalytic inhibitors, which interact non-covalently with enzyme (e.g. substrate competition) to impede enzymatic activity [14]. They do not result in DNA lesions and are typically less cytotoxic. (3) Non specific covalent modifying agents such as *N*-ethylmaleimide (NEM), which inactivate the enzyme by alkylating cysteine residues [15].

Topoisomerase poisons are often planar hydrophobic compounds that interact with free or enzyme bound DNA. Anthracyclines bind free DNA very tightly via intercalation between base pairs [9,10,13,16–18], while Hoechst 33358 is a minor groove binder [19]. In contrast, the poisons camptothecin and etoposide preferentially interact with the enzyme–DNA complex [6,7,11,12]. TU100 has structural similarity to both the anthracyclines and isoquinolines, containing a hydroquinone moiety and a benzopyridine derived functional group (see Fig. 4D). Cellular effects of anthracyclines are mediated in part by inhibiting topoisomerase II, while quinoline-based alkaloids like camptothecin target topoisomerase I [20–23]. Our previous work showed that TU100 functions as a dual topoisomerase I and II inhibitor but does not intercalate into DNA [24].

The present investigation further characterized TU100's unique mechanism of action using topoisomerase I. Pre-incubation of the drug and enzyme resulted in time dependent inhibition. Topoisomerase activity could not be recovered by diluting out TU100, suggesting it was covalently modifying the enzyme. This inactivation preferentially targeted free topoisomerase and was decreased in the presence of DNA. Inhibition could be alleviated by reducing agents, suggesting sulfhydryls on cysteine residues were being modified. This hypothesis was confirmed by showing that TU100 could protect topoisomerase I from inactivation by the sulfhydryl

* Corresponding author. Address: Department of Chemistry and Biochemistry, The University of Tulsa, 800 South Tucker Drive, Tulsa, OK 74104, United States.
E-mail address: robert-sheaff@utulsa.edu (R.J. Sheaff).

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modifying agent NEM. These results show TU100 is a novel topoisomerase I inhibitor whose mechanism of action involves enzyme inactivation via covalent modification of cysteine thiol(s).

2. Materials and methods

2.1. Chemicals

TU100. was synthesized as described previously [25] from the 3 + 2 dipolar cycloaddition of *N*-methyl-4-hydroxyisoquinolinium iodide and naphthoquinone, dissolved in 100% sterile DMSO, and stored at −20 °C. NEM, glutathione, L-cysteine, DTT, camptothecin, etoposide and hydrogen peroxide (30%) were all obtained from Sigma–Aldrich, St. Louis, MO. NEM was prepared fresh each use in 100% EtOH. Glutathione, L-cysteine, and DTT were dissolved in H_2O, while camptothecin and etoposide were re-suspended in 100% DMSO. Ethidium bromide was obtained from Fisher Scientific and dissolved in H_2O. Purified topoisomerase I was obtained from Topogen.

2.2. Cell culture

HeLa cells, a cervical cancer line, were obtained from the American Tissue Culture Collection (ATCC) and maintained in DMEM (Fisher Scientific) supplemented with 10% fetal bovine serum (FBS; Atlas Biologicals, Fort Collins, CO). They were maintained in a 37 °C water jacketed incubator with 5% CO_2 in 100 mm tissue culture dishes.

2.3. Topoisomerase purification

Topoisomerase was partially purified using a protocol from Topogen. Proliferating cells from two 100 mm plates were removed by scraping and pelleted at 800×*g* for 3 min at 4 °C. The cell pellet was washed with 3 ml TEMP buffer (10 mM Tris–HCl pH7.5, 1 mM EDTA, 4 mM $MgCl_2$, 0.5 mM freshly prepared PMSF) and re-suspended in 3 ml of the same buffer. After 10 min on ice the cell suspension was dounced with seven strokes in a glass homogenizer. Nuclei were pelleted at 1500×*g* for 10 min at 4 °C and washed with 1 ml cold TEMP. The nuclear pellet was re-suspended in TEP (same as TEMP minus $MgCl_2$) and an equal volume of 1 M NaCl added. The nuclei were vortexed three times for 15 s each and left on ice for 1 h. Insoluble material was removed by centrifugation at 12 k for 15 min at 4 °C. Supernatant containing topoisomerase activity was stored at −20 °C in small aliquots.

