Proteasome Inhibitor Bortezomib Increases Radiation Sensitivity in Androgen Independent Human Prostate Cancer Cells

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OBJECTIVES To investigate the effects of a strong proteasome inhibitor, bortezomib alone or in combination with radiotherapy on androgen-independent DU145 human prostate cancer cells. Proteasomes play important roles in cell cycle, proliferation, apoptosis, angiogenesis, and cellular resistance to chemotherapy and radiotherapy.

METHODS Increasing concentrations of bortezomib alone or in combination with radiation were applied to DU145 cells and IC_{50} values that inhibited cell growth by 50% were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium-bromide assay. Apoptosis was determined using annexin V staining by flow cytometry. mRNA levels of proapoptotic caspase-3 and antiapoptotic Bcl-2 genes were examined by reverse transcriptase polymerase chain reaction.

RESULTS The IC_{50} value of bortezomib was found to be 28 μM although 400- and 800-cGy radiation decreased the cell proliferation by 14% and 28%, respectively. In 400- and 800-cGy radiation applied DU145 cells, IC_{50} value of bortezomib decreased to 23- and 12 μM, respectively. Exposure to 5 μM bortezomib for 48 hours caused apoptosis in 35% of the population whereas 800-cGy radiation resulted apoptosis in 14% of cells. However, 42% of DU145 cells that were exposed to 800 cGy and 5 μM bortezomib underwent apoptosis. Reverse transcriptase polymerase chain reaction results showed a significant decrease in mRNA levels of antiapoptotic *Bcl-2* gene and an increase in proapoptotic caspase-3 gene expression in the combination group compared to control group.

CONCLUSIONS Bortezomib increases radiation sensitivity in androgen-independent human DU145 prostate cancer cells through inhibition of Bcl-2 and induction of caspase-3 genes. UROLOGY 75: 793–798, 2010. © 2010 Elsevier Inc.

Prostate adenocarcinoma is the most common noncutaneous malignancy diagnosed in males and the second leading cause of cancer death in North America.[1] Although clinically localized, prostate cancer can be treated effectively with surgery or radiation therapy, approximately 15% of patients present with locally advanced or metastatic disease, and recurrent disease develops after definitive local therapy in 40% of patients.[2-4]

Androgen ablation remains the main initial treatment of advanced prostate cancer and provides palliation of symptoms and survival benefit.[5] However, androgen-independent cells are eventually selected during androgen deprivation therapy, and progression to an androgen-independent state remains the primary cause of mortality in these patients within an average of 1.5 years.[5] Data from randomized trials of chemotherapy suggest an improvement in overall survival, pain relief, and quality of life with this form of therapy for hormone refractory prostate cancer.[6] Moreover, it is concluded, based on these data, that docetaxel should be considered for first-line treatment of metastatic hormone-refractory prostate cancer.[6] However, patients with hormone-refractory prostate cancer have not traditionally been offered chemotherapy as a routine treatment because of treatment-related toxicity and poor responses. Thus, there is a strong push to develop new compounds that interact with novel biological targets, either for use as single agents or more commonly in combination with front-line chemo- and radiotherapy or to modulate the response of established treatment regimens (i.e., restoring sensitivity to chemotherapy or radiotherapy).[7]

The proteasomes play a central role in regulation of cell cycle, proliferation, apoptosis, angiogenesis, metasta-

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sis, and resistance to chemotherapy and radiotherapy. Bortezomib, a potent inhibitor of the 26S proteasome, has shown significant activity against both androgen-dependent and androgen-independent prostate cancer in preclinical models.[8-12] A recently completed phase I/II clinical trial of bortezomib in patients with advanced prostate cancer showed its potential for this disease.[13] However, to date, there have been few reports on the combined use of bortezomib with either radiotherapy or chemotherapy to enhance the effects of these established treatment options. Therefore, the current study was designed to test the hypothesis that the combination of bortezomib with radiotherapy increases sensitivity in androgen-independent prostate cancer cell lines.

MATERIAL AND METHODS

Cell Lines and Culture Conditions

DU145 human prostate cancer cells (generously provided by Dr. Guray Saydam, Department of Hematology, Ege University School of Medicine) were maintained in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) growth medium supplemented with 10% fetal bovine serum (Life Technologies) and 1% penicillin–streptomycin (Invitrogen Corp., Carlsbad, CA) incubated at 37°C in a mixture of 5% CO_2 and 95% air. The cultures were free of Mycoplasma, reovirus type 3, pneumonia virus, K virus, Theiler's encephalitis virus, Sendai virus, minute virus, mouse adenovirus, mouse hepatitis virus, lymphocytic choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus (assayed by Science Applications International Corp., Frederick, MD).

