Bortezomib inhibits docetaxel-induced apoptosis via a p21-dependent mechanism in human prostate cancer cells

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Abstract

Bortezomib (PS-341, Velcade) is a peptide boronate inhibitor of the 20S proteasome that is currently being combined with taxanes in several clinical trials in patients with prostate cancer. Here, we report that bortezomib inhibited docetaxel-induced M-phase arrest and apoptosis in androgen-dependent LNCaP-Pro5 cells. Direct analysis of kinase activity in immune complex kinase assays revealed that docetaxel activated cyclin-dependent kinase (CDK) 1 (CDC2) and that bortezomib blocked this activation. The effects of bortezomib were associated with accumulation of p21 and mimicked by chemical CDK inhibitors or by transfecting cells with a small interfering RNA construct specific for CDK1. Transient transfection with p21 also inhibited docetaxel-induced apoptosis; conversely, p21 silencing reversed the antagonistic effects of bortezomib on docetaxel-induced apoptosis. Together, our data show that bortezomib interferes with docetaxel-induced apoptosis via a p21-dependent mechanism that is associated with CDK1 inhibition. These observations may have important implications for the ongoing bortezomib-docetaxel combination trials as well as trials using bortezomib and other cell cycle–sensitive agents. [Mol Cancer Ther 2006;5(8):2043–50]

Introduction

Prostate cancer is the most common cancer in men, with an estimated incidence of >230,000 new cases, and the second most lethal cancer in men, with ~30,000 new deaths (1). Advanced disease has a 5-year survival of only 30% (2). Historically, chemotherapy has been used with palliative intent but unclear survival benefit for these advanced-stage patients. Current practices for hormone-refractory, metastatic prostate cancer incorporate the use of taxanes. Docetaxel, in particular, is being incorporated in numerous current clinical trials either as a single or combination agent against androgen-independent prostate cancer and it is also being investigated for its use as a neoadjuvant or adjuvant agent in hormone sensitive, locally aggressive prostate cancer (3, 4). Combination regimens involving docetaxel and well-established agents, such as estramustine and prednisone, are being evaluated, but it seems that these strategies are not yielding a qualitative increase in anti-tumor activity. Thus, the development of novel combination approaches involving docetaxel and biological targeting agents is being pursued aggressively.

The activity of the 26S proteasome is required for cell cycle progression and cell survival, making it an attractive therapeutic target in cancer. Bortezomib (also known as PS-341 or Velcade) is a dipeptidyl boronic acid inhibitor of the chymotryptic activity of the proteasome that is currently the only proteasome inhibitor in clinical use (5). In preclinical studies, the drug displayed promising activity in the NCI-60 cancer cell line panel, with a mean IC50 of 7 nmol/L and a unique cytotoxic profile, compared with historical results of 60,000 other compounds (6). The drug has especially impressive activity in multiple myeloma (7) and recently received Food and Drug Administration approval for the treatment of this disease (7, 8). Bortezomib also exhibited activity in preclinical models of human solid tumors, especially when combined with standard chemotherapeutic agents, including docetaxel (9, 10). Bortezomib has been used in 60 clinical trials, most currently ongoing. It is being combined with docetaxel in five of these trials, including one for prostate cancer. Knowledge about the interactions of these two agents has current relevance for clinical application.

Here, we characterized the effects of bortezomib on docetaxel-induced apoptosis in androgen-sensitive LNCaP-Pro5 cells. The results show that bortezomib prevents docetaxel-induced accumulation of cells in M phase and subsequent apoptosis via a p21-dependent mechanism. Our findings represent a cautionary note for the development of this and other combinations of bortezomib with cell cycle–sensitive therapeutic approaches.

