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The combined effects of proteasome inhibitor bortezomib with topoisomerase I and II inhibitors on topoisomerase enzymes

Emine ÖKSÜZOĞLU*, Çiydem TIRINOĞLU, Barış KERİMOĞLU
Molecular Biology Division, Department of Biology, Faculty of Science and Letters, Aksaray University, Aksaray, Turkey

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Background/aim: DNA topoisomerases are ubiquitous enzymes that regulate conformational changes in DNA topology during essential cellular processes, and, for this reason, have been characterized as the cellular targets of a number of anticancer drugs. Bortezomib is a powerful proteasome inhibitor used in the treatment of hematological malignancies. In this study, we investigated the inhibitory effects of bortezomib on human topoisomerase I and II enzymes both alone and in combination modes with camptothecin and etoposide.

Materials and methods: The interactions of these drugs with topoisomerase enzymes were evaluated by relaxation assay in cell-free systems. IC_{50} values of the drugs on topoisomerase enzymes were calculated using the S probit analysis program.

Results: Bortezomib showed a very weak inhibition effect on topoisomerase I (IC_{50} = 87.11 mM). On the other hand, it had a strong inhibitory effect on topoisomerase II (IC_{50} = 1.41 mM). Our results indicated that bortezomib is effective not only on proteasome but also on topoisomerase II. In addition, bortezomib possesses an increased synergistic effect when used in combination with camptothecin and etoposide than when used alone.

Conclusion: The results of this study point out that these data may build a framework for combination studies with bortezomib, camptothecin, and etoposide in the treatment of cancer.

Key words: Bortezomib, proteasome inhibitor, DNA topoisomerase inhibitors, camptothecin, etoposide, drug combination, anticancer drugs

1. Introduction

Bortezomib (PS-341) is a dipeptide boronic acid analogue with antineoplastic activity. Bortezomib reversibly inhibits the 26S proteasome, a large protease complex that degrades ubiquitinated proteins. The ubiquitin–proteasome pathway plays an essential role in regulating the intracellular concentration of specific proteins, thereby maintaining homeostasis within cells. Inhibition of the 26S proteasome prevents this targeted proteolysis, which can affect multiple signaling cascades within the cell. This disruption of normal homeostatic mechanisms can lead to cell death. By blocking the targeted proteolysis normally performed by the proteasome, bortezomib disrupts various cell signaling pathways, leading to cell cycle arrest, apoptosis, and inhibition of angiogenesis. In vivo, bortezomib delays tumor growth and enhances the cytotoxic effects of radiation and chemotherapy (1,2). Tumor cells, that is, rapidly dividing cells, appear to be more sensitive to proteasome inhibition. Bortezomib (Velcade, Millennium Pharmaceuticals, Inc., Cambridge, MA, USA) is the first proteasome inhibitor anticancer drug approved in the USA by the Food and Drug Administration (FDA) for the treatment of newly diagnosed multiple myeloma and relapsed/refractory multiple myeloma and mantle cell lymphoma (3–5).

DNA topoisomerases are ubiquitous enzymes that control and modify the topological states of DNA. They can catalyze several interconversions between topological isomers of DNA by transiently breaking single or double strands, and resealing them after reorganization of the topology (6). Depending on the nature of the reactants and reaction conditions, topoisomerases can catalyze DNA relaxation/supercoiling, catenation/decatenation, and knotting/unknotting reactions (7,8). Based on their functional mechanisms, DNA topoisomerases have been classified into two types. Type I DNA topoisomerase breaks and rejoins only one of the two strands during catalysis, while type II DNA topoisomerase acts on both strands for each DNA strand-passing reaction and it requires ATP for full activity (9,10). Since the activity of topoisomerases is essential for several cellular processes such as replication, transcription, and chromosome condensation, inhibitory

* Correspondence: emineoksuzoglu@hotmail.com

activities of eukaryotic topoisomerases are widely used in anticancer drug development (11).

Topoisomerase inhibitors could be divided into two main groups, i.e. poisons and catalytic inhibitors. Topoisomerase poisons stabilize the DNA-enzyme cleavable complex, which is normally temporary, and breaks DNA ends bounded immediately after altering of topological state and they can trap this complex to cause DNA strand breaks. If the amount of these DNA breaks is intolerable, stability of DNA could be disrupted and apoptosis might be induced in the cells. Catalytic inhibitors block one or more steps of the catalytic cycle of the topoisomerases (12). Both kinds of inhibitors are commercially available for cancer treatment.

