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Proteasomal inhibition stabilizes topoisomerase IIα protein and reverses resistance to the topoisomerase II poison ethonafide (AMP-53, 6-ethoxyazonafide)

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ABSTRACT

Multiple myeloma (MM) is an incurable malignancy of plasma cells. Although multiple myeloma patients often respond to initial therapy, the majority of patients will relapse with disease that is refractory to further drug treatment. Thus, new therapeutic strategies are needed. One common mechanism of acquired drug resistance involves a reduction in the expression or function of the drug target. We hypothesized that the cytotoxic activity of topoisomerase II (topo II) poisons could be enhanced, and drug resistance overcome, by increasing the expression and activity of the drug target, topo II in myeloma cells. To test this hypothesis, we evaluated the cytotoxicity of the anthracene-containing topo II poison, ethonafide (AMP-53/6-ethoxyazonafide), in combination with the proteasome inhibitor bortezomib (PS-341/Velcade). Combination drug activity studies were done in 8226/S myeloma cells and its drug resistant subclone, 8226/Dox1V. We found that a 24-h treatment of cells with bortezomib maximally increased topo IIα protein expression and activity, and consistently increased the cytotoxicity of ethonafide in the 8226/S and 8226/Dox1V cell lines. This increase in cytotoxicity corresponded to an increase in DNA double-strand breaks, as measured by the neutral comet assay. Therefore, increasing topo IIα expression through inhibition of proteasomal degradation increased DNA double-strand breaks and enhanced the cytotoxicity of the topo II poison ethonafide. These data suggest that bortezomib-mediated stabilization of topo IIα expression may potentiate the cytotoxic activity of topo II poisons and thereby, provide a strategy to circumvent drug resistance.

1. Introduction

Multiple myeloma is a B-cell malignancy characterized by the accumulation of latent plasma cells in the bone marrow. Patients with multiple myeloma frequently respond to initial drug therapy, but later relapse with disease that is refractory to further treatment. In spite of recent advances in molecular therapeutics, the disease is uniformly fatal, and the median survival remains 36–60 months, with no significant improvements in long-term survival in the past 20 years [1].

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One class of chemotherapeutic agents classically used to treat patients with relapsed myeloma is drugs that inhibit topoisomerase II (topo II), including doxorubicin, etoposide, and mitoxantrone. Topo II is an ATP-dependent enzyme that catalyzes changes in DNA topology necessary for transcription, replication, and chromosome condensation and segregation [2,3]. Because topo II is essential for DNA replication, it has been identified as an important drug target in the treatment of cancer. Topo II exists in two isoforms: topo IIα is cell-cycle regulated and is necessary for chromosome condensation and segregation in mitosis, whereas topo IIβ is constitutively expressed and has been shown to be involved in DNA repair and transcription [4,5]. When administered as part of a combination chemotherapy regimen, topo II poisons contribute to the improved rates of remission and survival for many hematological malignancies including childhood acute lymphocytic leukemia (ALL), adult acute myelogenous leukemia (AML) and multiple myeloma [3]. Nevertheless, relapse associated with acquired resistance to topo II poisons remains a clinical challenge.

Known mechanisms of acquired drug resistance to topo II poisons include both modification of drug transport and alteration of the drug target. For example, the over-expression in tumor cells of the efflux transporter P-glycoprotein, encoded by the *mdr1* gene, can result in resistance to a wide range of anticancer drugs that vary structurally and functionally. This phenomenon is known as the multidrug resistance (MDR) phenotype [6,7].

Although resistance to topo II poisons is often observed in tumor cells that over-express P-glycoprotein, the frequency of clinical resistance cannot be explained by P-glycoprotein-mediated efflux alone. Reduction in topo II protein levels and activity have been proposed to be potentially more important mechanisms of resistance to topo II poisons [7]. Previously published studies of various cell lines that have been selected for resistance to topo II poisons indicates a variety of potential mechanisms leading to reduced topo II expression and activity. Topo II activity can be modulated by a decrease in expression of the gene due to either reduced transcription or translation, an alteration of the coding sequence leading to the production of an enzyme with modified activity, or post-translational modifications of the enzyme. These activities all may result in the observed phenotype of a reduction in topo II expression and activity [8].

Previous studies have investigated the emergence of the drug resistant-phenotype in the human multiple myeloma cell line RPMI 8226 (8226/S). When cells were selected for doxorubicin resistance (8226/Dox40), drug resistance was mediated by P-glycoprotein over-expression [9]. In contrast, when cells were selected for resistance to doxorubicin in the presence of the P-glycoprotein inhibitor, verapamil (8226/Dox1V), drug resistance was associated with reduced expression and activity of topoisomerase IIα with no induction of P-glycoprotein over-expression [10]. In the present study, the 8226/Dox1V cell line was used as a model to investigate potential strategies to reverse resistance to topo II poisons associated with a reduction in topo IIα expression and activity.