Materials and Methods

Animals, Cell Lines, and Antibodies

Male nude mice (BALB/c) were purchased from the Animal Production Area of the National Cancer Institute.
Frederick Cancer Research and Development Center (Frederick, MD). LNCaP-Pro5 human prostate cancer cells were derived from orthotopic recycling of the parental line (11). Briefly, the parental cell line (LNCaP, American Type Culture Collection, Rockville, MD) was orthotopically injected into the prostates of nude mice, and the resultant prostate tumors were harvested, mechanically dissociated, and regrown in culture. These cells were then reimplanted into the prostates of nude mice, and the recycling process was continued for a total of five cycles to produce the more locally aggressive variant LNCaP-Pro5. Cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum along with sodium pyruvate, nonessential amino acids, l-glutamine, vitamins, and antibiotics under conditions of 5% CO2 in air at 37°C. The HA-tagged p21 construct was obtained from Dr. Nora Navone (M.D. Anderson Cancer Center, Houston, TX). Antibodies were obtained from the following commercial sources: anti-p21 (BD PharMingen/Transduction Laboratories, San Diego, CA), FITC-conjugated anti-mouse (BD PharMingen), horse-radish peroxidase–conjugated sheep anti-mouse Ig and donkey anti-rabbit Ig (Amersham, Arlington Heights, IL), and anti-actin (Sigma Chemical Corp., St. Louis, MO).

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assays
Cells were plated in 96-well plates (5,000 per well) and allowed to adhere for 16 hours. The cells were then incubated with drugs for 48 hours, and 40 μL of a solution of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical) in PBS were added to each well. Cells were incubated for 1 hour at 37°C, and the medium was removed. MTT precipitates were then dissolved in DMSO (40 μL/well) for 20 minutes before quantification of optical densities (570 nm).

Propidium Iodide Staining and Fluorescence-Activated Cell Sorting Analysis
Cells were stained with propidium iodide (PI) to monitor cell cycle progression and to measure DNA fragmentation associated with apoptosis as described previously (12, 13). Cells were plated in six-well plates (1 × 10^4 per well). Following incubation with drugs for 24 hours, attached and floating cells were harvested, pelleted by centrifugation, and resuspended in PBS containing 0.1% Triton X-100, and 0.1% sodium citrate. Cells were incubated with the PI solution for at least 1 hour at 4°C before analyzing them by flow cytometry. Cells with a subdiploid DNA content were scored as apoptotic. In parallel studies, we fixed cells in ethanol before staining them with PI and obtained indistinguishable results.

Transfections and Immunoblotting
Cells (1 × 10^5) were transiently transfected with control vector, HA-tagged p21 (in pc-DNA3.1), nonspecific small interfering RNA (siRNA), or p21 siRNA (Cip1 siRNA/siAb Assay kit, Upstate, Charlottesville, VA) for 48 hours. Gene silencing was done according to the manufacturer’s instructions. Cells were then incubated with 100 nmol/L bortezomib, 120 nmol/L docetaxel (100 ng/mL), or the combination for an additional 24 hours. Floating cells were collected, plates were transferred to an ice slurry, and adherent cells were lysed directly on the plates by addition of 1% Triton X-100 buffer for 10 minutes. Adherent cells were then scraped and transferred to Eppendorf tubes containing pellets derived from the floating cells, where lysis continued for 1 hour at 4°C. Lysates were centrifuged for 15 minutes at 12,000 × g, and protein concentrations in the postnuclear supernatants were determined using the Bradford reagent (Bio-Rad, Hercules, CA). Approximately 20 μg from each sample were subjected to 12% SDS-PAGE, proteins were transferred to nitrocellulose membranes, and the membranes were blocked with 5% nonfat milk in a TBS solution containing 0.1% Tween 20 for 2 hours at 4°C. Blots were then probed overnight with the relevant antibodies, washed, and probed with an horseradish peroxidase–conjugated species-specific secondary antibodies coupled to horseradish peroxidase (Amersham). Immunoreactive material was detected by chemiluminescence (Amersham).

Two-Color Quantification of p21 Expression and Apoptosis
Cells were plated in six-well plates (1 × 10^4 per well). Following incubation with 100 nmol/L bortezomib, 120 nmol/L (100 ng/mL) docetaxel, or a combination of the two drugs, attached and floating cells were harvested, pelleted by centrifugation, washed in PBS, and resuspended in 1% paraformaldehyde for 10 minutes at room temperature. Cells were then washed in PBS, centrifuged, and resuspended in intracellular staining buffer containing 0.1% Triton X-100 and the anti-p21 monoclonal antibody (1:100 dilution) for 1 hour at 4°C. Cells were then washed in intracellular staining buffer, centrifuged, resuspended in intracellular staining buffer containing the FITC-conjugated rabbit anti-mouse Ig secondary antibody (1:50 dilution), and incubated for 30 minutes at 4°C under light protection. Stained cells were analyzed by two-color flow cytometry (FL1 and FL2 channels).