Camptothecin (CPT) is the best known topoisomerase I inhibitor. Camptothecin is a potent anticancer drug that has shown clinical activity in human tumors such as colon and ovarian cancers and is approved by the FDA. Studies suggested that camptothecin not only stabilized the cleavable complex, but also inhibited the relegation step of the catalytic cycle of topoisomerase I (13,14).

Etoposide is an important chemotherapeutic agent that is used to treat a wide spectrum of human cancers. It has been in clinical use for more than two decades and remains one of the most highly prescribed anticancer drugs in the world. The primary cytotoxic target for etoposide is topoisomerase II. Etoposide is a potent antitumor drug that was approved by the FDA for the treatment of various tumors such as small-cell lung cancer, breast cancer, leukemia, lymphoma, and the germ-line cancers (15).

Bortezomib, the potent therapeutic proteasome inhibitor, has been suggested for standard care in patients with newly diagnosed or relapsed multiple myeloma and several lymphoma subtypes (3–5). However, intrinsic and acquired resistance to bortezomib and toxicities may limit its efficacy. The discovery and development of new treatments are urgently needed due to the problems of current treatments, such as toxicities and drug-resistance. Drug combination is the most widely used approach in treating numerous dreadful diseases, such as cancer (16).

Bortezomib is a proteasome inhibitor anticancer drug. However, the inhibitory effect of bortezomib on human topoisomerase enzymes has remained insufficiently determined. In this study, bortezomib was applied in combination with topoisomerase I inhibitor camptothecin and topoisomerase II inhibitor etoposide. These inhibitors are widely used antitumor drugs that were approved by the FDA for the treatment of various cancers. However, the combined applications of these drugs were not studied sufficiently.

For this reason, we investigated the inhibitory effects of bortezomib on human topoisomerase I and II enzymes both alone and in combination modes with camptothecin and etoposide.

2. Materials and methods

2.1. Materials

Human topoisomerase I and II relaxation assay kits were purchased from Inspiralis UK. Bortozomib was obtained from Velcade (Johnson & Johnson Pharmaceutical Research and Development, Raritan, NJ, USA), camptothecin, etoposide, reagents for electrophoresis and the other chemicals were obtained from Sigma (Munich, Germany). Solutions of the tested compounds in 50% dimethyl sulfoxide (DMSO) were freshly prepared.

2.2. Topoisomerase I and II relaxation assay

We implemented the relaxation assay to test the topo I and II inhibition effect of the bortezomib and also combine the effect of proteosome and topoisomerase inhibitors in cell-free systems (13). Relaxation activity of topoisomerase enzymes was determined by measuring the conversion of supercoiled pBR322 plasmid DNA to its relaxed form (10). If the tested compounds interrupt this process, DNA remains in its supercoiled form. At this point, supercoiled DNA band intensities could be compared with its control.

For topo I the reaction mixture contained 1 μL of 10X assay buffer [20 mM Tris HCl pH = 7.5, 200 mM NaCl, 0.25 mM EDTA, 5% glycerol] , 0.3 μg of pBR322 plasmid DNA, 3 units of topo I enzyme, and different concentrations of test compounds in a total volume 10 μL.

For topo II the reaction mixture contained 1 μL of 10X assay buffer [50 mM Tris HCl pH = 7.5, 125 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT, 100 μg/mL albumin, 1 mM ATP], 0.3 μg of pBR322 plasmid DNA, 3 units of topo II enzyme, and different concentrations of test compounds in a total volume 10 μL. Both of the reaction mixtures were incubated for 1 h at 37 °C. After the incubation period, 2 μL of 6X loading buffer was added and the mixtures were subjected to electrophoresis on 1% agarose at 45 V for 3 h. After electrophoresis the gels were stained with ethidium bromide (1 μg/mL), photographed under UV light, and band distribution was analyzed with a gel analysis system (Bio-Rad) (8,10,13,17).

2.3. Statistical analysis

Optical intensity of the newly formed bands was used as a measure of enzyme activity. Moreover, topo I and II inhibition percentage of the compounds was calculated by comparing supercoiled DNA band intensities of the control (18). Optical intensity of each concentration of the compounds was compared with the control to calculate inhibition percentages. With these percentages, IC_{50} (50% inhibition concentrations) values of the compounds were calculated using the S probit analysis program. According to the literature, a compound with 50% inhibitory effect on topoisomerases could be regarded as an inhibitor (19).

