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14. ABSTRACT

Human topoisomerase IIα (*hs*topo IIα) is an essential enzyme that is the target of a number of anticancer drugs in clinical use. Clinically, resistance to anticancer drugs develops through various mechanisms, one of which can be found in a class of atypical multidrug resistant mutants (at-MDR) of *hs*topo IIα. Our goal is to clarify the biochemical basis of at-MDR among *hs*topo IIα mutants to shed more light on the topo II enzymatic mechanism. To this end, we have developed several footprinting techniques for studying the conformational changes of *hs*topo IIα. Additionally, we have identified sites of covalent thiol modification of *hs*topo IIα by anticancer drugs and chemopreventive agents. To this end, we developed a cysteine footprinting technique using liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) to assess the differential reactivities of the cysteine residues of *hs*topo IIα and compared their relative solvent accessibilities to a solvent contact surface (SCS) analysis of a structural model of *hs*topo IIα. In the presence of DNA, cofactors and anticancer drugs, we have seen changes in the relative thiol reactivities of *hs*topo IIα to thiol-alkylating reagents including monobromobimane and iodoacetamide. These changes, when examined in the context of the structural model of *hs*topo IIα, give insight into the conformational changes occurring in *hs*topo IIα. Additionally, using LC-ESI-MS, we have located sites of covalent cysteine modification of *hs*topo IIα by both anticancer drugs and chemopreventive agents, resulting in the first direct evidence of this novel poisoning mechanism by thiol alkylation of *hs*topo IIα.

15. SUBJECT TERMS
Protein footprinting, topoisomerase II, atypical multiple drug resistance (at-MDR), enzyme mechanism, teniposide

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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 using an alkylation/endoproteinase procedure coupled with liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). Structural changes of *hs*topo IIα 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 has allowed us to probe conformational changes of wild-type *hs*topo IIα in the presence and absence of anticancer drugs, DNA, and cofactors in order to further understand the *hs*topo IIα mechanism. Additionally, we will extend our studies to at-MDR mutants of *hs*topo IIα in order to better understand the biochemical basis of resistance among at-MDR mutants.

Using LC-ESI-MS, we have further demonstrated how newly identified topo II poisons and chemopreventive agents 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 using an endoproteinase/ mass spectrometry footprinting approach.

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Protein Conformational Changes

To address the questions at hand, we have made progress towards accomplishing our research goals. Previously, recombinant *hs*topo IIα was created by fusing an HMK site and H_6 tag to the N-terminus of *hs*topo IIα. 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 aforementioned construct was generated for the footprinting

procedure initially developed during the first year, in which HMK-H_6-*hs*topo IIα was treated with NTCB and subjected to alkaline denaturing conditions resulting in a ladder of peptide fragments which were radiolabeled, separated by SDS-PAGE and visualized by phosphorimaging. However, due to the difficulty encountered with the NTCB cysteine footprinting procedure, attempts were made to find an alternative footprinting procedure that could answer the same questions we began with. However, the HMK-H_6-*hs*topo IIα construct was retained for use in the current development of an alternative cysteine footprinting approach.

To this end, an endoproteinase footprinting procedure utilizing mass spectrometry was developed. Initial experiments were targeted at successfully identifying cysteine-containing peptides from a tryptic digest mixture by comparing spectra of reduced samples to those alkylated with iodoacetamide (IAM). For identification of alkylated cysteine-containing peptides, HMK-H_6-*hs*topo IIα was treated with IAM and denatured. Upon removal of excess denaturant and IAM, the sample was completely digested with trypsin and subjected to LC-ESI-MS. This sample was then compared to that of a tryptic digest of HMK-H_6-*hs*topo IIα which was not alkylated with IAM. The results from these experiments resulted in the successful identification of cysteine-containing peptide fragments covalently modified by IAM (Table 1).