Measurement of Cell Growth by 3-(4,5-Dimethylthiazol-2-yl)-2,5 Diphenyltetrazolium-Bromide (MTT) Assay

The IC50 values of bortezomib, radiation, and their combination that inhibited cell growth by 50% were determined from cell survival plots obtained by the MTT assay. Briefly, the first group of cells (2×10^4 cells/well) were plated into 96-well plates containing 100 μL of the growth medium and were treated with increasing concentrations of bortezomib (1-, 5-, 10-, 20-, and 50 μM) and incubated for 48 hours. The second group of cells were exposed to 50-, 200-, 400-, 600-, and 800-cGy gamma irradiation and incubated for 24 hours. The third group of cells were exposed to 400- and 800-cGy radiation, incubated for 24 hours, and were treated with increasing concentrations of bortezomib for 48 hours. They were then treated with 50 μL of MTT (5 mg/mL) for 4 hours and supernatant was removed. After that, the cells were incubated with 50 μL of 1 N isopropil alcohol for 4 hours, and the plates were read in a microplate reader at 570 nm. Then, as described previously, the IC_{50} values of all 3 were determined from cell survival plots.[14] To determine the interaction between bortezomib and radiation, isobologram plots were constructed using IC_{50} values obtained from MTT assays. The experiments were performed as triplicate in at least 3 independent experiments. Statistical significance was determined using two-way analysis of variance, and $P < .01$ was considered statistically significant.

Isolation of Total RNA and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted using RNeasy RNA isolation kit (Qiagen, Santa Clarita, CA) as described by the manufacturer. One microgram of total RNA was reverse transcribed using reverse transcriptase enzyme (Fermentas, Inc., Ontario, Canada). After 1-hour incubation at 42°C, the reactions were stopped by 70°C heating for 5 minutes. The resulting total cDNA was then used in PCR to measure the mRNA levels of Caspase-3, Bcl-2, and Beta actin genes. The mRNA levels of Beta actin were used as internal positive control. The primer sequences and PCR conditions were as follows: Bcl-2-forward (5′-GGTGAACTGGGGGAGGATTGT-3′), Bcl-2-reverse (5′-CTTCAGAGACAGCCAGGAGAA-3′); Caspase-3-forward (5′-CAAACTTTTTCAGAGGGGATCG-3′), Caspase-3-reverse (5′-GCATACTGTTTCAGCATGGCAC-3′), Beta actin-forward (5′-CAGAGCAAGAGAGGCATCCT-3′), and Beta actin-reverse (5′-TTGAAGGTCTCAAACATGAT-3′). Using these primers, 1 μL of the reverse transcriptase reaction was amplified for 35 cycles (94°C, 1 minute; 55°C, 2.5 minutes; 72°C, 2 minutes) with Taq DNA polymerase (Qiagen, Santa Clarita, CA), and their levels were normalized to that of Beta actin as described previously.[14]

Analysis of Cell Cycle Profiles in Bortezomib and Radiation Applied DU145 Cells

Apoptosis induced by 5 μM bortezomib alone, 800-cGy radiation or their combination on DU145 cells was examined by flow cytometry using AnexinV-FITC as described previously.[15]

RESULTS

Bortezomib Increased Radiation-Induced Cell Death in Du145 Cells

To determine the cytotoxic effect of bortezomib, radiation, and their combination, MTT assay was conducted. Radiation alone did not result in a significant decrease in cellular proliferation. As shown in Fig. 1, 400- and 800-cGy radiation decreased the cell proliferation by 14% and 28%, respectively (Fig. 1A). By contrast, the IC_{50} value of bortezomib was calculated from cell proliferation plots and was found to be 28 μM (Fig. 1B). In 400- and 800-cGy radiation applied DU145 cells, IC_{50} values of bortezomib were decreased to 23- and 12 μM, respectively. These results revealed that 800-cGy radiation increased sensitivity of DU145 cells to bortezomib about 2.5 times more compared to only bortezomib-applied cells. In 1 μM bortezomib-applied DU145 cells, there was 100% cell proliferation. However, application of 1 μM bortezomib with a combination of 400- and 800 cGy resulted in about 22% and 23% decrease in cell proliferation (Fig. 1B).