3. Results

Human topoisomerase I and II activities in cell-free systems were evaluated by the relaxation assay as described above. The relaxation assay utilizes supercoiled plasmid DNA as a substrate and has been used by many investigators to study inhibition of DNA topoisomerase I and II activities (20–23). The supercoiled substrate and its relaxed state can easily be distinguished in ethidium bromide stained gels since relaxed isomers migrate more slowly than the supercoiled isomer. The change in the molecular shape without a change in the molecular weight can be differentiated, since more compact molecules move faster compared with their more relaxed counterparts. If the DNA molecules were completely relaxed by topoisomerases and there is equilibrium between the different topological forms of the DNA molecules, then at the end of the electrophoresis several bands would be obtained. However, if the catalytic activities of topoisomerases were inhibited by the tested compounds, all the DNA molecules, therefore, were in the supercoiled form.

In this study, we primarily identified their inhibitory effects and IC_{50} values using different concentrations of topoisomerase I inhibitor camptothecin and topoisomerase II inhibitor etoposide, which are used as powerful drugs for therapeutic purposes in various cancers. Inhibition concentrations of these drugs were determined by applying a large dose range during experiment optimization. Inhibitory activities of the compounds were presented as mM concentrations that cause 50% inhibition per unit of enzyme (IC_{50}) under the assay conditions. Camptothecin and etoposide were found to be potent human DNA topoisomerase I and II inhibitors, having IC_{50} values of 0.29 mM and 3.77 mM, respectively (Table; Figures 1 and 2).

Bortezomib is a powerful proteasome inhibitor anticancer drug that is used in the treatment of hematological cancers. However, the inhibitory effect of bortezomib on human topo I and II enzymes has remained insufficiently determined so far in other studies. Therefore, it is interesting to undertake further studies to approach such effects. We investigated the inhibitory effects of bortezomib on human topoisomerase I and II enzymes applying four different concentrations (0.25, 0.5, 1, and 2 mM) in cell-free systems using the relaxation assay (Figures 3 and 4). The maximum soluble dose of bortezomib was 2 mM. Bortezomib showed a very weak inhibition effect on human topoisomerase I enzyme (IC_{50} = 87.11 mM) as seen in Figure 3. However, the IC_{50} value of bortezomib on human topoisomerase I enzyme was not in the range of tested concentrations. The IC_{50} value was calculated by plotting the percentage of inhibition versus the concentrations by using the S-probit program. On the other hand, the inhibitory effect of bortezomib on human topoisomerase II enzyme (IC_{50} = 1.41 mM) was strong, as seen in Figure 4.

In this study, we applied bortezomib in combination modes with topoisomerase I inhibitor camptothecin and topoisomerase II inhibitor etoposide. As a result, in both cases synergistic effects were observed (Figures 5 and 6).

Table. IC_{50} values of the tested compounds for human topoisomerase I and II enzymes.

Compounds	Topo I IC_{50} (mM)	Topo II IC_{50} (mM)
Camptothecin	0.29 mM	NE
Etoposide	NE	3.77 mM
Bortezomib	87.11 mM	1.41 mM

NE: Not effective

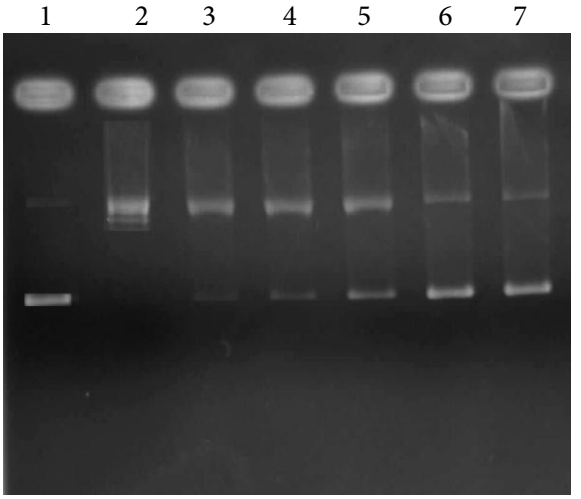

Figure 1. The inhibitory effect of camptothecin on human topoisomerase I. Lane 1: supercoiled pBR322 plasmid DNA (0.3 μg) without enzyme. Lane 2: plasmid DNA with 3 units of topo I enzyme (control). Lanes 3–7: plasmid DNA with 3 units of topo I enzyme in the presence of camptothecin at concentrations of 0.05, 0.1, 0.25, 0.5, and 1 mM, respectively. The relaxation assay in a cell-free system was performed as described in Materials and methods. ($IC_{50\ [campt.,\ topo\ I\ ]}$ = 0.29 mM).