Topoisomerase II is an ATP-dependent enzyme that catalyzes changes in DNA topology by passing an intact double helix through a transient double-stranded DNA break. A critical step in the reaction catalyzed by topo II involves the formation of a topo II-DNA covalent complex, referred to as the cleavable complex, in which each topo II homodimeric subunit is covalently linked to the 5′-phosphoryl ends of the broken DNA strand [11,12]. Under normal circumstances, the cleavable complex is a short-lived reaction intermediate. However, a persistence or stabilization of cleavable complexes leads to an accumulation of DSBs in the genome of the cell and therefore has cytotoxic effects [11]. Thus, anticancer activity of topo II poisons is directly associated with stabilization of the cleavable complex and resulting DNA strand breaks.

Previous studies have shown that the cell cycle-dependent expression of topo IIα is regulated by proteasomal degradation [13]. The 26S proteasome is a multicatalytic enzyme complex that is the primary component of the protein degradation pathway of the cell [14–16]. Inhibition of the proteasome is therefore a promising approach for cancer treatment. Bortezomib (PS-341/Velcade) is a dipeptide boronic acid inhibitor that is highly selective for the proteasome, having little affinity for other proteases (Fig. 1A). Bortezomib forms a covalent bond with the active site threonine in the core of the 20S proteasome, and inhibits the chymotryptic activity of the proteasome [17]. In 2003, bortezomib was approved in the

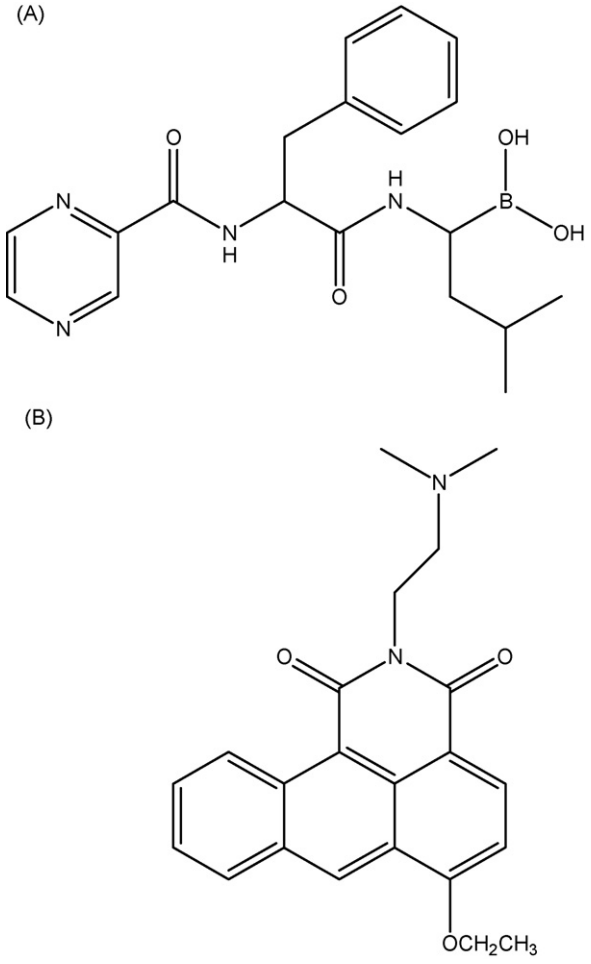

Fig. 1 – (A) Chemical structure of bortezomib (PS-341/Velcade). (B) Chemical structure of ethonafide (AMP-53/6-ethoxyazonafide).

United States for the treatment of relapsed and refractory multiple myeloma [18]. Patient responses to bortezomib-based combination therapy suggest the possibility of chemosensitization and synergy [19].

The hypothesis for the current study is that prevention of topo IIα degradation, by inhibiting the proteasome, and thereby increasing protein expression and activity will enhance the cytotoxic activity of topo II poisons and overcome drug resistance. To test this hypothesis, we evaluated the cytotoxicity of the novel topo II poison, ethonafide (AMP-53/6-ethoxyazonafide), in combination with the proteasome inhibitor bortezomib.

Ethonafide was developed at the University of Arizona as a potentially less cardiotoxic inhibitor of topo II (Fig. 1B). This agent is a member of the azonafide series of anthracene-based DNA intercalating antitumor agents [20]. Mechanistically, ethonafide inhibits topo II activity by stabilizing a covalent complex between topoisomerase II and DNA, known as the cleavable complex, making ethonafide a topo II poison [21]. In the present study, we examined the effects of ethonafide in combination with sub-cytotoxic doses of bortezomib as a strategy to potentiate the activity of ethonafide in drug resistant myeloma cells. We found that inhibition of the proteasome led to the intracellular accumulation of topo IIα, which in turn, increased the cytotoxic activity of the topo II poison ethonafide. These findings suggest that combinations of proteasome inhibitors with topo II poisons may provide a strategy for the treatment of patients with refractory disease.

