Biochemical and Biophysical Research Communications 408 (2011) 94–98

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Topoisomerase I/II inhibition by a novel naphthoquinone containing a modified anthracycline ring system

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ARTICLE INFO

Article history:
Received 25 March 2011
Available online 30 March 2011

Keywords:
Topoisomerase
Chemotherapeutic
Napthaquinone
Anthracycline
Enzyme inhibition

ABSTRACT

In an attempt to create more effective chemotherapeutic compounds, 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) was synthesized. Cell viability studies revealed TU100 is specific for eukaryotes and induces cell death. Based on its structural similarities to the anthracyclines and isoquinolines, the ability of TU100 to inhibit topoisomerase I and II was examined. TU100 was an effective inhibitor of both enzymes, as indicated by its ability to prevent topoisomerase-mediated relaxation of supercoiled plasmid DNA. The mechanism of action does not involve TU100 intercalation into DNA, unlike anthracyclines. Pre-incubation of topoisomerase with TU100 dramatically decreased the IC_{50}, suggesting the drug is a novel slow acting topoisomerase inhibitor that works in the absence of DNA. Taken together these results indicate the novel naphthoquinone adduct TU100 is a dual topoisomerase I/II inhibitor with a unique mechanism of action and chemotherapeutic potential.

1. Introduction

Cancer encompasses a diverse group of diseases resulting from accumulated genetic alterations, and remains one of the major health issues in the United States [1]. Many chemotherapy drugs target rapidly proliferating cells [2], but often exhibit a low therapeutic index resulting in adverse side effects [3]. Even if suppression of cancer cells is initially successful, expansion of a drug-resistant clone often compromises treatment [3]. For these reasons chemotherapeutics with distinct mechanisms of action are often combined in an attempt to maximize efficacy, limit side-effects, and prevent escape of resistant clones [4]. This paper describes the biologic characterization of a novel naphthoquinone adduct incorporating functionalities from the well known anthracyclines and isoquinoline drug classes.

Anthracyclines are some of the most effective and widely employed chemotherapeutic agents ever developed [5,6]. They are classified as anti-tumor antibiotics that damage DNA, and their mechanism of action can involve intercalation and/or generation of free radicals [7–9]. Unfortunately, their use is limited by serious side effects like vomiting and cardiotoxicity [10]. Chemically modified versions of anthracyclines have been synthesized in an attempt to avoid these complications, and exhibit dramatic effects on drug pharmacology [11,12]. Isoquinolines are heterocyclic aromatic compounds in which benzene is fused to a pyridine ring [13]. This benzopyridine forms the framework for natural alkaloids, anesthetics, antifungal agents, and disinfectants [14]. The potential of isoquinolines or their derivatives as chemotherapeutic agents has been less well explored. Given the efficacy displayed by the parent compounds, we hypothesized that combining functional groups from these drug classes would generate novel therapeutic compounds with improved efficacy and/or reduced side effects.

We therefore synthesized and characterized 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) [15]. The drug was cytotoxic to eukaryotic but not prokaryotic cells. *In vitro* assays using purified enzyme revealed TU100 blocks the activity of both topoisomerase I and II. The mechanism of inhibition did not involve DNA intercalation despite similarity to anthracyclines. Instead, TU100 appears to function as a novel slow acting topoisomerase poison even in the absence of DNA. Taken together these results indicate the naphthoquinone adduct TU100 is a dual topoisomerase I/II inhibitor with chemotherapeutic potential.

2. Materials and methods

2.1. Drugs

TU100 was synthesized as described previously [15] from the 3+2 dipolar cycloaddition of *N*-methyl-4-hydroxyisoquinolinium

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0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.03.126

iodide and naphthoquinone, dissolved in 100% sterile DMSO, and stored at −20 °C. For subsequent use in all assays dilutions were prepared in 100% sterile DMSO. Daunorubicin, camptothecin, luteolin, and etoposide were all obtained from Sigma–Aldrich, St. Louis, MO and dissolved in 100% sterile DMSO. Ethidium bromide was obtained from Fisher Scientific and dissolved in sterile water.