4. Discussion

In recent years, the outcome of multiple myeloma patients has been significantly improved due to the discovery of novel antimyeloma agents together with better knowledge of the pathophysiology of the disease. Among them, the proteasome inhibitor bortezomib (Velcade) represents an excellent drug that has quickly moved from the bench to the bedside and exhibits powerful antimyeloma activity. Nowadays, bortezomib-based therapies are suggested as standards of care in patients with newly diagnosed and relapsed multiple myeloma. However, intrinsic and

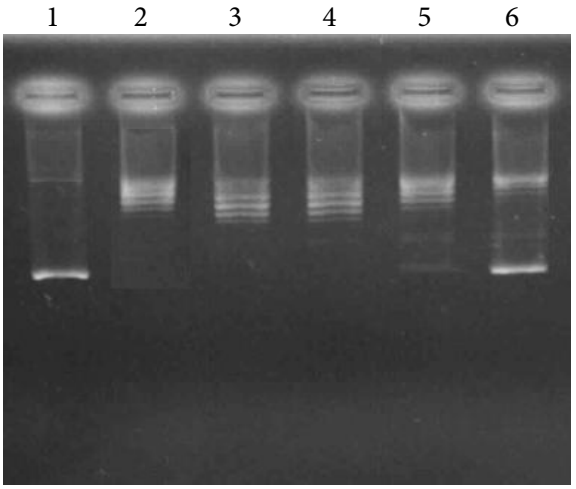

Figure 2. The inhibitory effect of etoposide on human topoisomerase II. Lane 1: supercoiled pBR322 plasmid DNA (0.3 µg) without enzyme. Lane 2: plasmid DNA with 3 units of topo II enzyme (control). Lanes 3–6: plasmid DNA with 3 units of topo II enzyme in the presence of etoposide at concentrations of 0.75, 1.5, 3, and 6 mM, respectively. The relaxation assay in a cell-free system was performed as described in Materials and methods ($IC_{50\ [etop,\ topo\ II\ ]}$ = 3.77 mM).

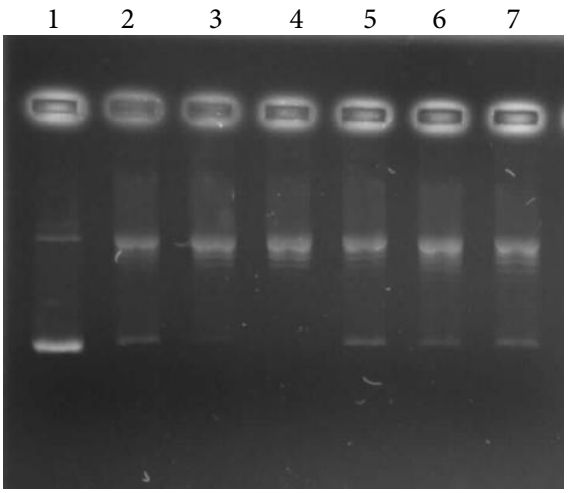

Figure 3. The inhibitory effect of bortezomib on human topoisomerase I. Lane 1: supercoiled pBR322 plasmid DNA (0.3 µg) without enzyme. Lanes 2 and 3: plasmid DNA with 3 units of topo I enzyme (control). Lanes 4–7: plasmid DNA with 3 units of topo I enzyme in the presence of bortezomib at concentrations of 0.25, 0.5, 1, and 2 mM, respectively. The relaxation assay in a cell-free system was performed as described in Materials and methods ($IC_{50\ [bort.,\ topo\ I]}$ = 87.11 mM).

acquired resistance to bortezomib and toxicities may limit its efficacy (24). The discovery and development of new treatments are urgently needed due to the problems of current treatment, such as toxicities and drug-resistance.

