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13. ABSTRACT (Maximum 200 Words)

Human topoisomerase IIa (hstopo IIa) is an essential enzyme that is the target of a number of anticancer drugs in clinical use, making the understanding of its catalytic mechanism very important. Clinically, resistance to anticancer drugs develops through various mechanisms, one of which can be found in a class of a typical multidrug resistant mutants (at-MDR) that has been identified. We will attempt to further clarify the biochemical basis of at-MDR among hstopo IIa mutants to shed more light on the topo II enzymatic mechanism. Additionally, we will identify sites of drug binding on hstopo IIa and the consequences of this action. To this end, we have probed the mechanism of hstopo IIa using cysteine footprinting and endoproteinase/mass spectrometry footprinting. With the cysteine footprinting technique, we have shown that menadione may induce conformational changes in hstopo IIa that cause it to remain in a configuration that is different from the wild-type populated state. Additionally, using an endoporteinase/mass spectrometry footprinting approach, we have located a potential cysteine residue on hstopo IIa that may be modified by menadione. These preliminary results provide the first direct evidence that menadione may act as an anticancer drug by binding to a specific position(s) on hstopo IIa causing it to adopt a conformation contrary to the wild-type form.

14. SUBJECT TERMS
Protein footprinting, topoisomerase II, atypical multiple drug resistance (at-MDR), enzyme mechanism, etoposide, adriamycin

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Table of Contents

Introduction

We are interested in the mechanism of *hs*topoIIα because it is the target of several drugs currently being used in cancer therapy such as etoposide and doxorubicin (Harris and Hochhauser 1992; Isaacs, Davies et al. 1995; Burden and Osheroff 1998). A number of these drugs work by trapping a DNA cleavage intermediate in which topo II is covalently attached to DNA. As a result, these drugs render the enzyme nucleolytic, inducing cytotoxicity. However, cancer cells become resistant over time (Harris and Hochhauser 1992). A unique class of at-MDR mutants has been discovered, some of which are characterized by a single amino acid change in topo II. These amino acid substitutions have been mapped to the Gyr B' and Gyr A' domains (Mao, Yu et al. 1999; Wang, Mao et al. 2001) While some of these mutants have undergone initial characterization, very few biochemical experiments have been carried out on at-MDR mutants of *hs*topo IIα. We will attempt to further clarify the biochemical basis of at-MDR among *hs*topo IIα mutants to shed more light on the topo II enzymatic mechanism. To accomplish this goal, we have employed protein footprinting at cysteine residues. Structural changes of *hs*topo IIα will likely lead to an alteration in the solvent-accessibility of the protein surface as domains open and close, resulting in varied sensitivity of amino acid residues towards footprinting reagents. This will allow us to probe conformational changes of wild-type and at-MDR mutants of *hs*topo IIα in the presence and absence of anticancer drugs, DNA, and cofactors in order to further understand the *hs*topo IIα mechanism and the biochemical basis of resistance among at-MDR mutants.

By utilizing cysteine footprinting, we will also attempt to further demonstrate how newly identified topo II poisons act to target the enzyme. Studies have shown that topo II can be poisoned by various mechanisms. (Liu, Rowe et al. 1983; Zechiedrich, Christiansen et al. 1989; Frydman, Marton et al. 1997; Kwok and Hurley 1998). A mechanism for topo II poisoning was recently discovered in which thiol alkylation of topo II stimulates topo II-dependent DNA cleavage (Frydman, Marton et al. 1997; Wang, Mao et al. 2001). A cysteineless mutant yeast topo II was found to be completely resistant to thiol-alkylating drugs, which implicates cysteine modification in the mediation of topo II-directed DNA cleavage (Wang, Mao et al. 2001). We have attempted to map the specific cysteine residue(s) involved in this mechanism by generating cysteine footprints of *hs*topo IIα. Moreover, we have used an endoproteinase/mass spectrometry footprinting approach in an attempt to identify the site(s) of drug binding on *hs*topo IIα.