2.4. Preparation of supercoiled plasmid DNA

Supercoiled plasmid DNA was purified from *E. coli* by alkaline lysis. The XL-1 Blue strain of *E. coli* was transformed with an Amp resistant plasmid and a single colony grown to log phase in 50 ml LB media. Cells were pelleted and re-suspended in DNA lysis buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris pH 8) followed by lysis with 10 ml 0.2 M NaOH + 1% SDS for 10 min on ice. Potassium acetate (7.5 ml of 3 M) was added for 10 min on ice followed by centrifugation to remove insoluble material. The plasmid was precipitated with 13.5 ml isopropanol for 5 min at room temp and pelleted by spinning 5 k for 20 min. Plasmid was re-suspended in 5 ml TE and the RNA digested with 100 μg RNase at 37 °C for 20 min. The plasmid was precipitated a final time with 550 μl NaOAc plus 3.5 ml isopropanol, then re-suspended in 0.5–1 ml TE and stored at −20 °C. The extent of supercoiling was determined by analysis on an 0.8% agarose gel run in the absence of ethidium bromide. Gels were post-stained with 2 μg/ml ethidium bromide, washed with distilled water, and plasmid migration visualized using the AlphaTech Imager.

2.5. Topoisomerase assays

Relaxation of supercoiled plasmid DNA (1 μg) was carried out in a 20 μl reaction at 37 °C for the indicated times with topoisomerase I (partially purified as described above or obtained in a highly purified form from Topogen) and topoisomerase I reaction buffer (10 mM Tris pH 7.9, 150 mM NaCl, 0.1% BSA, 100 μM spermidine, and 5% glycerol). The enzyme was diluted into TEP + 1 mg/ml BSA. Reactions were stopped by 5 μl of stop buffer (5% sarkosyl, 0.0025% bromophenol blue, 25% glycerol). The final DMSO concentration did not exceed 5% and had minimal effect on topoisomerase activity.

Pre-incubation of enzyme and drugs (in 5 μl) was carried out for indicated times and temperature in topoisomerase buffer, followed by the addition of reaction mix containing supercoiled plasmid DNA (15 μl) and incubation at 37 °C for 15–30 min. High concentrations of TU100 (100–500 μM) and camptothecin (10 μM) were utilized in these experiments to ensure complete enzyme inhibition. In Fig. 2C supercoiled plasmid DNA (0.25 μg) was included in the pre-incubation (on ice to allow topoisomerase binding but prevent relaxation). For pre-incubation of TU100 with TCEP-agarose, 500 μM TU100 and 50 μl 1:1 slurry of beads were incubated at room temperature for 10 min. The beads were removed by centrifugation and remaining TU100 analyzed in a relaxation assay.

Samples were separated on a 0.8–1% agarose gel in the absence of ethidium bromide, stained in 1X TAE with ethidium bromide (~2 μg/ml) for 30 min, and visualized using an Alpha-Tech imager. Topoisomerase inhibition or activity was quantified by determining the intensity of supercoiled and relaxed forms of plasmid DNA using the Alpha-Innotech imaging software. IC_{50} values were determined using Prism software.

2.6. Other enzymatic activities

Taq polymerase, restriction endonucleases (EcoR1), and the trypsin were obtained from either Fisher Scientific or Sigma, and their activity analyzed according to manufacturers' instructions. Bromelain (a cysteine protease) was obtained as a crude extract from pineapple. Briefly, ~5 g of fresh pineapple was flash frozen with liquid nitrogen and homgenized with mortar and pestle, then re-suspended in 5 ml PBS. The sample was sonicated 3× for 30 s each, then clarified by centrifugation. Supernatant contained highly active bromelain and was assayed using BSA as substrate.

3. Results

3.1. TU100 inhibits topoisomerase I activity

Topoisomerase activity was partially purified from HeLa cells as described in the Materials and Methods. Fig. 1A demonstrates its ability to relax supercoiled plasmid DNA substrate. Lane 1 is the no enzyme control, while lanes 2–5 show increasing cell extract decreased supercoiled DNA with a concomitant increase in the relaxed form. Quantitation showing disappearance of supercoiled DNA and appearance of the relaxed form is shown in Fig. 1B. We utilized the highly specific inhibitors camptothecin (targets topoisomerase I) and etoposide (targets topoisomerase II) to determine the enzyme responsible. Camptothecin completely inhibited the reaction while etoposide had no effect (data not shown), indicating the extract contained mainly topoisomerase I. We next confirmed that TU100 inhibits this reaction (Fig. 1C). Quantitation and graph-