Predicted Tryptic Peptide Fragment (sequence number range)	**MH_x^{x+} Calc. for Iodoacetamide Alkylation**	**MH_x^{x+} Exp. for Iodoacetamide Alkylation**	**MH_x^{x+} Calc. for monobromobimane Alkylation**	**MH_x^{x+} Exp. for monobromobimane Alkylation**
	m/z	*m/z*	*m/z*	*m/z*
MSCIR (102-106) $^{x=2}$	333.6531	333.6749	400.1424	400.1877
LCNIFSTK (169-176) $^{x=2}$	491.7513	491.7631	558.2406	558.2878
AGEMELKPFNGEDYTCITFQPDLSK (201-225) $^{x=4}$	723.3318	723.3589	756.5765	756.6298
WEVCLTMSEK (297-306) $^{x=2}$	641.7903	641.8027	708.2796	708.3358
SFGSTCQLSEK (387-397) $^{x=2}$	622.2812	622.2937	688.7704	688.8254
AAIGCGIVESILNWVK (401-416) $^{x=2}$	865.4653	865.5059	931.9545	932.0188
CSAVK (427-431) $^{x=2}$	282.6405	N.F.	349.1298	349.1762
NSTECTLILTEGDSAK (451-466) $^{x=2}$	869.9082	869.9336	936.3975	936.4589
VLFTCFK (729-735) $^{x=2}$	457.7402	457.7411	524.2295	524.2837
VEPEWYIPIIPMVLINGAEGIGTGWSCK (836-863) $^{x=3}$	1043.8596	1043.8671	1088.1857	1088.2478
LQTSLTCNSMVLFDHVGCLK (991-1010) $^{x=3}$	775.0414	775.0721	863.69	863.778
DELCR (1142-1146) $^{x=2}$	346.6516	N.F.	413.0705	N.F.

Table 1: Predicted and experimental mass values for the 13 cysteine-containing tryptic peptides.
X+ represents the charge state of the major ionic species observed.
m/z = mass to charge ratio, N.F. = not found

Once it was established that we could identify all of the tryptic peptides containing cysteine residues, we began assessing the differential reactivities of the cysteines of HMK-H_6-*hs*topo IIα. However, quantification of the alkylated cysteine-containing peptides is necessary for the validity of our approach. Thus, we developed a quantitiative mass spectrometric footprinting procedure utilizing both IAM as well as

deuterated iodoacetamide (D-IAM). Equivolume mixtures containing varying molar proportions of D-IAM and IAM modified peptides were mixed and analyzed by LC-ESI-MS. The quantitative nature of the procedure was verified by the linear relationship obtained from a plot of the relative peptide abundance (the percentage of intensity of the D-IAM modified peak divided by the sum of intensities of both D-IAM and IAM modified peaks = $P_{D\text{-}IAM} / (P_{D\text{-}IAM} + P_{IAM})$) versus its relative concentration ratio. Once the quantitative nature of the procedure was confirmed, D-IAM and IAM were used to evaluate the relative thiol reactivities of HMK-H_6-*hs*topo IIα. To this end, the enzyme was initially treated with various concentrations of D-IAM and was subsequently denatured and allowed to react with excess IAM. The protein was then dialyzed and digested with trypsin to create peptide fragments that were subjected to LC-ESI-MS. Because the protein was first treated with D-IAM before being denatured, a larger extent of D-IAM modification indicates elevated thiol reactivity, which likely correlates with increased solvent accessibility. For example, if a particular residue is located on the exterior of *hs*topo IIα resulting in high solvent accessibility, it is likely to react to a larger extent with the first reagent, D-IAM. More buried thiols may be less solvent accessible, resulting in less reaction with D-IAM, but increased reaction with IAM because this reagent is present after the protein has been denatured. Based on the relative ratios of cysteine-containing peptide modification with D-IAM versus IAM, 11 cysteines were divided into three groups according to their order of reactivity: Cys104 > Cys733, Cys170, Cys392, Cys300, Cys455, Cys405, Cys216 > Cys997-1008, Cys862