2. Materials and methods

2.1. Cell culture

The human multiple myeloma cell line 8226 was originally obtained from American Type Culture Collection (ATCC, Manassas, VA). The 8226/Dox1V cell line was originally established and generously provided by Dr. William Dalton (H. Lee Moffitt Cancer Center) and was maintained in the presence of 10 μg/ml verapamil and 10 nM doxorubicin. The 8226/siTIIα and 8226/siCT were established using RNAi technology [22]. Retroviral expression constructs expressing topoisomerase IIα or non-coding control siRNA sequences were obtained from Open Biosystems (Huntsville, AL). Replication-incompetent virions were produced by transfection of the PT67 packaging cell line (Clontech, Palo Alto, CA). Media containing infectious virions was collected, filtered in a 0.45 micron filter, and incubated with 8226/S cells for 72 h. Stably infected cells were selected in 0.5 μg/ml puromycin. All cell lines were maintained in RPMI 1640 (Cellgro, Herndon, VA) supplemented with 5% heat inactivated fetal bovine serum (Omega Scientific, Tarzana, CA), L-glutamine and penicillin/streptomycin (Gibco, Carlsbad, CA). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2.

2.2. Cytotoxicity assay

Cytotoxicity profiles of bortezomib and ethonafide were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye reduction assay as previously described [23]. To determine cytotoxicity of bortezomib alone, cells were seeded in 96 well plates and incubated 24 h in drug concentrations ranging from 1.56 to 800 nM. In subsequent studies, to determine the effect of bortezomib pre-treatment on the cytotoxic activity of ethonafide, cells were incubated with sub-cytotoxic concentrations of bortezomib, 2.5 or 5 nM, for 24 h prior to incubation for 72 h with serial dilutions of ethonafide ranging from 0.5 nM to 10 μM. Plates were incubated 4 h with MTT dye, solublized, and the absorbance at 540 nm was read with a μQuant microplate reader (BIO-TEK, Winooski, VT). The IC_{50} was determined by sigmoidal analysis of the dose response curve using Origin v6.1 software (Northampton, MA). Cytotoxicity was confirmed using flow cytometry of propidium iodide stained DNA content in cells exposed to bortezomib for 24 h, and in combination with ethonafide for an additional 72 h.

2.3. Topoisomerase II extraction and activity assay

Nuclear extracts for the topo II enzyme assay were prepared without the addition of protease inhibitors which may interfere with the enzymatic activity. All procedures were performed at 4 °C. Cells were collected by centrifugation and the cell pellet was resuspended in 5 ml of TEMP buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 4 mM $MgCl_2$, 0.5 mM PMSF). Centrifugation was repeated and the pellet was resuspended in 1 ml of TEMP and incubated on ice for 10 min. The cells were homogenized by 15–20 strokes in a tight-fitting Dounce homogenizer. Nuclei were pelleted by centrifugation at 12,850 × *g* for 10 min. The nuclear pellet was resuspended in 50–70 μl of TEP buffer (TEMP without $MgCl_2$) and an equal volume of 1 M NaCl and incubated on ice for 45 min. Nuclear extracts were collected by centrifugation at 100,000 × *g* for 1 h, and total protein concentrations were quantitated using bicinchoninic acid (BCA) colorimetric assay (Pierce, Rockford, IL).

Topoisomerase II activity was assessed by the decatenation reaction of kinetoplast DNA (kDNA) using the TopoGEN Topo II assay kit (Port Orange, FL). Reactions were performed in eppendorf tubes in a reaction mixture containing 2 μl of complete assay buffer (50 mM Tris–HCl (pH 8), 120 mM NaCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM ATP) and 0.137 μg of kDNA. Concentrations of 0.5–2 μg of either purified topoisomerase II purchased from TopoGEN or nuclear extracts was added to the reaction mixture and ddH_2O to bring the total volume to 20 μl. After incubation at 37 °C for 30 min, 4 μl of stop loading dye and 0.05 μg/ml proteinase K were added and samples were digested at 37 °C for 15 min. Samples were separated by electrophoresis at 45 V through a 1% agarose gel and visualized by staining with ethidium bromide under UV light.

2.4. Preparation of nuclear extracts for immunoblot analysis

Cells were treated with 2.5 nM bortezomib for the specified length of time. All subsequent procedures to obtain nuclear lysates were performed at 4 °C as previously described [24]. Briefly, cells were lysed in hypotonic buffer containing 0.2% NP-40 followed by high salt extraction of nuclear proteins.

Protein concentrations were quantitated by BCA assay (Pierce, Rockford, IL).