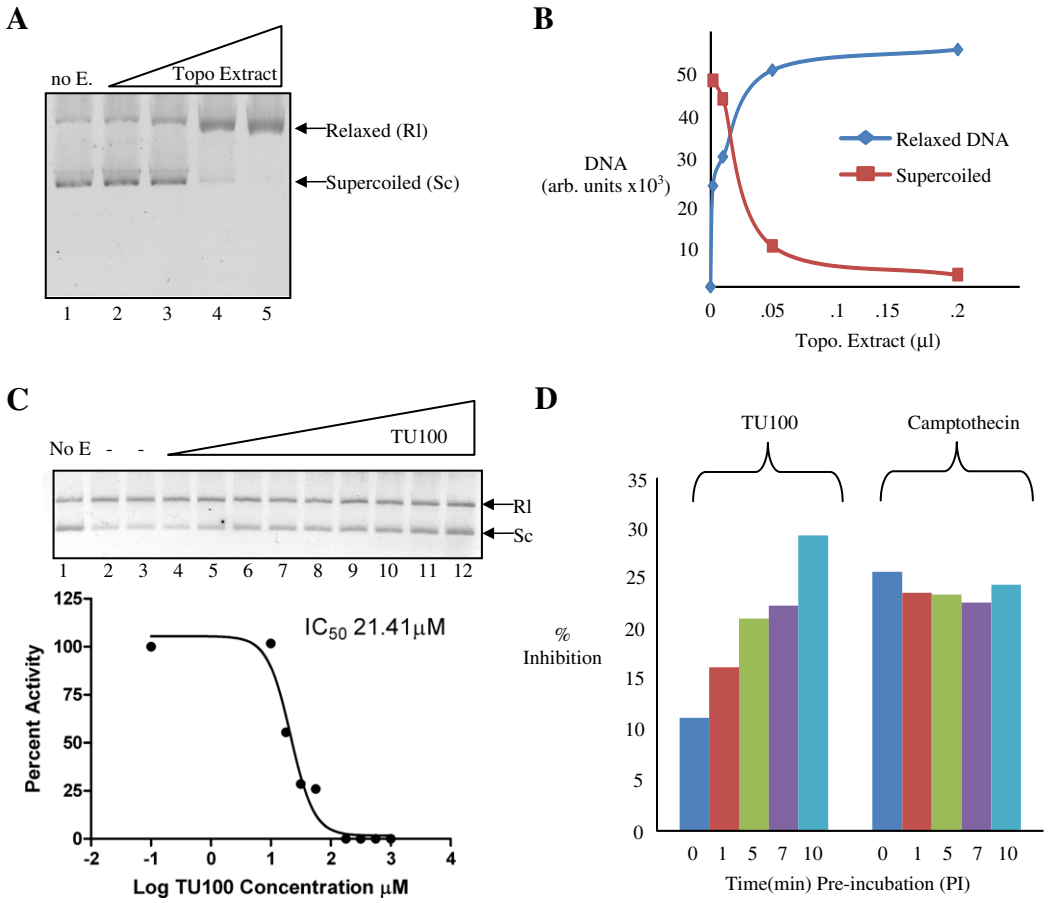

Fig. 1. (A) Relaxation of supercoiled plasmid DNA by partially purified topoisomerase I. A plasmid relaxation assay was carried out using topoisomerase I partially purified from HeLa cell extract. Products were analyzed by agarose gel electrophoresis. Lane 1 is the no enzyme control. (B) Quantitation of 1A. (C) TU100 inhibits topoisomerase I. A plasmid relaxation assay was carried out using the topoisomerase I extract in the presence of increasing TU100 concentrations. Products were analyzed by agarose gel electrophoresis and the IC_{50} determined. Lane 1 is the no enzyme control. (D) TU100 inhibition is time dependent. Topoisomerase I was pre-incubated for indicated times with TU100 or camptothecin before addition to a plasmid relaxation assay.

ing as% activity remaining vs. log TU100 concentration gave an IC_{50} of ~21 µM.

Previous results suggested TU100 inhibition is time dependent, so we analyzed the effect of pre-incubating drug and enzyme. In Fig. 1D TU100 or the camptothecin control was pre-incubated(PI) with topoisomerase I extract for the indicated times, then added to reaction mix containing DNA substrate. After 30 min at 37 °C products were analyzed by agarose gel electrophoresis. The amount of inhibition was determined relative to the no drug control. Increasing pre-incubation time dramatically enhanced TU100 potency, while camptothecin had no such effect (Fig. 1D). These results confirm TU100 is a slow acting inhibitor that can target topoisomerase I in the absence of DNA. We therefore further investigated its mechanism of action.