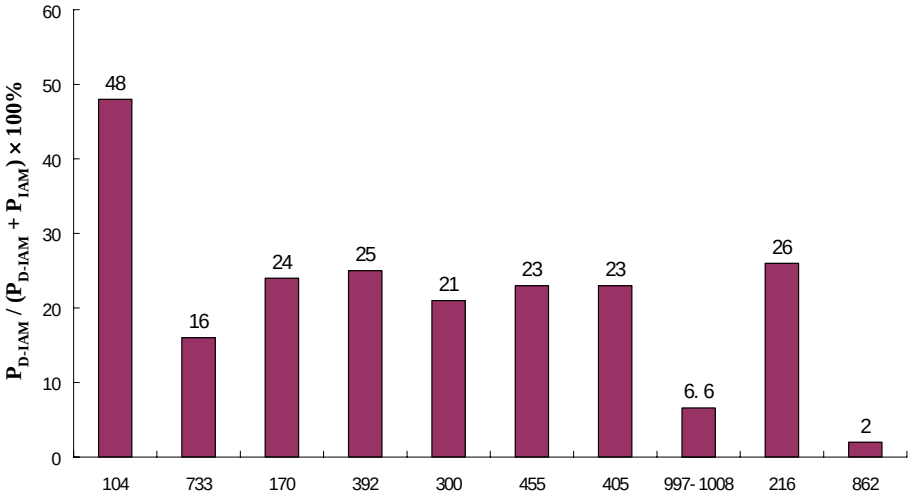

Figure 1: Percentages of D-IAM modification for different cysteines at 0.2 mM D-IAM

(Figure 1). To supplement and substantiate the biochemical data described thus far, an *hs*topo IIα structural model was constructed using *S. cerevisiae* topoisomerase II as a template. (Figure 2). Solvent contact surface analysis of the *hs*topo IIα homology model revealed four cysteines, Cys1008, Cys104, Cys216, and Cys405, as being the most solvent-accessible. These data agreed with that from the footprinting experiments, with the exception of Cys1008, which was shown to be one of the least reactive residues by our experimental data. One possible reason for this disagreement is that a sequence of 63 residues near Cys1008 was not modeled due to lack of *S. cerevisiae* template

structure in this region. It is possible that the missing 63 amino acids actually shielded Cys1008 from the solvent and thus made it less accessible. However, the *hs*topo IIα homology model appears to be a useful tool for visualizing the position of each thiol relative to the global structure of *hs*topo IIα. Further experiments utilizing this protein

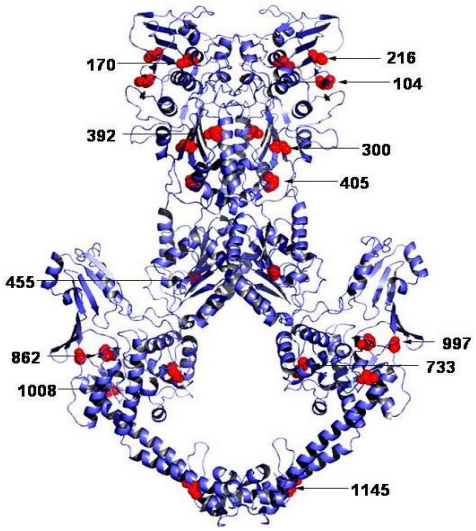

Figure 2: Model of Human topoisomerase IIα, with 12 cysteines emphasized in red. Cys427 was not modeled because corresponding structure was not present in the *S. cerevisiae* template.

footprinting method to detect protein conformational changes were attempted. In these experiments, the protein was preincubated in the presence or absence of DNA, anticancer drugs and cofactors and was subsequently treated with D-IAM to take a "snapshot" of the protein conformational state. The protein was then treated with IAM and denatured to expose and alkylate the remaining cysteine residues as a reference point for those cysteines that are less solvent-accessible. The protein was digested with trypsin and subjected to LC-ESI-MS. However, upon plotting the relative ratios of cysteines that reacted with D-IAM to those that reacted with IAM, no significant differences in cysteine reactivity were observed in the presence or absence of DNA, anticancer drugs and cofactors.