Body

To address the questions raised at hand, we have made progress towards accomplishing our research goals during the past year. To this end, recombinant *hs*topo IIα was created by fusing an HMK site and H_6 tag to the N-terminus of *hs*topo IIα (Fig. 1). The HMK motif was added to accomplish end-labeling of the protein; the HMK site contains the Protein Kinase A (PKA) consensus sequence, RRASV (Kennelly and Krebs 1991). The protein was truncated at amino acid 1405 because of the presence of an intrinsic PKA consensus sequence, RKPST, that would interfere with our results.

HMK-H_6-*hs*topo IIα was purified by affinity and ion exchange chromatography; the purified protein displayed activity comparable to the wild type protein. The amount of PKA required to radiolabel the HMK-tagged protein was empirically determined and footprinting experiments were then conducted.

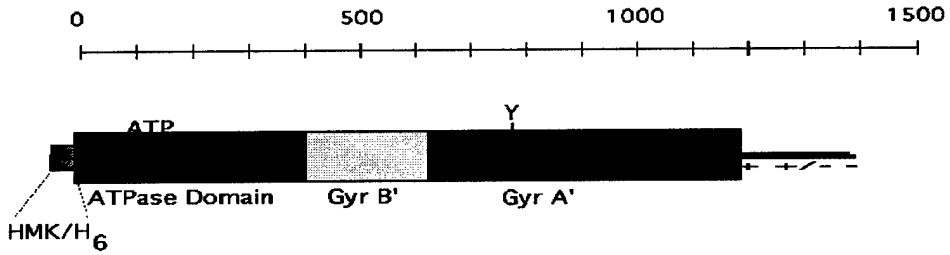

Figure 1: *Top*: represents the amino acid scale of *hs*topo IIα. *Bottom*: depicts the truncated construct created for cysteine footprinting experiments with the relative sizes and positions of each of the domains of *hs*topo IIα indicated. Y indicates the active site tyrosine residue. HMK and H_6 tags have been added to the N-terminus of *hs*topo IIα to aid in footprinting and purification. The hydrophilicC-terminus is represented as a bold line.

Cysteine footprinting entails generating a ladder of peptide fragments that can be visualized upon radiolabeling with PKA and γ-^{32}P-ATP. The presence and intensity of each band corresponds to the solvent accessibility of a particular cysteine residue, which may change in response to conformational alterations of *hs*topo IIα. To generate cleavage fragments, HMK-H_6-*hs*topo IIα was cyanylated at its cysteine residues with 5:1 and 0.5:1 ratios of NTCB: cysteine residues. Upon removal of NTCB, the protein was resuspended in an alkaline denaturing solution which facilitated cleavage of HMK-H_6-*hs*topo IIα into polypeptide fragments at S-cyanocysteine residues. An aliquot of the protein was radiolabeled at the N-terminal HMK site. The polypeptide fragments were then separated by SDS-PAGE and visualized by phosphorimaging.

The footprinting method described has been used to generate molecular weight markers in which the HMK-H_6-*hs*topo IIα protein was first denatured before NTCB was added. We have shown that the footprinting method works as expected because we have generated a ladder of polypeptides where each band can be attributed to partial cleavage at a specific cyanylated cysteine residue when *hs*topo IIα is first denatured (Fig. 2, left panel). Once it was established that the footprinting method was viable, we tested the effects of menadione on the footprinting pattern of native HMK-H_6-*hs*topo IIα. Menadione (vitamin K3) is a redox cycler capable of producing reactive oxygen species that can stimulate topo II-mediated DNA cleavage. However, menadione can also directly react with nucleophiles; Liu and coworkers have provided evidence that menadione may induce topo II-mediated DNA cleavage through a mechanism involving protein thiolation (Wang, Mao et al. 2001). Our results indicate a clear difference in the footprinting pattern in the presence of 500μM menadione. We see a marked increase in the intensity of the bands attributed to cleavage at C1008, C427, C405, C392, and C216 presumably as a result of increased solvent accessibility to NTCB (Fig. 2, right panel). Our preliminary data indicate that conformational changes are occurring within the GyrB' and GyrA' domains as a result of the action of a topoisomerase II poison.

Further studies are being conducted to determine the critical cysteine residue(s) involved in topo II poisoning by menadione and other compounds.