3.2. Covalent modification of topoisomerase I by TU100

The time dependency of TU100 inhibition could be due to slow binding, but this seems unlikely given the significant amount of pre-incubation required. We therefore considered the possibility TU100 was covalently modifying the enzyme since anthracyclines and isoquinolines can be involved in redox reactions [23,26]. Consistent with this hypothesis, increasing the pre-incubation temperature decreased the time required for maximal inhibition (data not shown).

As a more direct test we performed the dilution experiment shown in Fig. 2A. TU100 or camptothecin control was pre-incubated with the enzyme, then diluted four fold into a reaction mix containing supercoiled plasmid DNA. If topoisomerase I is covalently modified then it will still be inhibited despite drug dilution. Lane 1 is the no enzyme control. Lane 2 shows topoisomerase pre-incubated in the absence of drug retains activity. No inhibition occurred when enzyme was diluted into the final concentration of TU100 (lane 3) and camptothecin (lane 4), so any effect on topoisomerase activity would have to occur during pre-incubation. The pre-incubation conditions were adequate for drug:enzyme interaction because enzyme diluted into the pre-incubation drug concentrations (lanes 5 and 6) inhibited relaxation. Topoisomerase was then pre-incubated with TU100 (lanes7 and 8) or camptothecin (lanes 9 and 10) followed by dilution into the reaction mix. Only TU100 still inhibited plasmid relaxation, suggesting it was covalently modifying the enzyme during pre-incubation. Quantitation of the data is shown in Fig. 2B.

These results indicate TU100 can target free topoisomerase I, so we analyzed the effects of DNA substrate on this interaction. TU100 was pre-incubated with topoisomerase in the presence or absence of plasmid DNA and then diluted into the reaction mix (Fig. 2C). This pre-incubation was performed on ice to allow the topoisomerase I and DNA to interact but not undergo catalysis. TU100 alone blocked subsequent activity (compare lanes 2 and 3), while including DNA partially alleviated this inhibition (lane