Because no cysteine reactivity changes were observed using D-IAM followed by IAM, we attempted to carry out the footprinting procedure using a different reagent as the first alkylator. Kim et al. utilized a similar footprinting procedure to study tubulin-binding drugs and found that IAM was unable to satisfactorily locate sites of drug binding because the cysteines had little change in reactivity to IAM in the presence or absence of drug, similar to our results (Kim et al. 2004). However, when they employed an alternative thiol alkylator, monobromobimane (mBrB), they were able to distinguish the site of drug binding as a result of lowered cysteine reactivity towards mBrB in the presence of a tubulin drug. The presumed cause of this result is due to the fact that mBrB is larger in size than IAM and is therefore more selective in its ability to react with cysteines that are more sterically hindered. Thus, we modified our footprinting procedure to include mBrB as the first cysteine alkylator. Initially, we used mBrB and IAM to test the relative thiol reactivity of *hs*topo IIα (see Table 1 for a list of mBrB-modified peaks). Because the mBrB/IAM combination yielded similar results in terms of

relative cysteine reactivity, we used this technique to probe conformational changes of *hs*topo IIα. In our reactions, *hs*topo IIα was first preincubated in the presence or absence of anticancer drugs, DNA and cofactors and was subsequently treated with mBrB. Following this treatment, the enzyme was then alkylated with IAM under denaturing conditions and was digested with trypsin and subjected to LC-ESI-MS. The ratio of the intensity of the cysteine-containing trypsin fragments that reacted with mBrB versus the intensity of the fragments that reacted with IAM was determined and several notable changes in cysteine reactivity occurred. As seen in Figure 3, Cys392 is an

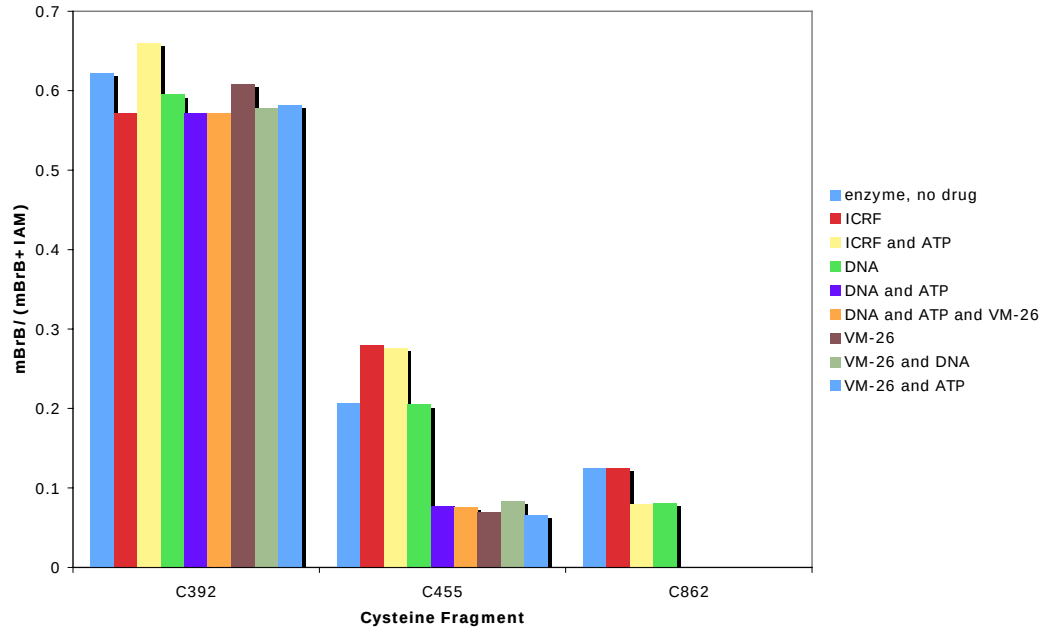

Figure 3: Cysteine Reactivity is affected by the presence anticancer drugs, DNA, and ATP. Cys 392 is an example of a cysteine residue whose relative reactivity to mBrB versus IAM did not change. Cys 455 is more solvent-inaccessible in the presence of VM-26. Cys 862 reactivity to mBrB is undected in the presence of VM-26, suggesting that this residue is also more solvent-inaccessible in the presence of VM-26.