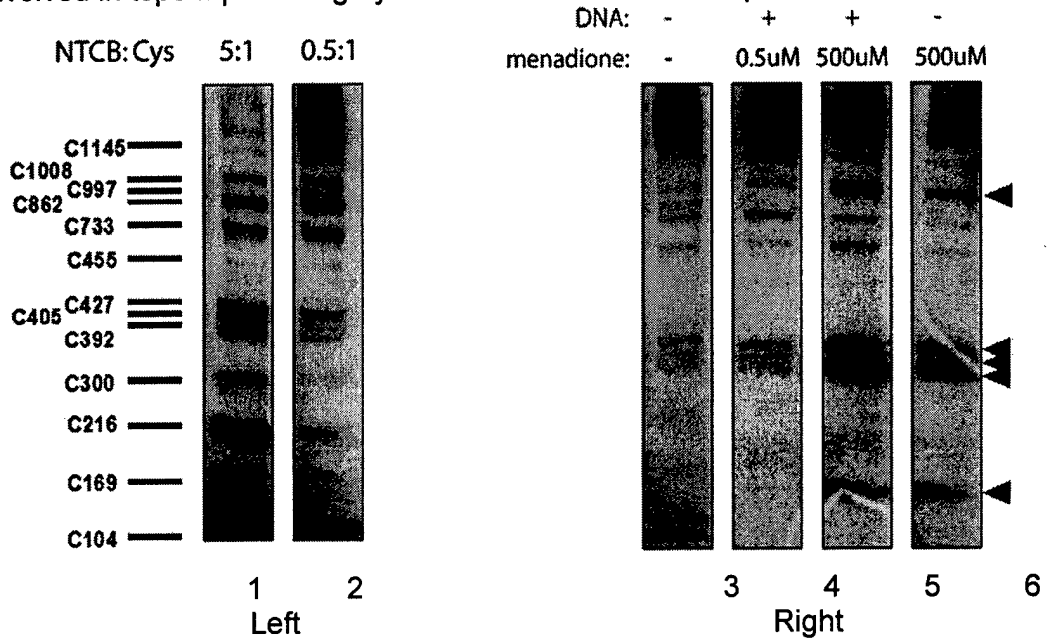

Figure 2: Protein footprinting at cysteine residues was carried out using 12 μg of protein in each reaction. *Left*: HMK-H_6-*hs*topo IIα was first denatured and treated with a 5:1 ratio of NTCB: cysteine residues (lane 1) or a 0.5:1 ratio (lane 2) before cleavage at the modified residues. The footprinting pattern can be attributed to cleavage at each cysteine residue of *hs*topo IIα. *Right:* All native HMK-H_6-*hs*topo IIα proteins were treated with a 0.5:1 ratio of NTCB: cysteine residues. In lane 3, no additional species were added. In lanes 4-6, HMK-H_6-*hs*topo IIα was incubated with the indicated amount of menadione and 6 μg DNA, or DNA was omitted from the reaction (lane 6). Treatment with 500μM menadione results in an increase in the intensity of the bands marked: C1008, C427, C405, C392, C216. The presence of DNA does not seem to have a significant effect on the cleavage pattern induced by menadione.

Difficulty was encountered when attempting to carry out the cysteine footprinting. Attempts at cysteine footprinting were conducted for months with no success. However, it was discovered that protein loss occurred at several steps during the footprinting procedure. Upon these discoveries, the cysteine footprinting procedure was modified until reproducible results were obtained.

Due to the difficulty encountered with the cysteine footprinting procedure, attempts were made to find an alternative method to the footprinting procedure that could answer the same questions we began with. To this end, an endoproteinase footprinting procedure utilizing mass spectrometry was developed. In this procedure, HMK-H_6-*hs*topo IIα was incubated in the presence or absence of DNA and anticancer drugs. The reaction was terminated by dialysis into ammonium bicarbonate and HMK-H_6-*hs*topo IIα was proteolyzed overnight with a series of residue-specific endoproteinases. Proteolysis results in a collection of fragments that can be attributed to cleavage of HMK-H_6-*hs*topo IIα at specific sites that can be mapped using matrix-assisted laser desorption ionization