2.5. Immunoblot analysis

Protein amounts of 30 μg per sample were separated by electrophoresis on a 10% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The membrane was first incubated with anti-topo IIα antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and then with the corresponding secondary antibody (Jackson ImmunoResearch, West Grove, PA). The constitutive nuclear protein, lamin A/C (Cell Signaling, Danvers, MA) was used as a loading control. Antibody binding was visualized using Pierce Supersignal chemiluminescence substrate.

2.6. Comet assay

The comet assay was used to detect and quantitate DNA double-strand breaks in myeloma cells (CometAssay Kit, Trevigen, Gaithersburg, MD). Cells were pre-treated for 24 h with 2.5 nM bortezomib followed by 1 or 0.5 μM ethonafide for 1 h. Cells and media were collected, resuspended in cold PBS and added to 1% low melting agarose. The cell-agarose suspension was layered onto a glass slide and incubated for 45 min at 4 °C in the dark. Slides were then placed in ice cold lysis solution with proteinase K (80 μg/ml) for 1 h and incubated at 37 °C in the same lysis solution plus proteinase K overnight. Following lysis, the samples were washed 4 times for 30 min each with cold 1× Tris–Borate–EDTA (TBE) buffer and subjected to electrophoresis at 23 V for 18 min. Slides were briefly rinsed with ddH_2O and fixed in 70% ethanol for 5 min. The slides were then allowed to dry in the dark and stained with SYBR Green I (Molecular Probes, Eugene, OR) for 30 to 45 min and washed for 5 min with 1× TBE. Images of 50 cells were captured using a Nikon PCM 2000 confocal microscope at an excitation/emission wavelength of 494 nm/521 nm, respectively. The comet moment measures the length of the comet tail and fluorescent intensity to determine the degree of DNA strand breakage. It was quantitated using CometScore software (TriTek, Sumerduck, VA) and represents the mean of 3 independent experiments, 50 cells per treatment.

3. Results

3.1. Ethonafide inhibits topoisomerase II in multiple myeloma cells

Using purified topoisomerase IIα in a cell free assay, ethonafide inhibits enzyme activity with slightly less potency than mitoxantrone (Fig. 2A). In order to investigate the effect of ethonafide on cellular topoisomerase II, nuclear extracts were prepared from the 8226/S multiple myeloma cell line and incubated with kinetoplast DNA in the presence of increasing concentrations of ethonafide. Fig. 2B demonstrates that ethonafide inhibits the decatenation activity of native topoisomerase II in a dose-dependent manner.

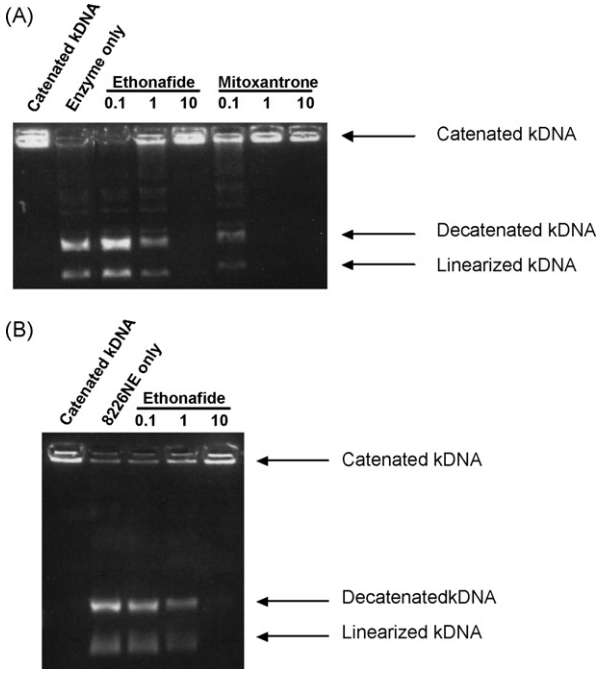

Fig. 2 – Inhibition of topoisomerase II activity by ethonafide. (A) kDNA is incubated with purified topoisomerase II in the presence of increasing concentrations of ethonafide (AMP-53) or mitoxantrone (Mitox). Inhibition of topoisomerase II activity is represented by a decrease in decatenated/relaxed kDNA and, subsequently, an increase in high molecular weight (HMW) kDNA. (B) Native topo II activity in 8226/S cell nuclear lysates is inhibited by ethonafide in a dose-dependent manner, demonstrated by a decatenation assay. Data shown are representative of $n = 3$.

3.2. Myeloma cells with reduced topoisomerase IIα expression are resistant to ethonafide

Previous studies have demonstrated that ethonafide has significant cytotoxic activity in a variety of tumor types, including myeloma [20]. To further examine the activity of ethonafide in myeloma, and investigate mechanisms of resistance, we established cytotoxicity profiles for the parental 8226/S cell line, and the drug resistant 8226/Dox1V cell line (Table 1). The 8226/Dox1V cell line displays 3.8-fold resistance to ethonafide compared to the parental cell line. These data correlate well with topo IIα protein expression (Fig. 3). The

Table 1 – Cytotoxicity of ethonafide in human multiple myeloma cell lines

Cell line	Mean IC_{50} in μM ± S.E.M.	Fold resistance
8226/S	0.182 ± 0.045	–
8226/Dox1V	0.692 ± 0.173	3.80
8226/SiTIIα	0.308 ± 0.144	2.01
8226/SiCT	0.153 ± 0.045	–

Cytotoxicity was determined by MTT assay following 72 h drug exposure. $n = 8–13$.