example of a residue whose relative reactivity to mBrB is not affected by the presence of anticancer drugs, DNA or ATP. Cys455, on the other hand, is a residue that has a marked decrease in reactivity to mBrB whenever the anticancer drug VM-26 (teniposide) is present. This could be the result of VM-26 locking *hs*topo IIα in a conformation that sterically hinders Cys455, or it could be the result of VM-26 directly blocking the site where Cys455 is located. It is interesting to note that Cys455 is near to the region in which *hs*topo IIα binds to and cleaves DNA, and VM-26 is a compound that affects *hs*topo IIα in such a way as to promote cleavage of DNA. Also of note is Cys862, which is also close to the region in which *hs*topo IIα binds DNA and is near the active site tyrosine residue involved in breaking the DNA backbone in a trans-esterification reaction. When VM-26 is present, we cannot detect reaction of Cys862 with mBrB, indicating that this region of *hs*topo IIα is either sterically hindered by the protein conformational change, or is blocked by binding of VM-26.

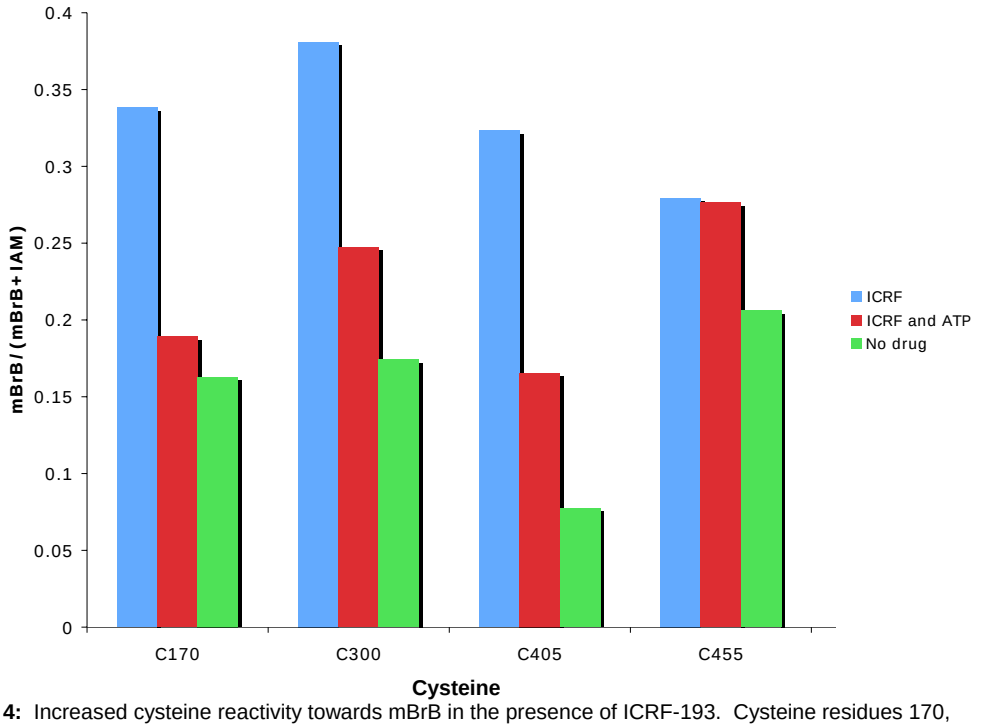

Figure 4: Increased cysteine reactivity towards mBrB in the presence of ICRF-193. Cysteine residues 170, 300, 405, and 455 have increased reactivity towards mBrB in the presence of ICRF-193.