2.2. Cell culture

Cells 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) unless specified otherwise. H293 are kidney epithelial cells, NIH3T3s are mouse embryo fibroblast, and HeLa are cervical cancer cells. Cultures were maintained in a 37 °C water jacketed incubator with 5% CO_2.

2.3. Trypan blue dye exclusion assay

Proliferating cells were plated in 60 mm plates and exposed to various concentrations of TU100 for the indicated times. Media containing any detached cells was then removed and combined with cells from the plate that were removed using trypsin. An aliquot was withdrawn (10 μl) and mixed with an equal volume trypan blue (Fisher Scientific), followed by counting of live and dead (blue) cells using a hemocytometer. At least 100 cells were counted for each point in order to give a good representation of the fraction of cell death.

2.4. Escherichia coli and yeast growth curves

E. coli (XL-1 Blue containing an Ampicillin (Amp) resistant plasmid) was grown overnight in Circle Grow broth (a richer version of LB) plus 50 μg/ml Amp. The overnight culture (10 μl) was diluted into 21 ml of fresh Circle Grow plus Amp (50 μg/ml). Aliquots (3 ml) were withdrawn, treated with either DMSO or TU100, then placed at 37 °C with shaking to initiate cell growth. Aliquots (0.5 ml) were sampled at the indicated times and the OD^{595} determined using the Varian 50Bio UV/Vis spectrophotometer. For the yeast growth curves *Saccharomyces cerevisiae* was grown overnight in YPD broth. The next morning a fraction was diluted into 15 ml fresh YPD and divided into 3 ml aliquots containing DMSO or TU100. Samples were incubated at 26 °C with shaking for the indicated times, at which point 1 ml aliquots were withdrawn and the OD^{595} determined using the Varion 50Bio UV/Vis spectrophotometer.

2.5. Cell Titer Blue viability assay

Approximately 1500 cells were then plated in a 96 well plate containing 100 μl DMEM plus 10% FBS and treated with the indicated concentration of drug or DMSO control. Final DMSO concentration was typically 1% and had minimal effects on cell viability. Cell viability was determined by the addition of 10 μl Cell Titer Blue resazurin reagent (Promega) and fluorescence measurement after 3 h at $560^{ex}/590^{em}$ using a Tecan Safire plate reader.

2.6. Fluorescence microscopy

For visualizing nuclei proliferating HeLa cells were plated in 60 mm plates containing sterile coverslips in 10% FBS at 37 °C. Individual coverslips were treated with DMSO control or the indicated concentrations of drug overnight at 37 °C. Media was aspirated and the cells washed with PBS, then treated with DAPI (0.5 μg/ml) for 5 min to stain DNA. Cells were again washed with PBS and photographed at 20× and 40× using fluorescence microscopy.

2.7. Intercalation assays

Plasmid DNA (1 μg) was incubated with the indicated drugs at room temperature for 15 min, then resolved on 0.8–1% agarose gels (TBE) in the absence of ethidium bromide. After electrophoresis gels were stained in 1× TBE with ethidium bromide (~2 μg/ml) for 30 min and visualized using an Alpha-Tech imager.

2.8. Topoisomerase assays

Supercoiled plasmid DNA (1 μg) was incubated in a 20 μl reaction with the indicated topoisomerase obtained from a commercial supplier (Topogen). For assaying topoisomerase I activity the reaction buffer (final concentration) contained 10 mM Tris pH 7.9, 150 mM NaCl, 0.1% BSA, 100 μM spermidine, and 5% glycerol. The topoisomerase II reaction buffer was composed of 50 mM Tris pH 8, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, and 0.5 mM DTT. Reactions were carried out at 37 °C then halted by the addition of 5 μl of stop buffer (5% sarkosyl, 0.0025% bromophenol blue, 25% glycerol). Reactions were initiated with the addition of enzyme. Pre-incubation of enzyme and TU100 (10 min) was carried out at room temperature and the reaction initiated by the addition of plasmid and transfer to 37 °C. Samples were separated on a 0.8–1% agarose gel in the absence of ethidium bromide, followed by staining in 1X TBE with ethidium bromide (~2 μg/ml) for 30 min and visualization using an Alpha-Tech imager. Extent of topoisomerase inhibition was quantified by determining the intensity of supercoiled or relaxed forms of plasmid DNA using the Alpha-Innotech imaging software.