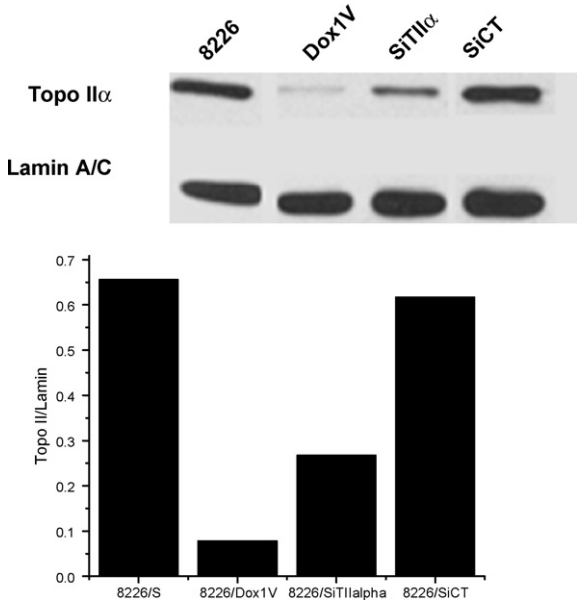

Fig. 3 – Basal expression of topoisomerase IIα. (A) Nuclear lysates were prepared from the multiple myeloma cell lines 8226/S, 8226/Dox1V, 8226/SiTIIα, and 8226/SiCT, and analyzed for topoisomerase IIα expression by immunoblot analysis. Lamin A/C was used as a loading control. (B) Densitometric analysis of topo IIα expression normalized to lamin A/C. Data are representative of $n = 3$.

8226/Dox1V cell line shows 88% reduction in topo IIα expression compared to the parental cell line, and is 7.3-fold resistant to doxorubicin, as previously reported [10].

To further investigate the activity of ethonafide in topo IIα-deficient cell lines, multiple myeloma cell lines with reduced expression of topoisomerase IIα were created using siRNA (8226/siTIIα). Scrambled siRNA was used as a control to account for off-target effects of the transfection. The 8226/siTIIα cell line showed a 59.2% reduction in topo IIα expression compared to the parental cell line, whereas topo IIα expression was unchanged in 8226/siCT cells (Fig. 3). Cytotoxicity assays demonstrate that the 8226/siTIIα cell line is consistently 2-fold resistant to ethonafide compared to the 8226/S and 8226/siCT cell lines (Table 1). These data establish a strong correlation between the expression and activity of topo II, and the cytotoxic activity of ethonafide.

3.3. *Bortezomib treatment increases topo IIα expression and activity*

Topo IIα expression is known to be regulated by proteasomal degradation, with a reported half-life of approximately 6 h in the MCF7 breast cancer cell line [13]. To determine the kinetics of topo IIα expression and activity in our system, 8226/S and 8226/Dox1V cells were treated with 2.5 nM bortezomib for 6, 14, or 24 h. Control experiments established that this concentration of bortezomib induced only 2–5% cell death over the course of the treatment (data not shown). Propidium iodide staining for DNA content demonstrated 3% increase in sub-G1 fraction of control cells treated with 2.5 or 5.0 nM bortezomib alone for 72 h (data not shown). Topo IIα protein expression levels were analyzed using Western blot analysis, and topo II activity was determined by the topo II decatenation assay. The time course analysis demonstrated an increase in topo IIα protein levels that peaked at 14 h and was maintained through 24 h. This increase was more dramatic in the 8226/Dox1V cell line (Fig. 4B), which has very low endogenous topo IIα expression compared to the parental cell line 8226/S (Fig. 4A). Additionally, the increase in topo IIα protein expression in the 8226/Dox1V cell line was associated with a similar increase in enzyme activity (Fig. 4C).