In addition to VM-26, we also treated *hs*topo IIα with the compound ICRF-193, which is known to bind to the N-terminus of *hs*topo IIα and lock it in a closed conformation. It is interesting to note that several residues in the N-terminus are affected by the presence of ICRF-193. Particularly, Cys405, Cys300, and Cys170 are all residues on the exterior of the N-terminus of *hs*topo IIα that become more reactive to mBrB in the presence of ICRF-193 and ATP. When no ATP or ICRF-193 are present, the N-terminus adopts an open conformation, but when ICRF-193 and ATP are present, the N-terminus closes as seen in Figure2. Thus, it follows that these residues would react more readily with mBrB when ICRF-193 is present, because it will lock the N-termiumus closed and keep these residues more solvent-accessible. Cys455 is also more solvent accessible in the presence of ICRF-193, possibly as a result of the N-terminus being locked in the closed conformation, resulting in loss of steric hindrance of Cys455. Now that the protein footprinting method chosen is able to detect differences in cysteine reactivity in response to various reagents, we will extend our studies to further refine the method and then apply it to the study of at-MDR mutants of *hs*topo IIα in an attempt to better understand the biochemical basis of resistance of these mutants. For example, are the N-terminus or C-terminus of the at-MDR mutants adopting different conformational states than the wild type protein? How is the DNA binding domain affected in the at-MDR mutants? These are additional questions that we will address with the protein footprinting technique in hand.

Cysteine Binding sites

Using mass spectrometry, we have also identified sites of thiol alkylation of *hs*topo IIα by both anticancer drugs such as menadione and chemopreventive compounds such as

diallyl trisulfide (DAT), which has been shown to induce topoisomerase II-mediated DNA cleavage. In this procedure, we directly observed thiol modification by identifying the appearance of new tryptic peptide fragments in alkylated samples. In this procedure,

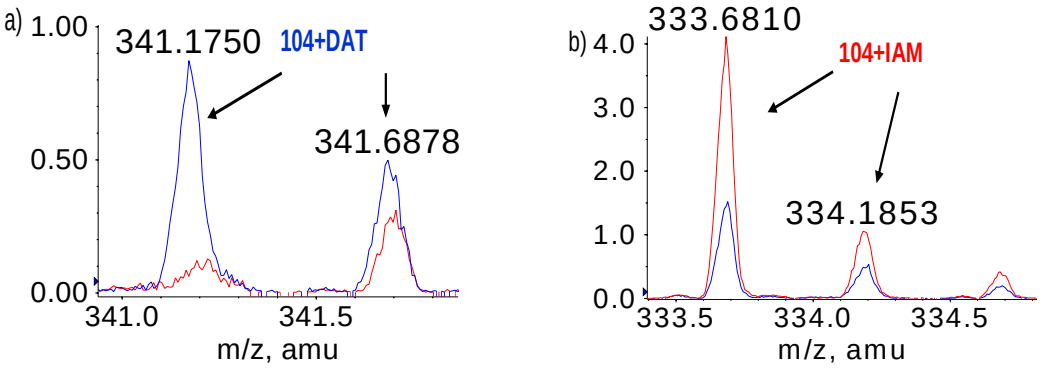

Figure 5: Mass Spectra demonstrating the modification of Cys104 by DAT a) Direct evidence: appearance of a novel peak occurs in the DAT-treated sample (blue, upper spectrum), but is not present in the untreated sample (red, lower spectrum). b) Indirect evidence: the cysteine-containing peptide MSCIR pretreated with DAT (blue, lower spectrum) was not modified with IAM to the same extent as the sample not treated with DAT (red, upper spectrum) because it had previously reacted with DAT, preventing reaction with IAM. Arrows indicate different isotopes of the peptide, which are in the 2+ charged state.