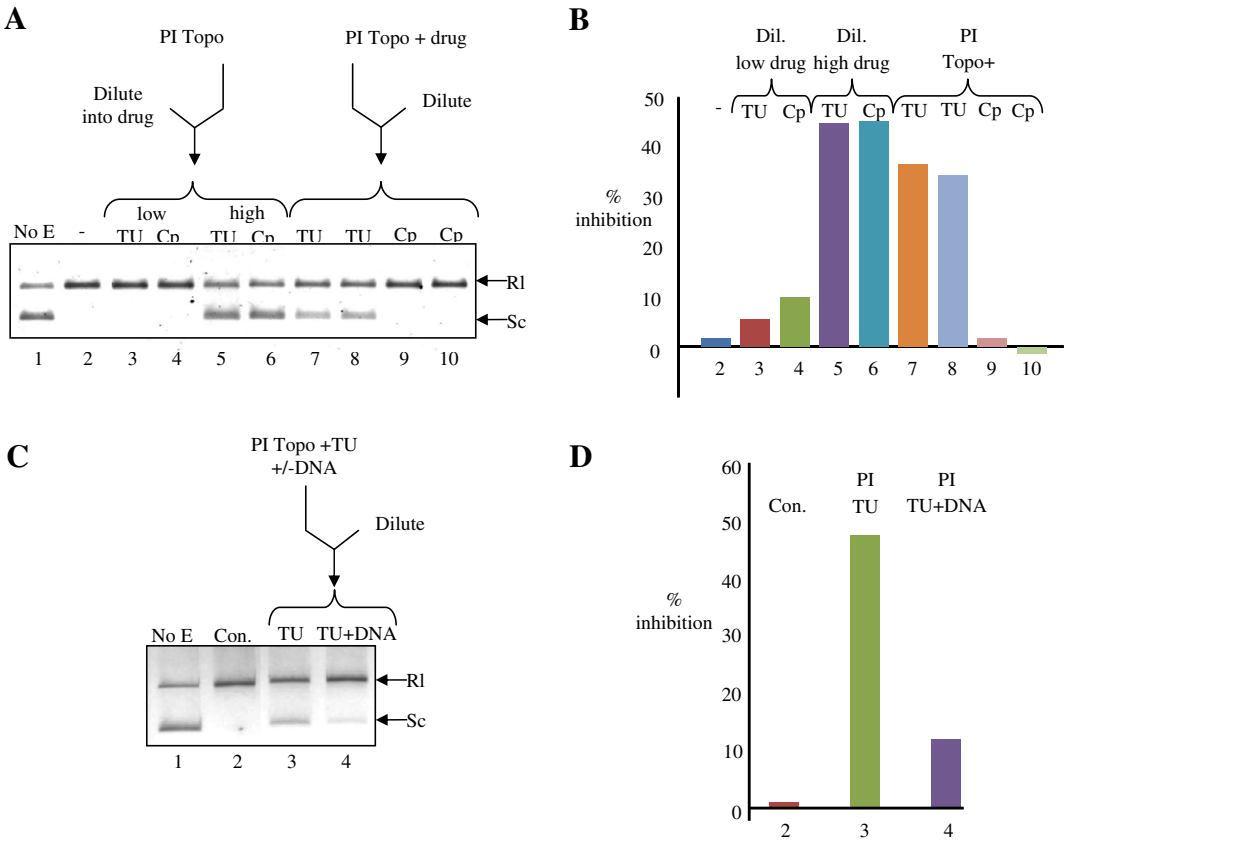

Fig. 2. (A) TU100 inhibits topoisomerase by covalent modification. Enzyme was pre-incubated with TU100 or camptothecin and diluted 4-fold into reaction mix (lanes 7–10). As controls, enzyme was pre-incubated in DMSO and diluted into high drug concentrations (lanes 5 and 6) or low concentrations (lanes 3 and 4). (B) Quantitation of 2A. (C) Presence of DNA in pre-incubation reduces TU100 inhibition. Enzyme was pre-incubated with TU100 in the presence or absence of plasmid DNA and then diluted into reaction mix. (D) Quantitation of 2C.

4). Quantitation of the data is shown in Fig. 2D. These results suggest TU100 preferentially targets free topoisomerase, and that formation of the enzyme:DNA complex either prevents TU100 binding or blocks access to the site of covalent modification.

3.3. Reducing agents reverse TU100 inhibition

A likely target for covalent modification are reactive sulfhydryls of cysteine residues, some of which have been identified as critical for topoisomerase I activity using the thiol modifying agent *N*-Ethylmaleimide [15]. To test this idea we analyzed TU100 effects in the presence of the reducing agent DTT. Increasing DTT prevented topoisomerase inhibition, as did the reducing agents glutathione and L-cysteine (Fig. 3A). A DTT titration against camptothecin had no such effect (data not shown). TU100 has a hydroquinone ring which can undergo oxidation/reduction, so DTT could inactivate TU100. We examined this possibility using TCEP-agarose, which is a strong reducing agent attached to agarose beads. TU100 was pre-incubated with this reagent followed by its removal via centrifugation. The resulting TU100 was still able to inhibit topoisomerase I (Fig. 3B), and this inhibition could be alleviated by DTT (lane 5). These data indicate reducing agents are not inactivating TU100, but rather overcoming its inhibitory effects.

Results to this point were obtained by analyzing topoisomerase I activity in a partially purified extract. To confirm that similar outcomes would be obtained with highly purified enzyme, we utilized topoisomerase I from a commercial source. Fig. 3C shows that pre-incubating this purified enzyme with TU100 caused a dramatic decrease in the IC_{50}, confirming TU100 is a slow acting inhibitor that covalently modifies topoisomerase I. Likewise, including DTT alleviated TU100 inhibition (Fig. 3D). These observations support our hypothesis that TU100 is covalently modifying topoisomerase I by targeting reactive sulfhydryls of cysteine residues.

3.4. TU100 specifically targets the thiol group of cysteine(s)

To directly determine if reducing agents can re-activate inhibited topoisomerase I, the enzyme was pre-incubated with TU100 and then exposed to DTT (Fig. 4A). DTT successfully rescued enzyme activity when it was added directly to the inactivated enzyme (lane 3), or when included in the subsequent reaction mix (lane 4). Thus, while TU100 covalently modifies and inactivates topoisomerase I, this reaction can be alleviated by reducing agents. Given their well known role in redox chemistry, these observations are consistent with TU100 alkylation of cysteine thiols. However, anthracyclines can also participate in redox chemistry by generating reactive oxygen species, so TU100 could indirectly inactivate topoisomerase via such an intermediate that targets cysteines. We think this explanation unlikely, however, because hydrogen peroxide failed to inhibit enzyme activity even at high levels, and it had no effect on TU100 inhibition (Fig. 4B). TU100 also failed to inhibit other enzyme activities (e.g. polymerases, nucleases, and proteases), as might be expected for a compound generating highly reactive and nonspecific reactive oxygen species (data not shown).