3. Results

3.1. TU100 synthesis and structure

Enhancing the therapeutic index of chemotherapeutic agents by chemical modification is a productive avenue of new drug development [16]. We therefore incorporated functional elements from the anthracyclines with those from isoquinoline based drugs to synthesize the naphthoquinone adduct *N*-methyl-5*H*-benzocycloheptanaphthalene-5,12-imine (TU100) (Fig. 1A) [15]. LC–MS demonstrated purity and confirmed the compound exhibited the expected molecular weight (data not shown).

3.2. TU100 specifically targets eukaryotic cells

Given that anthracyclines were originally discovered as antibiotics and target DNA metabolism in diverse organisms, we examined TU100 effects on yeast and bacterial growth curves [5,6]. *E. coli* (XL1-Blue strain) and yeast (*S. cerevisiae*) cultures were grown overnight and diluted in the appropriate liquid media plus TU100 or DMSO control. Aliquots were withdrawn at indicated times and absorbance determined at 595 nm. TU100 had no effect on bacteria growth (Fig. 1B) but completely inhibited yeast proliferation (Fig. 1C), suggesting it is specific for eukaryotic cells. A trypan blue dye exclusion assay was utilized to determine TU100 effects on mammalian tissue culture cells. A fixed number of proliferating cells were plated in 60 mm tissue culture dishes and allowed to attach overnight. Cells were treated with increasing concentrations of TU100 or DMSO control for up to 20 h. Trypan blue staining revealed the accumulation of dead cells, consistent with the observed effects on cell adherence. Cell death was time dependent and occurred rapidly, with stained cells evident after only 2 h exposure to the drug (Fig. 1D). In marked contrast the chemotherapeutic drugs daunorubicin and camptothecin had little