HMK-H_6-*hs*topo IIα was incubated in the presence or absence of DNA and anticancer drugs or chemopreventive compounds. The reaction was quenched and HMK-H_6-*hs*topo IIα was treated with excess IAM followed by trypsin digestion and LC-ESI-MS. In this procedure, it is feasible to discover sites of covalent modification by DAT, for example, by identifying fragments in DAT-treated samples that are not present in untreated samples. If the unique fragment corresponds to a molecular weight increase consistent with a reaction between DAT and a cysteine residue, then the site of modification can be identified. Moreover, the IAM-modified peptide will decrease in intensity or disappear altogether for samples that were pretreated with DAT, because their reaction with DAT precludes their reaction with IAM. Our preliminary data provides direct evidence that the chemopreventive compound DAT is reacting with thiol groups on HMK-H_6-*hs*topo IIα. For example, as seen in Figure 3a, treatment with DAT results in the appearance of a new fragment consistent with a reaction between DAT and Cys104 contained in the peptide fragment MSCIR. Additionally, in the sample pretreated with DAT, the amount of IAM-modified peptide decreased (Figure 3b) because Cys104 had reacted with DAT, the first reagent it encountered, resulting in less reaction with the second reagent added, IAM, and hence a diminished signal for the IAM-modified peptide fragment. While data from LC-ESI-MS suggests that DAT modifies Cys104 to the greatest extent, additional thiols are modified as well. Thus, experiments are in progress to help determine the cysteine residues that are critical for this novel poisoning mechanism of *hs*topo IIα; site directed mutagenesis coupled with *in vitro* DNA cleavage assays will help to answer these remaining questions.

During the first year, we putatively identified menadione as having reacted with Cys427 by matrix-assisted laser desorption ionization (MALDI) MS. Preliminary results from LC-ESI-MS suggest that menadione reacts with additional thiol residues, albeit through indirect evidence. The indirect evidence is similar to that mentioned previously in which samples first treated with menadione followed by treatment with IAM exhibited

diminished signals for cysteine-containing peptides modified with IAM, suggesting prior reaction with menadione precluding reaction with IAM. Additionally, chemopreventive compounds such as that found broccoli, benzyl isothiocyanate (BITC), have been tested, and indirect evidence also suggests reaction with thiol residues. Experiments are in progress to test a range of potential topoisomerase II poisons that act to inhibit the enzyme by thiol alkylation.

Key Research Accomplishments

- Overexpression of HMK-H_6-hstopoIIα
- Purification of active HMK-H_6-hstopoIIα
- Successful development and implementation of a footprinting technique using LC-ESI-MS
- Reproducible identification of tryptic peptides containing cysteine residues
- Establishment of the relative thiol reactivity of *hs*topo IIα in native conditions
- Identification of conformational changes occurring in HMK-H_6-hstopoIIα using cysteine footprinting
- Development of structural model of *hs*topo IIα based on homology to the *S. cerevisiae* topoisomersase II structure
- Identification of thiol modification sites by anticancer drugs and chemopreventive compounds
- Putative verification of a new means by which thiol alkylators poison *hs*topo IIα

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 *hs*topo IIα using mass spectrometric cysteine footprinting in a procedure utilizing tryptic digestion coupled with LC-ESI-MS. Using LC-ESI-MS cysteine footprinting, we have assessed the differential thiol reactivities of the cysteine residues of *hs*topo IIα and have identified conformational changes occurring in the wild type enzyme. To complement the thiol reactivity results obtained by LC-ESI-MS, we have compared our data to a solvent contact surface analysis of an *hs*topo IIα structural model based on homology with its yeast counterpart. Moreover, we have found direct evidence of covalent thiol modification of *hs*topo IIα using LC-ESI-MS. These results exhibit the first direct evidence that thiol alkylation is indeed a novel mechanism of *hs*topo IIα poisoning. In the future, we will further clarify the biochemical basis of resistance among at-MDR mutants and we expect to complement our drug binding data with site-directed mutagenesis to pinpoint those cysteines most important in the thiol alkylation poisoning mechanism of *hs*topo IIα.

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