Results
Effects of Bortezomib and Docetaxel on Cell Proliferation and Apoptosis
We compared the concentration-dependent effects of bortezomib, docetaxel, or both agents on the proliferation of LNCaP-Pro5 cells using MTT assays. Consistent with previous work (14), bortezomib inhibited cell proliferation at low nanomolar concentrations (IC₅₀ 3 nmol/L; Fig. 1A). Docetaxel also inhibited cell proliferation in a concentration-dependent fashion (IC₅₀ 2 nmol/L; Fig. 1B). However, when cells were incubated with 2 nmol/L docetaxel in the presence of increasing concentrations of bortezomib (1.5–25 nmol/L), no significant change in growth suppression was seen compared with that observed with docetaxel or bortezomib alone (Fig. 1A). Likewise, when cells were incubated with 3 nmol/L bortezomib in the presence of increasing concentrations of docetaxel, the level of growth suppression was similar to that observed in response to incubation with bortezomib or docetaxel alone (Fig. 1B). Therefore, no additive or synergistic growth inhibition was observed.
observed in cells exposed to docetaxel plus bortezomib versus either drug alone.

We then measured the effects of the bortezomib, docetaxel, or bortezomib plus docetaxel on cell cycle progression and DNA fragmentation characteristic of apoptosis by PI staining and fluorescence-activated cell sorting (FACS) analysis. Cells incubated with either docetaxel (12 nmol/L) or bortezomib (10 nmol/L) displayed significant DNA fragmentation 48 hours after drug exposure (68% and 45%, respectively; Fig. 1C). However, when cells were incubated with docetaxel in the presence of increasing concentrations of bortezomib (1–10 nmol/L), a significant reduction in docetaxel-induced apoptosis occurred as the concentration of bortezomib increased (Fig. 1C). Likewise, when cells were incubated with bortezomib (10 nmol/L) in the presence of increasing concentrations of docetaxel (1.2–12 nmol/L), the levels of DNA fragmentation decreased compared with the levels observed in cells incubated with docetaxel alone (Fig. 1D). In both cases, the maximal levels of DNA fragmentation decreased by at least 50% in a manner that seemed to be limited by the extent of bortezomib-induced cell death (Fig. 1C and D).

**Role of Cyclin-Dependent Kinase 1**

Docetaxel-induced apoptosis occurs during the M phase of the cell cycle (15, 16). While we were conducting our PI/FACS analyses of DNA fragmentation, we noted that bortezomib completely blocked the accumulation of cells in M phase that was observed in LNCaP-Pro5 cells incubated with docetaxel alone (Fig. 2A, arrows). In previous work, we showed that bortezomib blocks DNA synthesis (17), and other work showed that it also arrests cell cycle progression in G2 (6), and both effects would be expected to prevent entry into M phase. To study the molecular mechanisms of the observed cell cycle blockade in more detail, we assessed the effects of bortezomib and docetaxel on cyclin-dependent kinase (CDK) 1, the CDK required for entry into mitosis. Docetaxel induced strong activation of CDK1 in the LNCaP-Pro5 cells, and bortezomib completely blocked docetaxel-induced CDK1 activation (Fig. 2B), consistent with our previous observations in human pancreatic cancer cells (13). Similarly, chemical inhibitors of CDKs [olomoucine (Fig. 2A) and roscovitine (data not shown)] also blocked docetaxel-induced M-phase arrest.
and inhibited docetaxel-induced apoptosis (Fig. 2C). Finally, siRNA-mediated knockdown of CDK1 (Fig. 2D, bottom) also blocked apoptosis (Fig. 2D, top), directly showing the obligate role of CDK1 in docetaxel-induced cell death. Together with the previous study (13), these results establish that bortezomib is a potent inhibitor of CDK1 activation, which seems to explain its antagonistic effects on docetaxel-induced M-phase accumulation and cell death.