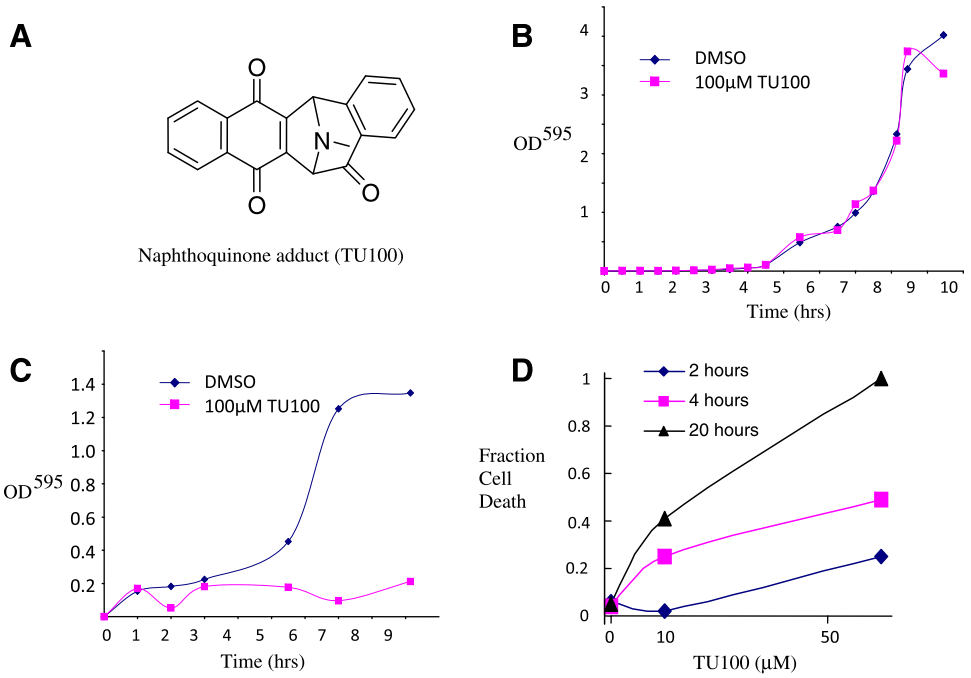

Fig. 1. (A) Structure of 12,13-dihydro-N-methyl-6,11,13-trioxo-5H-benzo[4,5]cyclohepta[1,2-b]naphthalen-5,12-imine (TU100); (B) TU100 does not inhibit *E. coli* growth. An overnight culture of *E. coli* was diluted and two aliquots incubated at 37 °C in the presence of DMSO control or 100 μM TU100. The optical density at 595 nM (OD^{595}) was determined at the indicated time points and plotted as OD^{595} vs. time; (C) TU100 inhibits yeast growth. An overnight yeast culture was diluted and two aliquots incubated at 26 °C in the presence of DMSO control or 100 μM TU100. The optical density at 595 nm (OD^{595}) was determined at the indicated time points and plotted as OD^{595} vs. time; (D) time course of TU100-induced mammalian cell death. Proliferating H293 kidney epithelial cells were treated with the indicated concentrations of TU100 for 2, 4, or 20 h followed by determination of cell death by trypan blue dye exclusion. The graph shows fraction of dead cells vs. TU100 concentration at the indicated times.

observable effect on cell viability at these early time points (data not shown).

3.3. TU100 cytotoxicity is comparable to known chemotherapeutic agents

Effects of TU100 on cell morphology were examined by DAPI staining of cell nuclei followed by fluorescence microscopy [17]. Fig. 2A shows that increasing TU100 results in distinct alterations in both nuclei shape and DNA staining, similar to those seen with the apoptosis-inducing drug camptothecin [18]. In order to better quantify cell death we utilized the Cell Titer-Blue Cell Viability Assay®, which is based upon the conversion of resazurin to the fluorescent resorufin by living cells [19]. Proliferating cells were plated in 96 well plates and treated the next day with TU100, daunorubicin, camptothecin, or luteolin for 24 h. Resazurin was then added for 3 h and cell viability analyzed at $560^{ex}/590^{em}$ using a microplate reader. This comparison indicated TU100 exhibits potency similar to these known cytotoxic agents (Fig. 2B).

3.4. TU100 inhibits topoisomerase I and II

Anthracyclines inhibit DNA metabolism in part by directly targeting topoisomerase II [9,20]. Conversely, quinoline alkaloids like camptothecin preferentially inhibit topoisomerase I [18]. Since TU100 has structural characteristics of both these classes of drug we examined its ability to inhibit highly purified topoisomerase I and II obtained from a commercial source. An enzymatic assay was established in which topoisomerase activity was measured by monitoring the mobility of plasmid DNA by agarose gel electrophoresis. Negatively supercoiled is more compact and will migrate faster in the gel than DNA that has been relaxed by topoisomerase. Fig. 3A shows that the plasmid DNA substrate alone mainly exists in the faster migrating supercoiled form (lane 2). Incubation with purified topoisomerase I at37 °C for 30 min removed negative supercoils and converted the plasmid to the relaxed form, as indicated by its slower migration in the agarose gel (lane 3). The reaction was then repeated in the presence of increasing concentrations of TU100, and revealed that the drug inhibits the ability of topoisomerase I to catalyze the relaxation of supercoiled DNA (lanes 4–7). A similar experiment was performed using topoisomerase II, which has a quite different mechanism of action (Fig. 3B). Nevertheless, TU100 effectively inhibited topoisomerase II (lanes 5–8). In fact, TU100 was more potent than the well known topoisomerase II inhibitor etoposide (lanes 9–12) [21]. The ability of TU100 to inhibit both topoisomerase I and II is consistent with its structural similarities to anthracyclines and quinoline alkaloids, so we investigated whether the mechanism of inhibition was also similar.