We therefore investigated whether TU100 was directly targeting topoisomerase I and alkylating cysteine thiols. Previous work showed the thiol modifying agent NEM inhibits topoisomerase I by such a mechanism [15]. If TU100 were targeting these cysteines, then it might be able to protect them from NEM. Enzyme was first

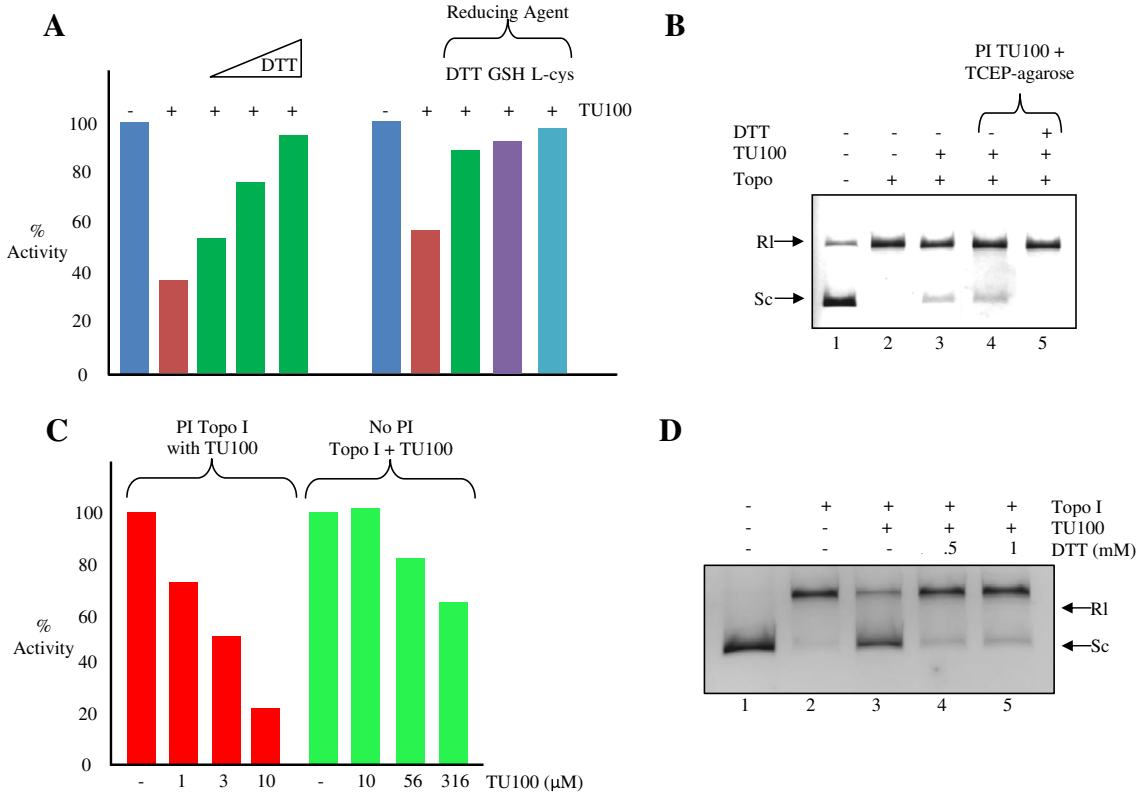

Fig. 3. (A) Reducing reagents prevent TU100 inhibition. A plasmid relaxation assay was carried out in the presence of TU100 plus indicated concentrations of DTT (left side). GSH and L-cysteine (1 mM) had similar effects (right side). (B) Reducing agents do not inactivate TU100. TU100 was pre-incubated with TCEP-agarose beads and then assayed for inhibitory activity against topoisomerase I. (C) TU100 covalently modifies highly purified topoisomerase I. TU100 was analyzed for inhibition with (left side) and without (right side) pre-incubation with highly purified topoisomerase I. Note that much lower TU100 was required with pre-incubation. (D) TU100 inhibition of highly purified topoisomerase I can be prevented by DTT. A plasmid relaxation assay was carried out using highly purified topoisomerase in the presence of TU100 with and without DTT.

pre-incubated with or without TU100 and then exposed to NEM (Fig. 4C). Supercoiled plasmid DNA was added to initiate the reaction in conjunction with 5 mM DTT to alleviate TU100 inhibition and inactivate free NEM. Conditions were established such that there was not sufficient time nor DTT to alleviate any NEM – modified topoisomerase I generated during the pre-incubation. Thus, exposing topoisomerase to 1 mM NEM inhibited activity despite subsequent dilution into reaction mix containing DTT (lane 1). In marked contrast, pre-incubating the enzyme with TU100 significantly protected it from NEM exposure (lanes 2 and 3). The last lane confirmed TU100 interacted with and inhibited topoisomerase in the absence of DTT. Additional controls ruled out the possibility TU100 was directly interacting with NEM (data not shown). These results provide strong evidence that TU100 is covalently modifying the same cysteine thiol(s) as NEM, leading to enzyme inactivation.