**Role of p21**

In previous studies, we and others showed that bortezomib inhibits S phase and promotes the accumulation of p21 (13, 14, 17, 18). It therefore seemed reasonable that p21 might contribute to the bortezomib-induced inhibition of cell cycle progression and apoptosis in docetaxel-treated cells. In a first series of experiments, we exposed LNCaP-Pro5 cells to bortezomib, docetaxel, or a combination of both agents for 24 hours and measured p21 expression. Levels of p21 were markedly elevated in cells exposed to bortezomib or bortezomib plus docetaxel as measured by immunoblotting (Fig. 3A) and intracellular staining and FACS analysis (Fig. 3B). By FACS, 70% of cells treated with bortezomib or bortezomib plus docetaxel for 24 hours expressed elevated p21 levels (Fig. 3B). At the 24-hour time point, the bortezomib-mediated antagonism of docetaxel-induced apoptosis was more obvious than it was at 48 hours (Fig. 3A).

To further explore the role of p21 in the effects of bortezomib, we used two-color FACS to distinguish the p21-positive and p21-negative subsets of LNCaP-Pro5 cells so that we could measure levels of apoptosis within each subset (Fig. 4A). The control cells and those exposed to docetaxel displayed identical levels of DNA fragmentation in the ungated population and the p21-negative subset because the percentage of p21-positive cells within the bulk population was too low to affect the determination by FACS analysis.

**Figure 2.** Effects of bortezomib and chemical CDK inhibitors on docetaxel (D)–induced M-phase arrest and apoptosis. A, effects of bortezomib or olomoucine on docetaxel-induced M-phase arrest. Cells were incubated for 24 h with 12 nmol/L docetaxel in the absence or presence of 100 nmol/L bortezomib (PS-341) or 50 μmol/L olomoucine, and cell cycle progression was measured by PI staining and FACS analysis. Results are representative of three independent replicates. B, effects of bortezomib on docetaxel-induced activation of CDK1. Cells were incubated with 12 nmol/L docetaxel, 100 nmol/L bortezomib, or both agents (D + BZ) for 24 h, and CDK1 activity was measured in immune complex kinase assays using histone H1 as a substrate. The CDK1 content of the immunoprecipitates was also measured in parallel by immunoblotting (‘‘CDK1 input’’; bottom). Note that docetaxel increased CDK1-mediated histone H1 phosphorylation without affecting total CDK1 levels, whereas the inhibition of CDK1 activity observed in cells incubated with bortezomib is associated with decreased CDK1 expression. C, effects of chemical CDK inhibitors on docetaxel-induced DNA fragmentation. Cells were incubated with 12 nmol/L docetaxel in the absence or presence of 25 μmol/L roscovitine of 50 μmol/L olomoucine for 48 h, and DNA fragmentation was measured by PI staining and FACS analysis as described in Materials and Methods. Columns, mean (n = 3); bars, SD. *p < 0.05, significantly different from docetaxel alone. D, effects of CDK1 knockdown on apoptosis. Cells were transiently transfected with a siRNA construct specific for CDK1 or an off-target control (against firefly luciferase) for 48 h. Cells were then incubated with 12 nmol/L docetaxel, 100 nmol/L bortezomib, or both agents for 24 h, and DNA fragmentation was quantified by PI/FACS. Columns, mean (n = 3); bars, SD. Note that equivalent levels of DNA fragmentation were observed in cells transfected with the control siRNA construct under all conditions, which contrasts with what we observed in untransfected LNCaP-Pro5 cells; we attribute this to the fact that nonspecific silencing sensitizes the LNCaP-Pro5 cells to bortezomib (45). Nonetheless, exposure to bortezomib plus docetaxel did not increase DNA fragmentation compared with exposure to either agent alone.
population was low in the absence of bortezomib (Fig. 4B).
However, in cells treated with either bortezomib alone or bortezomib plus docetaxel, very little apoptosis was detected in the p21-positive subset, whereas levels of apoptosis were high in the 30% of cells that did not express p21 (Fig. 4B). Therefore, expression of p21 correlated directly with resistance to apoptosis in cells treated with either bortezomib alone or bortezomib plus docetaxel.