3.5. TU100 does not intercalate into DNA

Given the well-known ability of anthracyclines to directly bind DNA, it was possible that TU100 inhibits topoisomerases indirectly by intercalation [9]. We therefore analyzed TU100 effects on the mobility of supercoiled plasmid DNA. Intercalating agents bind and relax negatively supercoiled plasmid DNA, causing it to run slower in an agarose gel [5,6]. In Fig. 4A, the negatively supercoiled plasmid alone runs as a single band on the agarose gel (lane 1). Increasing concentrations of the well known anthracycline daunorubicin (lanes 3–6) caused an upward shift in mobility, consistent with its well known role as a DNA intercalator. In contrast, the topoisomerase inhibitor camptothecin binds DNA poorly and hence did not influence plasmid mobility (lanes 8–11). Fig. 4B shows a similar experiment using the ethidium bromide, which as expected caused plasmid relaxation (lanes 3–6). In marked contrast to ethidium bromide and despite its similarity to the anthracycline daunorubicin, TU100 did not shift plasmid mobility (lanes

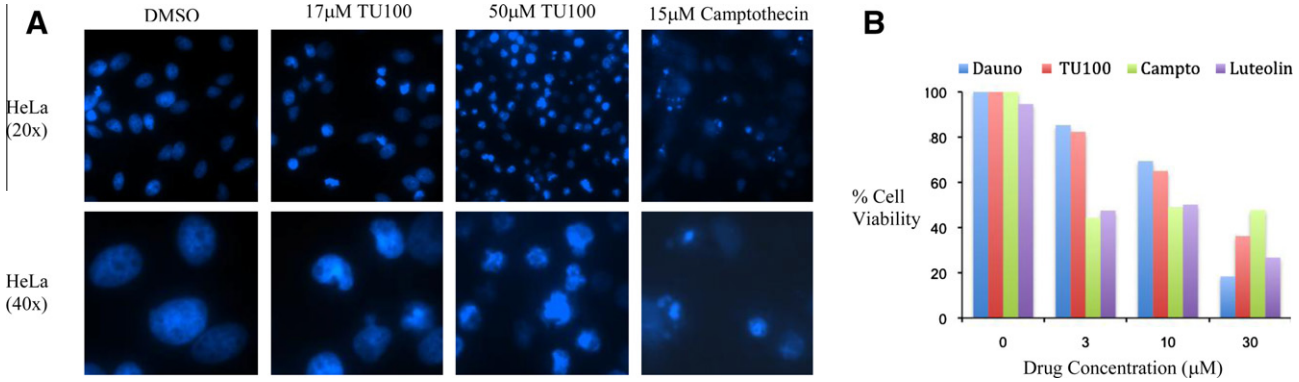

Fig. 2. (A) Fluorescence microscopy of TU100-treated cells. Proliferating HeLa cells were plated on coverslips and treated with DMSO control or the indicated concentrations of drug overnight. Cells were exposed to DAPI (0.5 μg/ml) for 5 min to stain nuclei, then washed and photographed at 20× (top panels) and 40× (bottom panels) using fluorescence microscopy. Cells treated with drug exhibit the punctate DNA staining and deformed nuclei indicative of apoptotic cells; (B) TU100 potency is comparable to other well known cytotoxic agents. Proliferating NIH3T3 fibroblasts cells were treated with the indicated concentrations of daunorubicin, TU100, camptothecin, and luteolin for 15 h. Cell viability was then determined using the Cell Titer Blue assay. The graph plots % cell viability vs. drug concentration, and shows that TU100 decreases cell viability at concentrations similar to other drugs.