Synergistic Effect of Bortezomib and Radiation on DU145 Cells

Apoptosis is characterized by different morphologic properties one of which is the translocation of the membrane phospholipid phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane. Once PS moves from internal part of the membrane to extracel-

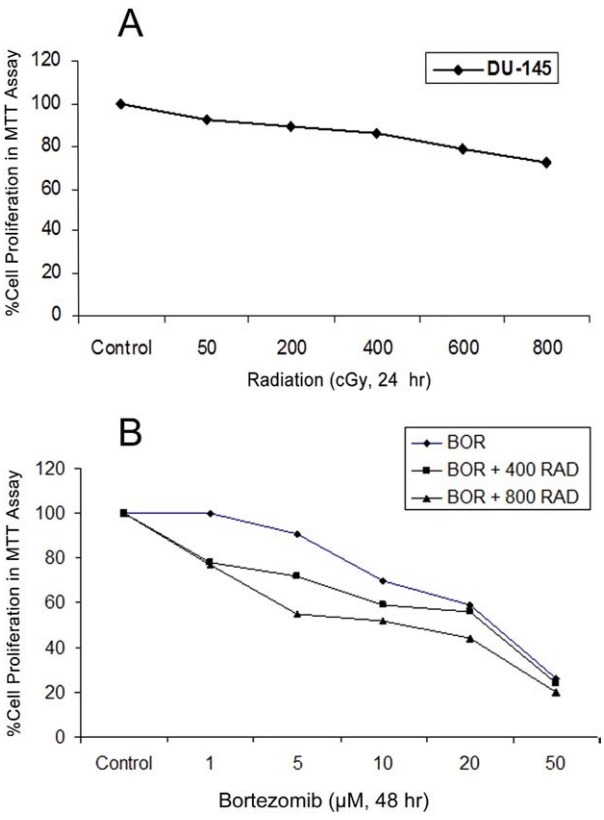

Figure 1. (A) Effects of radiation on the growth of DU145 cells, in situ. The MTT assays were performed using triplicate samples in at least 3 independent experiments. **(B)** Effects of bortezomib and radiation on the growth of DU145 cells, in situ. The IC_{50} concentrations of bortezomib and bortezomib with 400- and 800-cGy radiation were determined by MTT assay for DU145 cell line, as described in materials and methods.

lular environment, PS becomes available for annexin V binding. Cells that are positive for annexin V and negative for FITC are in early apoptosis as PS movement has occurred, but cell membrane is still intact whereas the cells that are positive for both annexin V and FITC dye are in late stages of apoptosis because of PS movement to the outer site of membrane and loss of cell membrane integrity.

Apoptotic death of DU145 cells treated with bortezomib alone or in combination with radiation were examined by AnexinV-FITC flow cytometry. The data revealed that exposure to 5 μM bortezomib for 48 hours caused apoptosis in 35% of the population whereas 800-cGy radiation resulted apoptosis in 14% of the cells (Fig. 2). However, in 800 cGy and 5 μM bortezomib applied DU145 cells, 42% of the cells underwent apoptosis. The combination index of isobologram analyses for the effects of bortezomib and radiation on DU145 cells showed strong synergism (combination index < 1) (Fig. 3). These data demonstrate that bortezomib increased the radiosensitivity of human DU145 prostate cancer cells to radiation.

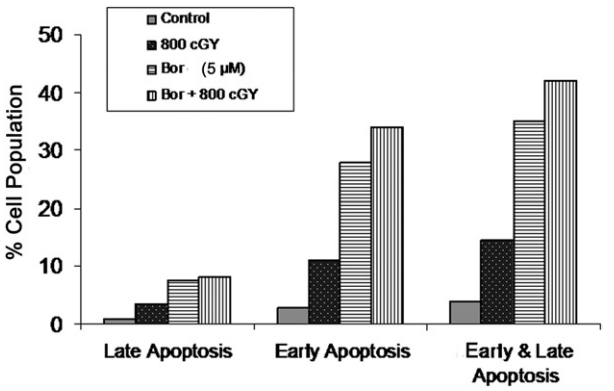

Figure 2. Effects of bortezomib and radiation on apoptosis of DU145 cells. Early, late, and cumulative percentages of apoptosis of DU145 cells treated with 5 μM bortezomib, 800 cGy radiation, and their combination were determined by flow cytometry.