NEM, however, is a nonspecific modifying agent that will react with exposed thiols. Thus, it will typically inactivate any enzymatic activity dependent on cysteine residues (e.g. cysteine proteases). To determine if TU100 was specific for topoisomerase we analyzed its ability to inhibit the cysteine protease bromelain. The drug had no effect on bromelain activity even at high concentrations (data not shown), indicating TU100 specifically targets and inactivates topoisomerase I by binding the enzyme and alkylating cysteine thiol(s).

4. Discussion

The naphthoquinone *N*-methyl-5*H*-benzocycloheptanaphthalene-5,12-imine (TU100) is a novel drug candidate with structural similarity to both anthracyclines and tetrahydroisoquinoline antibiotics. Previous work suggested TU100 is a slow acting, dual topoisomerase I and II inhibitor that does not strongly interact with or intercalate into free DNA [24]. We now show that TU100 specifically inactivates free topoisomerase I. TU100 does not appear to be a topoisomerase I poison in the sense of stabilizing DNA cleavage intermediates, and it is not a classical catalytic inhibitor because it covalently modifies the enzyme. Instead, TU100 represents a novel topoisomerase inhibitor with a unique mechanism of action. It covalently modifies cysteine residue(s) via alkylation like NEM, but displays a strong specificity for topoisomerase since the cysteine dependent protease activity of bromelain was not affected. This suggests TU100 first binds specifically to the free enzyme and then is oriented for alkylation of cysteine thiol(s).

A model for how enzyme alkylation might occur is depicted in Fig. 4D. Due to symmetry about the naphthoquinone portion of TU100, a thiol on a critical cysteine could react with TU100 via Michael addition at either bridge position between the heterocyclic ring and the hydroquinone. The time dependence of inactivation could be explained by a slow conformational change required for this chemical step, resulting in formation of the indicated product in the keto or enol configuration. It is unclear which form would be more thermodynamically stable, but the enol form might better explain reversibility by reducing agents. This mechanism is consistent with previous work showing that naphthoquinones can react with sulfides in the manner shown [27]. Regardless, reversibility of this reaction with reducing agents indicates TU100 modification does not irreparably alter topoisomerase I function. Given our previous work demonstrating that TU100 targets both topoisomerase I and II, efforts are ongoing to determine if inhibition of topoisomerase II involves a similar mechanism.