3.4. *Bortezomib pre-treatment enhances the cytotoxicity of ethonafide*

Combination MTT cytotoxicity assays were done to determine the effects of a 24 h pre-treatment with bortezomib on the cytotoxic activity of ethonafide in the 8226/S, 8226/Dox1V, 8226/SiTIIα and SiCT multiple myeloma cell lines. Minimally cytotoxic concentrations of bortezomib (2.5 or 5 nM) were used for these experiments. Bortezomib pre-treatment consistently enhanced the activity of ethonafide in all four cell lines (Fig. 5). In the 8226/S cells, pre-treatment with 2.5 nM bortezomib was associated with a mean 23% decrease in the IC_{50} of ethonafide while 5 nM pre-treatment was associated with a mean 36% decrease in the IC_{50}. In the 8226/Dox1V cells, pre-treatment with 2.5 nM bortezomib was associated with a mean 15% decrease in the IC_{50} of ethonafide while 5 nM pre-treatment was associated with a mean 21% decrease in the IC_{50}. In the 8226/SiTIIα cells, pre-treatment with either 2.5 or 5 nM bortezomib was associated with a mean 25% decrease in the IC_{50} of ethonafide. In the 8226/SiCT cells, pre-treatment with 2.5 nM bortezomib was associated with a mean 8% decrease in the IC_{50} of ethonafide while 5 nM pre-treatment was associated with a mean 21% decrease in the IC_{50}. These data show that bortezomib pre-treatment enhances the cytotoxicity of ethonafide in a dose-dependent manner. Importantly, pre-treatment with bortezomib reduces the IC_{50} of ethonafide in the drug resistant 8226/Dox1V and 8226/SiTIIα cell lines to a value comparable to the IC_{50} in the parental cell line.

3.5. *Bortezomib pre-treatment increases ethonafide-induced DNA double-strand breaks*

Topo II poisons stabilize the cleavable complex leading to the accumulation of double-stranded DNA breaks, the critical event in mediating drug cytotoxicity. To determine if increased ethonafide activity was associated with increased DNA damage in myeloma cells, we examined DNA double-strand breaks (DSBs) by neutral comet assay. Cells were incubated for 24 h with 2.5 nM bortezomib followed by a 1-h incubation with ethonafide and analysis of DNA DSBs, defined by the comet moment. In the 8226/S cell line, bortezomib pre-treatment followed by exposure to either 0.5 or 1 μM ethonafide produced a 9 and 16% increase in DNA DSBs, respectively, as compared to treatment with ethonafide alone (Fig. 6A). Additionally, in the 8226/Dox1V cell line, bortezomib pre-treatment followed by exposure to 0.5 or 1 μM ethonafide corresponded to a 85 and 24% increase in DNA DSBs, respectively (Fig. 6B). These data demonstrate that pre-treatment with bortezomib correlates