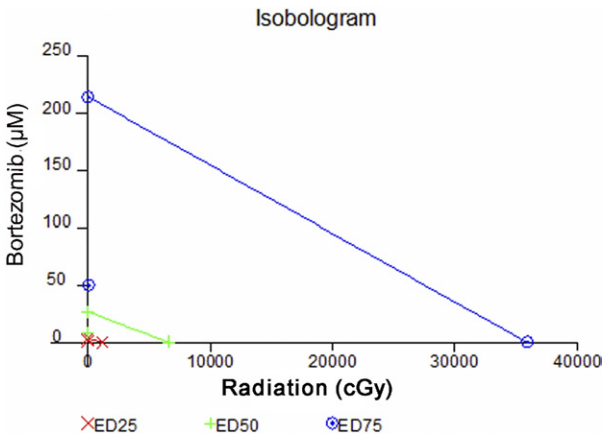

Figure 3. Isobologram analysis of bortezomib and radiation interaction in DU145 cells. The effective 25%, 50%, and 75% inhibition doses (*25TH ED., ED_{50}, and 75TH ED.*, respectively) were plotted. Points below the line indicate the synergistic effect.

Expression Analyses of Antiapoptotic Bcl-2 and Apoptotic Caspase-3 Genes in Bortezomib and Radiation Applied DU145 Cells

RT-PCR results showed that there were parallel decreases in mRNA levels of β-actin gene, used as internal positive control, in 5 μM bortezomib or radiation or combination of both applied cells compared to untreated controls. By contrast, it is known that expression levels of β-actin gene never changes in response to internal or external factors.[16] However, since there were significant decreases in cell number in bortezomib or radiation alone or combination of both applied samples, the mRNA levels of β-actin gene in these samples were also decreased. As we normalize PCR products of antiapoptotic Bcl-2 and caspase-3 genes with β-actin levels in these samples, we have determined significant decreases in Bcl-2 and increase in caspase-3 genes in cells treated with bortezomib or 800-cGy radiation, and combination of these, compared to the control group (Fig. 4).

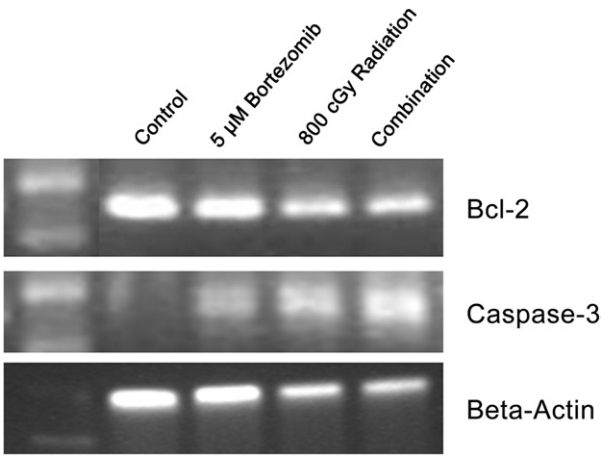

Figure 4. mRNA levels of antiapoptotic Bcl-2, proapoptotic Caspase-3 and Beta actin genes in control, 5 μM bortezomib, 800 cGy radiation, and the combination of 5 μM bortezomib and 800-cGy radiation applied DU145 cells, determined by RT-PCR. Beta actin mRNA levels were used as internal positive control. The first lane is the DNA ladder.

COMMENT

Proteosome inhibitors are being evaluated in clinical and preclinical trials in different malignancies. In preclinical studies, it has been reported that bortezomib (previously known as PS-341), which is a strong proteasome inhibitor, induces apoptosis in many solid tumors including prostate cancer cell lines.[8-11,17,18] Therefore, inhibition of proteasome activity could be a new strategy for the treatment of prostate cancer. In our study, we investigated the effects of bortezomib alone or in combination with radiation on androgen-independent DU145 human prostate cancer cells.

The proteasome is a large multiprotein complex present in all cells that degrades ubiquitinated proteins.[19] This is a highly regulated process that controls the expression of numerous cellular targets such as cyclins, cyclin-dependent kinase inhibitors (p21 and p27), pro- and antiapoptotic factors such as Bax and Bcl-2, tumor suppressors such as p53, and inhibitor of nuclear factor-κB (NF-κB).[20] Bortezomib is a potent and selective inhibitor of the chymotryptic-like activity of the proteasome.[17] Inhibition of proteolysis through disregulation of proteasomal function results in induction of apoptosis in tumor cells and cells overexpressing the antiapoptotic Bcl-2 gene.[18,21] In vitro and in vivo studies with bortezomib in androgen-dependent (LNCaP) and androgen-independent (DU145, PC3) prostate cancer cell lines resulted in growth arrest and apoptosis.[8-11]