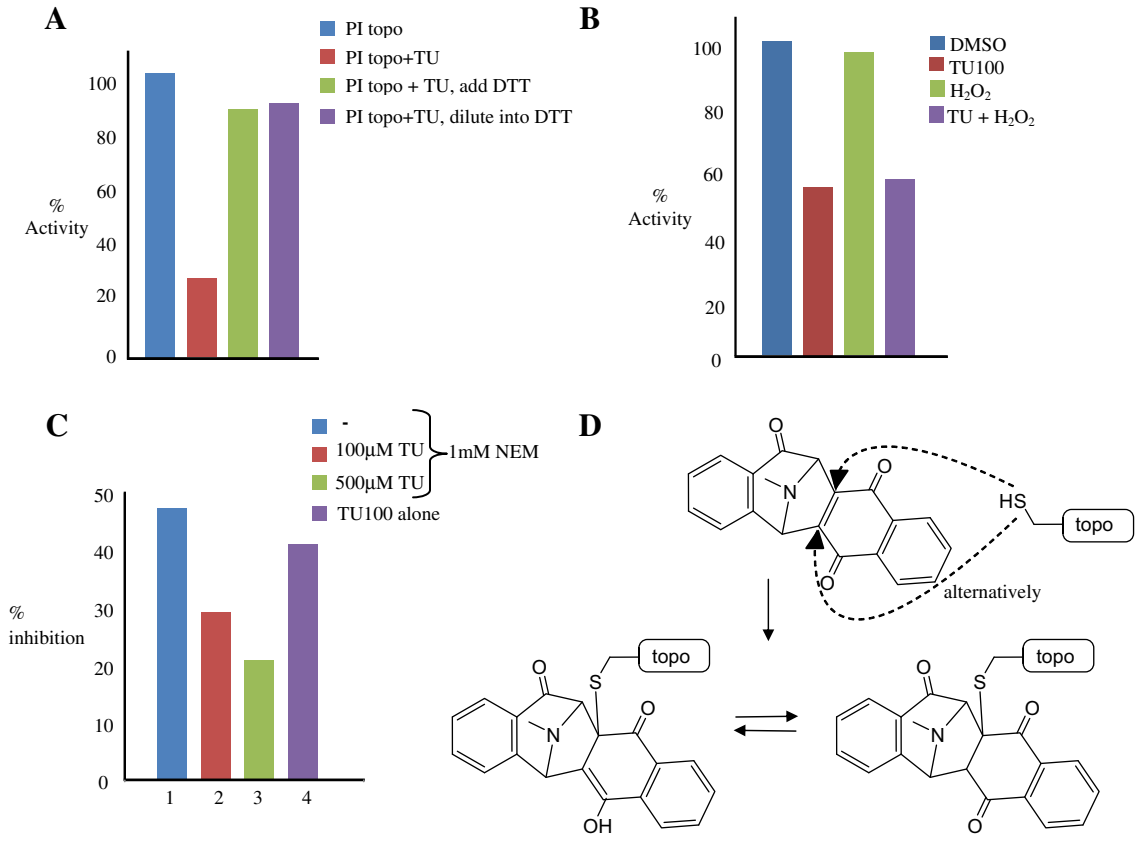

Fig. 4. (A) Reducing reagents reverse TU100 inhibition. Topoisomerase I extract was pre-incubated with TU100 followed by addition of or dilution into reaction mix containing DTT (1 mM). (B) Hydrogen peroxide does not inhibit topoisomerase I. A plasmid relaxation assay was carried out in the presence of TU100, hydrogen peroxide (1 mM), or both. (C) TU100 protects topoisomerase I from NEM inactivation. Enzyme was pre-incubated with indicated TU100 concentrations (10 min room temp) followed by addition of *N*-ethylmalaimide. Enzyme was then diluted into reaction mix containing supercoiled plasmid and 5 mM DTT (lanes 1–3). Lane 4 is a no DTT control showing TU100 inhibits during the pre-incubation. (D) Proposed Model for topoisomerase I inactivation by TU100. Due to symmetry about the naphthoquinone portion of TU100, the cysteine thiol can attack via Michael addition at either bridge position between the heterocyclic ring and the hydroquinone, resulting in cysteine alkylation and enzyme inactivation. The product could exist in the keto or enol form.

How might alkylation by TU100 inhibit topoisomerase I? Previous work showed NEM likely inhibits by modifying vicinal cysteines 504 and/or 505 [15]. Given that TU100 can protect from NEM inactivation, these cysteines are probable targets for the drug. The crystal structure of enzyme bound to DNA shows that these cysteines are located outside the catalytic active site in the base of a loop near the lip 2 region. This region interacts with a corresponding loop in lip 1 to generate the closed clamp holding DNA [15]. Mutational analysis revealed these cysteines are not required for DNA binding or catalysis [15], suggesting that their alkylation with a bulky group (like TU100) might prevent required clamp closure around duplex DNA. This model is consistent with our observation that pre-forming the enzyme–DNA complex partially abrogated TU100 inhibition, such that either drug binding or accessibility to cysteines is decreased. We are currently investigating the specific targeting of these cysteines using topoisomerase mutants.

The readily reversible nature of topoisomerase I inactivation by TU100 is of interest with respect to its consideration as a potential chemotherapeutic agent. Normal cells have robust redox regulatory systems that maintain a reducing environment [28]. Cancer cells tend to be under more oxidative stress and contain compromised redox regulatory pathways, thus existing in a more oxidizing state than normal cells [29]. Such a distinction would seem to favour TU100 inhibition of topoisomerase in the oxidizing environment of cancer cells, potentially increasing its putative therapeutic index. This effect could perhaps be further exploited by pharmacological alteration of the cellular redox environment, temporal control of TU100 reactivity, or its combination with other chemotherapeutic agents. Given these possibilities, further chemical modification of TU100 to enhance its unique functional characteristics and advance its development as a chemotherapeutic agent are currently underway.

5. Conflicts of interest

None declared.

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