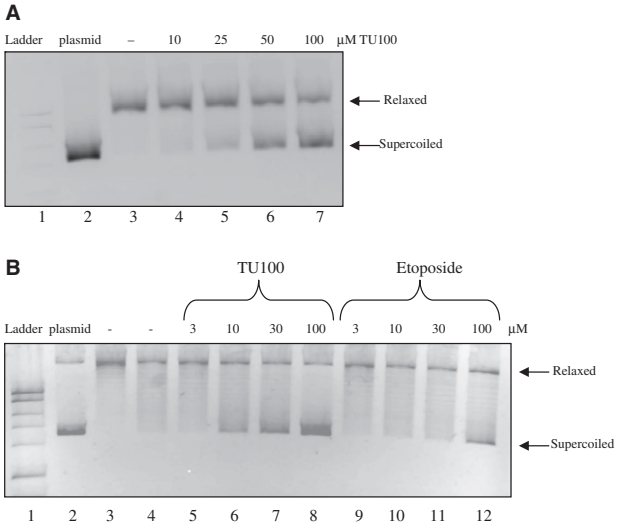

Fig. 3. (A) TU100 inhibits purified topoisomerase I. A plasmid relaxation assay was carried out using purified topoisomerase I obtained from a commercial supplier in the presence or absence of the indicated TU100 concentrations. Products were then analyzed by agarose gel electrophoresis. Lane 2 is the no enzyme control; (B) TU100 is a potent topoisomerase II inhibitor. A plasmid relaxation assay was carried out using purified topoisomerase II obtained from a commercial supplier in the presence or absence of TU100 or the well known topoisomerase II inhibitor etoposide. Products were then analyzed by agarose gel electrophoresis. Lane 2 is the no enzyme control.

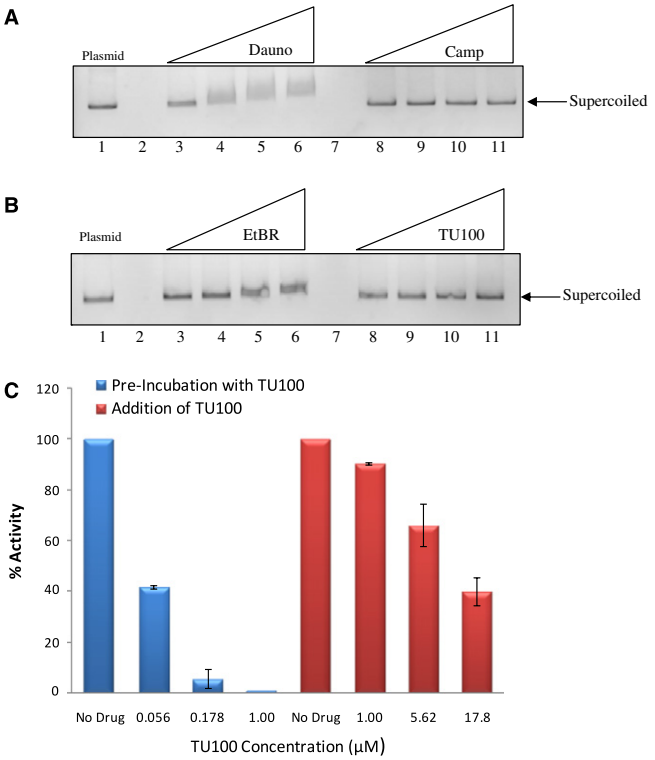

Fig. 4. (A) DNA intercalation by the anthracycline daunorubicin. Negatively supercoiled plasmid DNA was incubated with increasing concentrations of daunorubicin or camptothecin and analyzed by agarose gel electrophoresis. Control lane 1 shows plasmid alone. (B) TU100 fails to alter mobility of plasmid DNA. Negatively supercoiled plasmid DNA was incubated with increasing concentrations of ethidium bromide or TU100 and analyzed by agarose gel electrophoresis. Control lane 1 shows plasmid alone. (C) TU100 is a slow acting inhibitor. Topoisomerase II activity was measured at the indicated TU100 concentrations with and without pre-incubating drug with enzyme. On the left (blue) topoisomerase II and TU100 were pre-incubated for 10 min and the reaction initiated by addition of DNA substrate. On the right (red) enzyme pre-incubated with DMSO carrier was added to DNA substrate and TU100. Reactions were allowed to proceed for 30 min at 37 °C and products analyzed by agarose gel electrophoresis. Graph shows percent topoisomerase activity remaining relative to the no drug control. Note that the TU100 concentrations utilized in the pre-incubation reactions (blue) are significantly lower due to their enhanced potency. (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.)

8–11). These data indicate TU100 is not a strong intercalating agent, and hence suggest its ability to inhibit topoisomerase is not mediated through direct interaction with DNA. We therefore turned our attention to the possibility that TU100 directly interacts with and inhibits topoisomerase.