Studies have shown that bortezomib induces apoptosis of multiple myeloma and prostate cancer cells via inhibition of NF-κB.[8,9,22] NF-κB is known to be a survival pathway that is overexpressed in androgen-independent prostate cancer cells.[9,11,23] Growth inhibition of androgen-independent prostate cancer cells mediated by bortezomib occurs via inhibition of NF-κB. This results in the inability of NF-κB to translocate to the nucleus and bind to the promoters of multiple genes including proinflammatory proteins (IL-6, tumor necrosis factor-α), and antiapoptotic proteins (Bcl-2 family).[20,24] Thus, blockage of NF-κB activity in prostate cancer cells is associated with suppression of angiogenesis, invasion, and metastatic spread via downregulation of various antiapoptotic genes including Bcl-2 and Bcl-xL.[23-26] In our study, combined exposure of DU145 cells to bortezomib and radiation was associated with a decrease in antiapoptotic protein bcl-2 and an increase in proapoptotic caspase-3 gene. Our results suggest that disruption of the NF-κB cascade with bortezomib results in sensitization of prostate cancer cells to the lethal actions of radiation.

Proteasome inhibition may also overcome resistance mechanisms in response to chemotherapy and radiotherapy. Thus, bortezomib can enhance the antitumor activity of these therapeutic modalities by either restoring sensitivity or by showing additive or synergistic effect which in turn results in reduced dosage and toxicity. Bortezomib, in combination with tumor necrosis factor-α (TNF-α) or TNF-related-apoptosis-inducing ligand shows synergistic effect to induce apoptosis in human prostate and bladder cancer cells.[8,9] Enhanced radiation sensitivity with proteasome inhibition via downregulation of NF-κB was first reported by Russo et al[27] in colorectal cancer cells. Pervan et al[28] demonstrated increased radiosensitivity by proteasome inhibition in the TRAMP-C1 prostate cancer tumor model. Mice with TRAMP-C1 tumors received either bortezomib or placebo before radiation treatment. Animals receiving bortezomib treatment showed enhanced radiosensitivity and delayed tumor growth compared to the controls. In our study, we also showed that combination of bortezomib with radiation improved the growth inhibitory effect of radiation against DU145 cells. The data showed that the combination of radiation with increasing concentrations of bortezomib for 48 hours decreased growth synergistically, as detected by the shift of the IC_{50} values in the isobologram to the left of the line plot joining the x and y axes that represent the IC_{50} of bortezomib and radiation, respectively. Considered together, these data suggest that bortezomib sensitizes cells to the effects of radiation and increases tumor cell apoptosis.

Bcl-2 overexpression in human prostate cancer cells is known to be related to increased resistance to radiotherapy and chemotherapy, and this is associated with increased rates of treatment failure in prostate cancer patients treated with radiation therapy.[29] For that reason, downregulation of Bcl-2 activity is essential to enhance sensitivity to irradiation. Cao et al[12] reported that combination of docetaxel and bortezomib can effectively sensitize *Bcl-2*-overexpressing human prostate cancer cells (PC-3-*Bcl-2*) to the effects of radiation by modulating the expression of members of the *Bcl-2* family. In the current study, the effect of radiation induced apoptosis by

bortezomib was reflected by a significant decrease in mRNA levels of antiapoptotic Bcl-2 gene and an increase in proapoptotic caspase-3 gene.

These preclinical studies provided a rationale to further study the effects of bortezomib in combination with other cytotoxic agents. In a phase I/II dose escalation study, the antitumoral activity of bortezomib in combination with docetaxel was evaluated to determine the dose limiting toxicities and maximum tolerated dose (MTD) in patients with androgen-independent prostat cancer.[13] They have reported no dose limiting toxicities at any dose level, despite dose escalation to 1.6 mg/m^2 of bortezomib and 40 mg/m^2 of docetaxel. Prostate- specific antigen levels declined by $\geq$ 50% in 28% of the patients, and partial response was achieved in 11% of the patients whereas 67% had stable disease. These findings were consistent with other clinical studies evaluating bortezomib alone or in combination with docetaxel.[30,31] However, to our knowledge no clinical studies have been designed to determine the synergistic effects of bortezomib and external beam radiation.

CONCLUSIONS

In summary, preclinical in vitro and in vivo studies showed that bortezomib treatment resulted in increased survival and tumor apoptosis with decreased tumor growth, angiogenesis, and metastatic spread. It has also been shown to enhance the antitumor properties of antineoplastic treatment modalities in phase I and II clinical trials. These findings provide a rationale to investigate the effects of bortezomib in combination with chemotherapy, radiation therapy, or novel agents in patients with solid tumors. Our results also suggest that proteasomes can be a novel target for the treatment of androgen-independent prostate cancer. Inhibition of proteasome activity in combination with radiation results in radiosensitization of tumor cells. Thus, combination treatment can be a promising strategy for the treatment of prostate cancer patients. Further clinical studies are required to understand the effects of bortezomib in combination with radiation.

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