(MALDI) mass spectrometry. In this procedure, it is feasible to discover sites of drug modification by identifying fragments in the drug-treated sample that are not present in the untreated sample. If the unique fragment corresponds to a molecular weight increase that coincides with the molecular weight of the anticancer drug used, then the site of drug modification can be identified. Our preliminary data indicate that menadione may be modifying cysteine residue 427 based on footprinting with the endoproteinase Lys-C and MALDI mass spectrometry. Figure 3 depicts the presence of a Lys-C generated fragment in the menadione-treated sample with a mass/charge ratio of 679 which corresponds to the molecular weight. This may represent the fragment CSAVK (MW = 507) with menadione (MW = 172) covalently bound to the cysteine residue to give the fragment with MW = 679 (507+ 172 = 679).

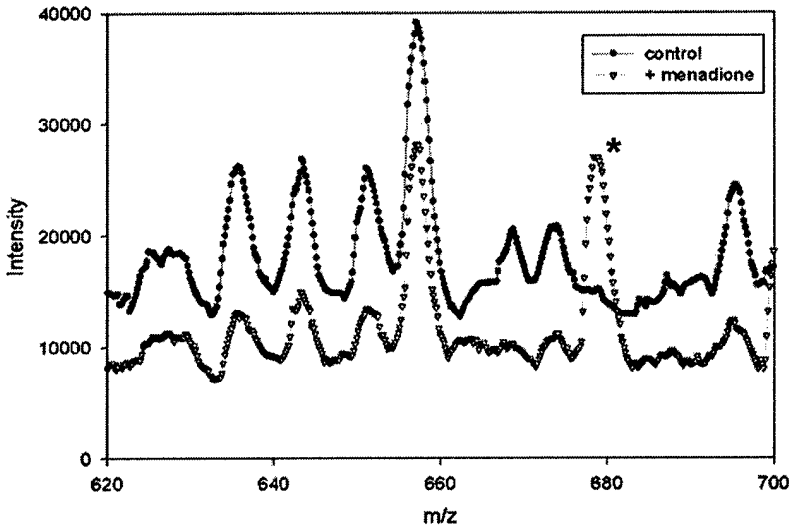

Figure 3: 50μg of HMK-H_6-hstopoIIα was treated in the presence (open triangles) and absence (closed circles) of 500μM menadione. The samples were dialyzed into 50mM ammonium bicarbonate and digested overnight with 1μg of the endoproteinase Lys-C. The samples were subjected to MALDI mass spectrometry to obtain the spectrum shown. The menadione-treated sample contains a fragment with an m/z ratio of 679 (indicated with an asterisk) that may be due to covalent modification of Cys 427 in the CSAVK fragment (507g/mol) by menadione (172g/mol).

Key Research Accomplishments

- Overexpression of HMK-H_6-hstopoIIα
- Purification of active HMK-H_6-hstopoIIα
- Fine-tuning and troubleshooting the development of an NTCB-based cysteine footprinting technique for use with HMK-H_6-hstopoIIα
- Identification of conformational changes occurring in HMK-H_6-hstopoIIα using cysteine footprinting
- Development of an alternative technique to footprinting by using mass spectrometry and endoproteinases
- Putative identification of drug modification sites using mass spectrometry

Reportable Outcomes

- Successful completion of Ph.D. candidacy exam based on work supported by this grant

Conclusions

Human topoisomerase IIα is an essential enzyme that is the target of a number of anticancer drugs in clinical use, making the understanding of its catalytic mechanism very important. Thus, we are examining the mechanism of *h*stopo IIα using cysteine footprinting and the newly developed endoproteinase mass spectrometry footprinting technique. Using cysteine footprinting, we have shown that menadione may induce conformational changes in *h*stopo IIα different from the wild-type populated state. Moreover, we have found a potential site of drug modification on *h*stopo IIα using the endoproteinase/mass spectrometry footprinting approach. In the future, we will further clarify the biochemical basis of resistance among at-MDR mutants and we expect to unambiguously pinpoint the positions of drug binding on *h*stopo IIα.

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