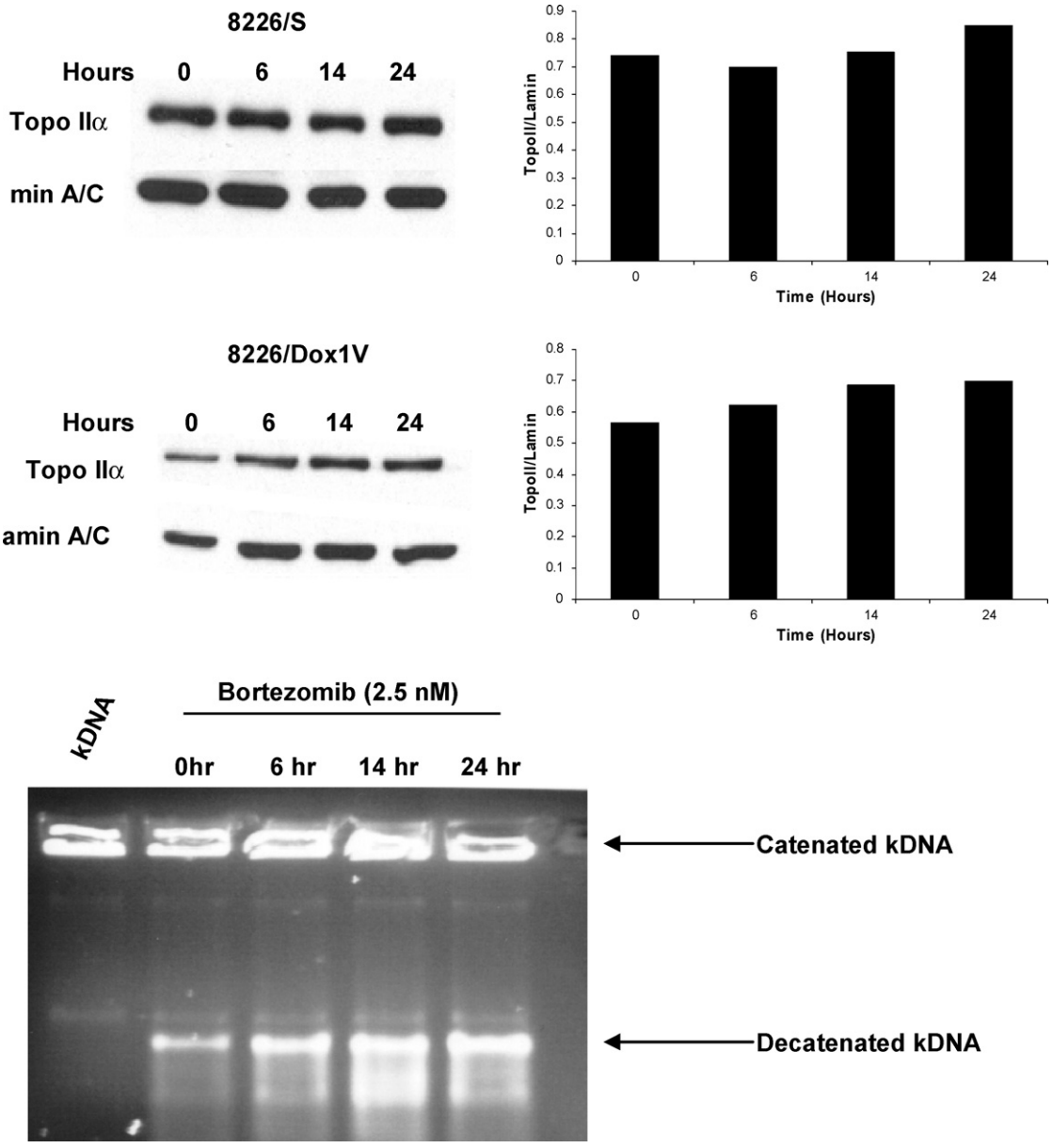

Fig. 4 – Bortezomib treatment increases topoisomerase IIα expression and activity. (A) 8226/S and (B) 8226/Dox1V cells were incubated with 2.5 nM bortezomib for 6, 14 and 24 h and nuclear lysates were prepared. Immunoblot analysis was used to examine topo IIα expression and densitometric analysis used for quantitation of topo II lamin ratios. (C) 8226/Dox1V cells were incubated with 2.5 nM bortezomib for 6, 14 and 24 h and nuclear lysates were prepared and analyzed for topoisomerase IIα activity by the topoisomerase II decatenation assay. Increased topo II activity is represented by decatenated/relaxed kDNA. Data shown are representative of *n* = 3.

with an increase in ethonafide-induced DNA DSBs. Furthermore, these data support the hypothesis that increasing topo II protein expression allows increased DNA damage and restores sensitivity to topo II posions.

4. Discussion

The presence of topo II is required to generate topo II poison-stabilized cleavable complexes and subsequent DSBs, and resistance to topo II poisons has been associated with reduced enzyme expression and activity. Using a rational schedule of bortezomib pre-treatment, we demonstrated that preventing topo II degradation by proteasomal inhibition increases both the expression and the activity of topo IIα, and enhances the activity of the topo II poison ethonafide. Furthermore, this treatment protocol effectively reverses drug resistance associated with reduced topo IIα expression and activity.

Increasing the expression and activity of topoisomerase as a strategy to enhance topo II poison efficacy has been the subject of ongoing studies [25]. Barker et al. recently demonstrated that inhibition of heat shock protein 90 (Hsp90) with geldanamycin

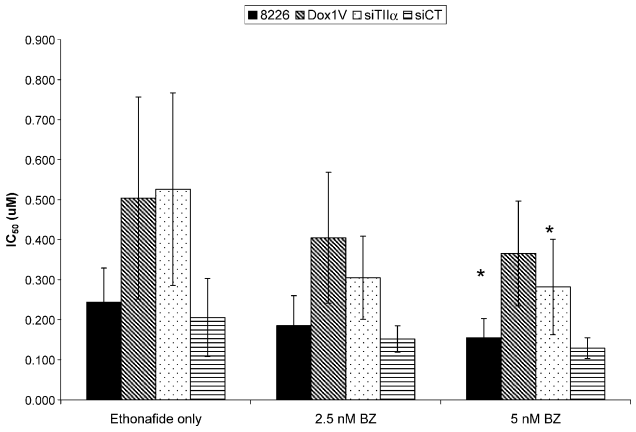

Fig. 5 – Pre-treatment with bortezomib (BZ) enhances the cytotoxicity of ethonafide. Cells were exposed to 2.5 or 5 nM bortezomib for 24 h followed by a 72 h incubation with ethonafide. Cell viability was analyzed by MTT dye reduction, and the IC_{50} calculated using Origins software. Data were corrected for the cytotoxic activity of bortezomib alone (≤5% in all experiments). Data shown is the mean and S.E.M. of at least $n = 3$ for each condition. *Indicates $p < 0.05$.