3.6. TU100 is a novel slow acting topoisomerase inhibitor that works in the absence of DNA

Topoisomerase inhibitors are generally classified according to their specificity and whether they act via noncovalent binding or as a poison that covalently attaches and irreversibly inactivates the enzyme. A kinetic analysis of topoisomerase activity in the presence of TU100 indicated the amount of inhibition was

increasing over time (data not shown). We therefore investigated the possibility TU100 was a slow acting inhibitor by determining the effect of pre-incubating the drug with enzyme (Fig. 4C). Purified topoisomerase II was allowed to react with various concentrations of TU100 or DMSO vehicle for 10 min, followed by addition of DNA substrate and transfer to 37 °C to initiate the reaction. As the control enzyme pre-incubated with DMSO was diluted into the indicated concentrations of TU100. Reactions were allowed to proceed for 30 min followed by analysis of plasmid mobility on an agarose gel. Pre-incubating topoisomerase II and TU100 dramatically enhanced drug potency, decreasing the IC_{50} from approximately 11.5–.01 μM. Inhibition of topoisomerase I also significantly increased when the enzyme was pre-incubated with TU100 (not shown). Taken together these observations show that TU100 is a slow acting inhibitor that can target both topoisomerase I and II in the absence of DNA.

4. Discussion

Synthesis of the naphthoquinone *N*-methyl-5*H*-benzocycloheptanaphthalene-5,12-imine (TU100) yielded a novel drug candidate with structural similarity to both anthracyclines and tetrahydroisoquinoline antibiotics. TU100 inhibited yeast and mammalian cell growth but had no effect on *E. coli,* indicating it does not have antibiotic properties like the anthracyclines. Thus, it appears TU100 specifically targets eukaryotic cells rather than simply damaging general biomolecules like DNA or protein, and its potency was comparable to that of other well known chemotherapeutic agents. TU100 has a ring skeleton similar to anthracyclines, which are known to cause DNA damage directly or disrupt its metabolism [5,8]. Despite this structural similarity TU100 does not intercalate into DNA. In this regard it will be interesting to determine if TU100 displays the cardiotoxicity associated with some anthracycline derivatives.

Anthracyclines are also known to inhibit DNA metabolism by targeting topoisomerase II, while quinoline alkaloids like camptothecin preferentially inhibit topoisomerase I [9,18,20]. TU100 has structural characteristics of both drug classes and was found to function as a dual topoisomerase I and II inhibitor. The failure of TU100 to block *E. coli* growth is interesting in this regard because it utilizes gyrase, a topoisomerase II type enzyme that is inhibited by antibiotics such as the aminocoumarins (e.g. novobiocin) and the quinolines (e.g. nalidixic acid and ciprofloxacin) [22,23]. The inability of TU100 to affect *E.* coli growth suggests that it is not inhibiting gyrase, and so may be targeting structural features only present in eukaryotic topoisomerase I and II rather than the evolutionarily conserved active sites.

Consistent with this interpretation, the mechanism of action appears to be unique and involve targeting the enzyme in the absence of DNA. Pre-incubation of topoisomerase and TU100 significantly enhanced drug potency, which suggests TU100 is a slow acting inhibitor that preferentially targets free enzyme. In contrast, most topoisomerase poisons characterized to date bind and stabilize various forms of the enzyme:DNA complex. This temporal feature of TU100 inhibition could be due to slow binding of the drug, slow reactivity, or perhaps sequestration of enzyme in an inactive state from an unfavorable equilibrium. Given the dramatic increase in drug potency with pre-incubation, it is tempting to speculate that TU100 is a slow acting poison that causes irreversible enzyme inactivation. Taken together our results indicate TU100 joins a small class of compounds that function as dual topoisomerase inhibitors, displays a unique mechanism of action, and thus has potential for further development as a chemotherapeutic agent.

5. Conflicts of interest

None declared.

Acknowledgments

This study was supported in part by University of Tulsa Faculty Development Awards to J.C.D. and R.J.S. and a Merck-AAAS Undergraduate Research Award to R.J.S. J.C.D .carried out the synthesis of TU100 used in this study while a professor of chemistry at The University of Tulsa. The authors would like to thank, Dr. Paul Baures for use of his LC–MS and comments on the manuscript, Dr. William Potter for helpful suggestions regarding experimental design, and Dr. Razi Hussaini for help generating figure of TU100 chemical structure.

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