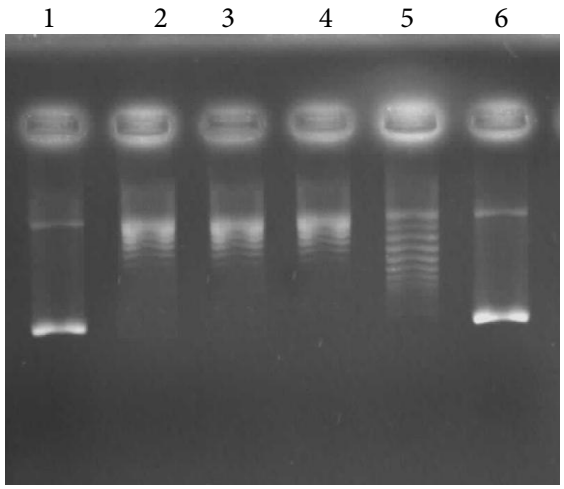

Figure 4. The inhibitory effect of bortezomib on human topoisomerase II. Lane 1: supercoiled pBR322 plasmid DNA (0.3 µg) without enzyme. Lane 2: plasmid DNA with 3 units of topo II enzyme (control). Lanes 3–6: plasmid DNA with 3 units of topo II enzyme in the presence of bortezomib at concentrations of 0.25, 0.5, 1, and 2 mM, respectively. The relaxation assay in a cell-free system was performed as described in Materials and methods ($IC_{50\ [bort.,\ topo\ II\ ]}$ = 1.41 mM).

Drug combination is the most widely used approach in treating numerous dreadful diseases, such as cancer. The main aims are to achieve synergistic therapeutic effects and dose and toxicity reductions, and to minimize or delay the induction of drug resistance (16). Most cancers have multiple genetic alterations and molecular abnormalities. It is seldom very useful to use only one anticancer drug owing to refractory and drug resistance of cancer tissues. Using anticancer drugs in combinations instead of singly might dramatically promote the control of cancer progresses and metastasis in different patients (25).

Combination therapy has been the standard of care, especially in cancer treatment, since it is a rationale strategy to increase response and tolerability and to decrease resistance. Currently, there is growing interest in combining anticancer drugs aiming at maximizing efficacy while minimizing toxicity through the delivery of lower drug doses (26,27).

There are a limited set of principles that underlie drug combinations in cancer treatment. Briefly, the drugs used in combination should possess the following features: to use drugs with nonoverlapping toxicities so that each drug can be administered at near-maximal dose; combine agents with different mechanisms of action and minimal cross-resistance in order to inhibit the emergence of broad spectrum drug resistance; preferentially use drugs with proven activity as single drugs and administer the combination at early stage disease and at a schedule with

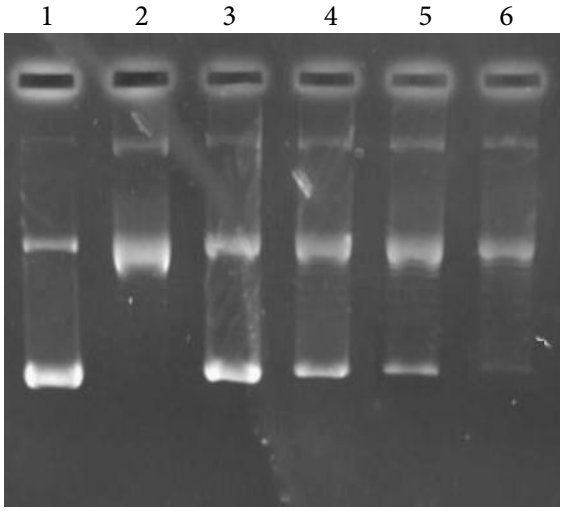

Figure 5. The combined effects of bortezomib and camptothecin on human topoisomerase I. Lane 1: supercoiled pBR322 plasmid DNA (0.3 μg) without enzyme. Lane 2: plasmid DNA with 3 units of topo I enzyme (control). Lanes 3–6: plasmid DNA with 3 units of topo I enzyme in the presence of bortezomib and camptothecin at concentrations of 2 mM bort. + 1 mM camp., 1 mM bort. + 0.5 mM camp., 0.5 mM bort. + 0.1 mM camp., and 0.25 mM bort. + 0.05 mM camp., respectively. The relaxation assay in a cell-free system was performed as described in Materials and methods.