To more directly evaluate the role of p21 in the process, we transiently transfected LNCaP-Pro5 cells with an HA-tagged form of p21 or empty vector and compared the effects of docetaxel in the transfectants. Parallel control experiments with a green fluorescent protein expression plasmid showed that transfection efficiency was approximately 30% to 40% (data not shown). Cells transfected with p21 displayed significantly lower levels of apoptosis than were observed in cells transfected with empty vector (Fig. 5). Immunoblotting studies confirmed that the transfected cells expressed endogenous and tagged p21 (Fig. 5, inset).

Requirement for p21 in Bortezomib-Mediated Inhibition of Docetaxel-Induced Apoptosis

The results presented above strongly suggested that p21 was involved in the inhibition of apoptosis observed in LNCaP-Pro5 cells treated with docetaxel plus bortezomib.
To directly test this possibility, we examined the effects of silencing p21 expression on apoptosis in cells treated with bortezomib plus docetaxel. In control cells, significant inhibition of docetaxel-induced apoptosis was seen when 100 nmol/L bortezomib was combined with 120 nmol/L docetaxel after 24 h (Fig. 6). However, in p21-silenced cells, the antagonism was reversed and the levels of apoptosis observed in cells incubated with bortezomib plus docetaxel were equivalent to those observed in cells exposed to docetaxel alone (Fig. 6). Immunoblotting confirmed that the siRNA construct eliminated p21 expression under all conditions (Fig. 6, bottom). These results corroborate the other findings and show that bortezomib-induced accumulation of p21 is at least partly responsible for the antagonistic effects bortezomib on docetaxel-induced apoptosis.

**Discussion**

Given the challenge of advanced prostate cancer therapy, novel agents with promising activity are quickly incorporated into trials that often include conventional agents as well. Bortezomib, a potent inhibitor of the 26S proteasome, has shown significant activity against both androgen-sensitive and androgen-independent prostate cancer in preclinical models (6, 14, 19). A recently completed phase I clinical trial of bortezomib in patients with advanced prostate cancer showed its potential for this disease (20). Paclitaxel has a well-established role in advanced prostate cancer (21–23), and docetaxel is currently under investigation as an equivalent or superior substitute (21, 24–26). Results of phase II clinical trials of docetaxel-based combination regimens have shown clear palliative responses, decreases in PSA levels by at least 50% in at least 60% of patients, and suggestions of improved survival (24). Docetaxel also holds promise in the early stages of prostate cancer progression and is currently under investigation for its role as a neoadjuvant or adjuvant agent in aggressive, androgen-sensitive prostate cancer (3, 4, 27–29). Furthermore, docetaxel-based regimens are actively being developed to incorporate novel investigational agents, such as a current clinical trial with bortezomib in advanced prostate cancer. Docetaxel affects many cellular pathways and is therefore thought to have excellent potential for synergy with novel anticancer agents (30). Promising targets have included the epidermal growth factor receptor (31), HER-2 (32–35), the farnesyl transferase pathway (36), and Bcl-2 (37, 38). Bortezomib is under current investigation in five clinical trials with combination docetaxel but is as yet of unproven benefit.

