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

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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.

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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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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

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