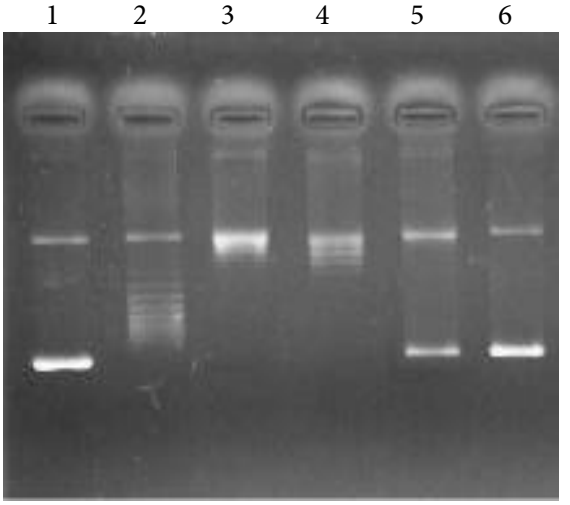

Figure 6. The combined effects of bortezomib and etoposide on human topoisomerase II. Lane 1: supercoiled pBR322 plasmid DNA (0.3 μg) without enzyme. Lane 2: plasmid DNA with 3 units of topo II enzyme (control). Lanes 3–6: plasmid DNA with 3 units of topo II enzyme in the presence of bortezomib + etoposide at concentrations of 0.25 mM bort. + 0.75 mM etop., 0.5 mM bort. + 1.5 mM etop, 1 mM bort. + 3 mM etop., and 2 mM bort. + 6 mM etop., respectively. The relaxation assay in a cell-free system was performed as described in Materials and methods.

a minimal treatment-free period between cycles but still allowing the recovery of sensitive target tissues (26,27).

Nowadays, drugs combination studies for cancer therapy have been tried by many researchers (24,28–30). Combinations of topo I inhibitor camptothecin and topo II inhibitor doxorubicin have been found to synergistically inhibit cancer cell growth in vitro (28). Etoposide and cisplatin is the most used combination chemotherapy regimen in extensive-stage disease small-cell lung cancer and usually achieves a high initial response rate (29).

Combination studies of bortezomib with various anticancer drugs, including dexamethasone, DNA-damaging drugs (such as melphalan, cyclophosphamide, and doxorubicin), thalidomide, and lenalidomide, are currently ongoing in patients with both relapsed/refractory and newly diagnosed multiple myeloma (24). The triplet combination of bortezomib and thalidomide plus dexamethasone regimen was one of the highly effective and well tolerated induction therapies for multiple myeloma patients (30).

Over the past decade, new insights into the biology of multiple myeloma have provided the framework for the development of novel therapies to overcome drug resistance. Remarkable activity seen with bortezomib as a single agent in advanced refractory or relapsed multiple myeloma has validated proteasome as an effective target in the treatment of cancer and has led to approval for bortezomib use in patients for whom prior therapies have failed. Based on preclinical and clinical data demonstrating that bortezomib adds to the anticancer activity of other chemotherapeutic drugs, studies of combination therapies have been initiated more recently in patients with both refractory/relapsed and newly diagnosed multiple myeloma in an attempt to expand the therapeutic armamentarium for this still-devastating malignancy. Studies similar to these will provide important information concerning the best combination of drugs and, it is hoped, will soon reveal the optimal treatment strategies to improve the prognosis of patients with cancer (24).

In the present study, bortezomib was applied in combination with topoisomerase I inhibitor camptothecin and topoisomerase II inhibitor etoposide. These inhibitors are widely used antitumor drugs that were approved by the FDA for the treatment of various cancers. However, the combined applications of these drugs were not studied sufficiently. For this reason, we investigated their combined implementations and, as a result, in both cases synergistic effects were observed. When applied alone 0.5 mM bortezomib and 0.1 mM camptothecin were ineffective on topoisomerase I (Figures 1 and 3) while they were effective when used in combination at these concentrations (as seen from lane 5 of Figure 5). Likewise, 1 mM bortezomib

and 3 mM etoposide were found to be ineffective when applied alone on topoisomerase II enzyme (Figures 2 and 4) while they were effective when used in combination at these concentrations (as seen from lane 5 of Figure 6). Our results suggest that bortezomib possesses an increased effect at lower doses when used in combination with topoisomerase I and II inhibitor drugs than when used alone. After all, the observation of synergistic effects of the drugs with maximizing efficacy while minimizing toxicity through the delivery of lower drug doses indicates that combinations of these drugs can be tried in future in vivo applications.

Taken together, our data demonstrated that bortezomib is effective not only on proteasome but also on topoisomerase II. In addition, the combined use of proteasome inhibitor bortezomib with topo I inhibitor camptothecin or topo II inhibitor etoposide exhibited a synergistic effect on the inhibition of human topoisomerase enzymes. Combinations of these drugs are more effective than any single drug. The results of this study indicate that these data may build a framework for combination studies with bortezomib, camptothecin, and etoposide in the treatment of cancer.

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