The results of this study suggest that the activity of bortezomib as a cell cycle inhibitor can produce antagonistic effects on apoptosis when it is combined with taxanes. The effects of bortezomib are at least partially mediated by accumulation of the CDK inhibitor, p21, and our experiments with chemical CDK inhibitors (roscovitine and olomoucine) or CDK1 silencing strongly suggest that CDK1 inhibition is also involved. Previous studies have concluded that taxanes initiate apoptosis in cells that are initially arrested at M phase (15) and that taxane-induced apoptosis in other models also requires CDK1 activation (13, 16). Our observations in the LNCaP-Pro5 cells are entirely consistent with these previous results.
Although the drug has clear antagonistic effects on docetaxel-induced apoptosis in this model and others (13), other studies have reported that bortezomib sensitizes certain other cell types to taxane-induced apoptosis, either via inhibition of the antiapoptotic transcription factor, nuclear factor-κB (39), or via stabilization of the proapoptotic BH3-only member of the BCL-2 family, Bim (40). Thus, it seems that the mutually antagonistic effects of bortezomib and docetaxel that we have observed in the human prostate and pancreatic cancer cells we have studied are not universal. Importantly, we have observed very similar antagonistic effects in human DU-145 prostate cancer cells exposed to bortezomib plus docetaxel, and in human PC-3 cells, exposure to the combination provides no additive benefit compared with exposure to either single agent alone. Furthermore, we have knocked down Bim expression in the LNCaP-Pro5 and PC-3 cells and find no impairment of either bortezomib- or docetaxel-induced apoptosis, indicating that Bim is not required for cell death induced by these agents in human prostate cancer cells. Thus, the rules governing the interactions between bortezomib and docetaxel seem to vary in a cell type–dependent fashion, and it will be necessary to better define the biological mechanisms that produce one or the other phenotype if this combination is to be maximally effective in patients.

Silencing p21 produced a general increase in docetaxel- and bortezomib-mediated cell death (Fig. 6), whereas silencing CDK1 inhibited both pathways (Fig. 2D). Thus, it does not seem that p21 is required for bortezomib-induced apoptosis, and in fact, strategies to inhibit p21 expression in tumors will probably enhance the activity of the drug (41, 42). Our data are consistent with other studies that have concluded that cell cycle promoters (Myc and CDKs) tend to globally sensitize cells to apoptosis (43), whereas cell cycle inhibitors (including p21) tend to inhibit cell death (41, 44). Precisely why this is the case is unclear but is under active investigation by many laboratories. However, we showed recently that stabilization of p21 partially underlies the ability of bortezomib to synergize with tumor necrosis factor–related apoptosis–inducing ligand (TRAIL) to induce apoptosis in LNCaP-Pro5 cells (45). Thus, it may be possible to exploit the effects of drug on p21 and cell cycle progression by combining it with agents such as TRAIL, which preferentially induce apoptosis under these conditions.

A better understanding of the molecular mechanisms underlying bortezomib-induced apoptosis could also facilitate the design of more effective combination regimens. Candidates include inhibition of nuclear factor-κB (39, 46), p53 activation (18), production of reactive oxygen species (47–49), and the accumulation of misfolded and/or damaged proteins resulting in a phenomenon termed “endoplasmic reticulum stress” (50–54). With respect to the latter, our group and another found that, like tumor necrosis factor–related apoptosis–inducing ligand, histone deacetylase inhibitors synergize with bortezomib to promote cell death by enhancing the effects of bortezomib on endoplasmic reticulum stress (51, 52).

It should be emphasized that tumor cell apoptosis is only one of the mechanisms underlying drug-mediated tumor growth inhibition in vivo. For example, in our own previous work, we showed that treatment of human L3.6pl pancreatic cancer cells with bortezomib plus docetaxel resulted lower levels of clonogenic survival (measured at 14 days) compared with those observed in cells treated with either bortezomib or docetaxel alone (13). Treatment with the combination also resulted in stronger inhibition of tumor growth than did therapy with either single agent in orthotopic L3.6pl tumor xenografts, effects that were linked to strong inhibition of tumor cell proliferation and angiogenesis (13). Thus, the fact that bortezomib and taxanes inhibit cell cycle progression at different points and inhibit angiogenesis via different molecular mechanisms probably explains why combination therapy is more potent when the assays of drug activity are highly sensitive to changes in tumor cell proliferation. However, if the goal of therapy is to regress established bulky disease, then caution should be exercised before proceeding with this or other combinations of bortezomib and cell cycle–sensitive agents. Although inhibition of proliferation and/or angiogenesis is expected to produce disease stabilization, it does not seem that these effects are generally sufficient to produce tumor regression. Conversely, levels of therapy-induced apoptosis seem to predict response in tumors treated with cytotoxic agents (55). Apparently, antagonism of docetaxel-induced apoptosis of bortezomib can be overcome (e.g., by silencing p21 expression). Alternatively, it may be better to optimize scheduling or identify combination regimens that better exploit the complex effects of bortezomib on the cell cycle.

References
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