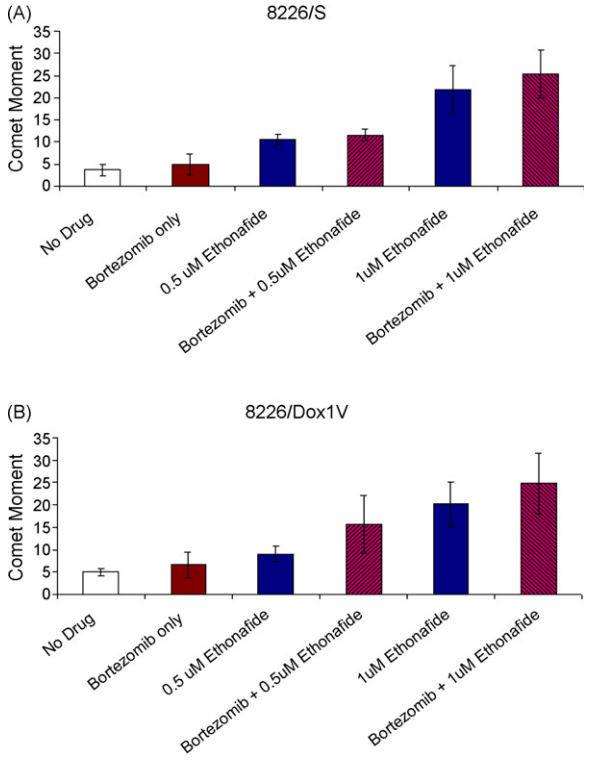

Fig. 6 – Pre-treatment with bortezomib increases ethonafide-mediated DNA DSBs in both 8226/S and 8226/Dox1V cell lines. (A) 8226/S or (B) 8226/Dox1V cells were incubated for 24 h with 2.5 nM bortezomib followed by a 1 h incubation with either 0.5 or 1 μM ethonafide. Samples were subjected to single-cell electrophoresis and the comet moment calculated using CometScore software. Data represent the mean of 3 independent experiments, 50 cells per treatment.

disrupts the Hsp90-topo II interaction leading to an increase of unbound topo II. In the presence of a topo II poison the elevated unbound topo II levels resulted in an increase in cleavable complexes and a corresponding increase in DNA damage and cell death [25]. Similarly, in the present study, we demonstrate that when a topo II poison is present, increased levels of enzymatically active topo II protein results in increased DNA damage and increased cell death.

The use of a proteasome inhibitor to enhance the activity of a topo II poison is an interesting strategy, in that some recent studies point to a specific requirement for the proteasome pathway for processing topo II-DNA covalent complexes into DNA damage [12]. Zhang et al. propose that proteasomal degradation of topo II cleavable complexes transforms topo II-concealed DSBs into protein-free DSBs. In support of this hypothesis, they demonstrate down regulation of topo IIβ by etoposide is reversed by co-treatment with the proteasome inhibitor MG132. Furthermore, they show that mouse embryonic fibroblasts treated concurrently with MG132 and etoposide incur less DSBs than cells treated with etoposide alone. Conversely, our findings demonstrate that pre-treatment with a sub-cytotoxic dose of a proteasome inhibitor, for a length of time resulting in an accumulation of topo IIα, greatly enhances the activity of the later administered topo II poison. One of the primary differences between our study, and that of Zhang et al. is the scheduling of drug exposure. Because we were interested in the effects of bortezomib on topoII expression and activity, protein levels were monitored and the topoII poison was administered at the time of peak protein accumulation. In contrast, Zhang et al. utilized concurrent drug exposure to investigate the activity of proteasomal degradation in topoII-DNA covalent complexes. Although neither study specifically examined the conformation or the localization of the enzyme, it is possible that allowing an accumulation of active protein prior to cell cycle arrest resulted in increased complex formation. Together, these data suggest administering bortezomib prior to a topo II poison may be an effective strategy to overcome clinical drug resistance.

A second factor that may contribute to the discrepancy in these studies is the specific topoII isoform examined. Ethonafide has been previously shown to stabilize both topoIIα- and topoIIβ-DNA complexes [21], however the primary DSB formation and cytotoxicity of the agent has been attributed to inhibition of topoIIα, whereas proteasomal processing of topoII-DNA covalent complex processing is more efficient in topoIIβ complexes.

Recently, Biehn et al. conducted a phase I trial of bortezomib and pegylated liposomal doxorubicin (PegLD) in patients with advanced hematologic malignancies [26]. The combination of bortezomib and PegLD showed significant antitumor activity against advanced multiple myeloma, with 36% of heavily pre-treated patients having a complete or near-complete response and 73% having a partial response or better. The liposomal formulation of doxorubicin provides a prolonged half-life for the drug, potentially resulting in tumor exposure similar to the

sequential dosing presented here. An extended follow-up study of multiple myeloma patients treated on this phase I study confirms that a regimen of bortezomib and PegLD provides not only superior response rates but also prolongs time to progression [26]. These results support the hypothesis that bortezomib may enhance sensitivity to topo II poisons and overcome topo II poison resistance in a clinical setting. Our work provides a mechanistic basis for these clinical studies, and further demonstrates the importance of drug scheduling. Additionally, the sub-cytotoxic doses of bortezomib required for this potentiation may be beneficial in the clinical management of combination therapies with overlapping toxicities.

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