ABSTRACT

Advanced prostate cancer is resistant to current therapeutic strategies. Bortezomib (Velcade; previously called PS-341) is a potent and specific inhibitor of the 26S proteasome that is currently in clinical trials for treatment of various malignancies, including prostate cancer. We investigated the effects of bortezomib on p53 in the LNCaP-Pro5 prostate cancer cell line. Bortezomib induced strong stabilization of p53, but it did not promote phosphorylation on serines 15 and 20, and p53 remained bound to its inhibitor, mdm2. Nonetheless, bortezomib stimulated p53 translocation to the nucleus (not mitochondria) and enhanced p53 DNA binding, accumulation of p53-dependent transcripts, and activation of a p53-responsive reporter gene. Furthermore, stable LNCaP-Pro5 transfectants of LNCaP-Pro5 expressing the p53 inhibitor human papillomavirus-E6 displayed reduced bortezomib-induced p53 activation and cell death. Together, our data demonstrate that bortezomib stimulates p53 activation via a novel mechanism.

INTRODUCTION

Prostate cancer is the most prevalent tumor detected in men, with rates of incidence and mortality of about 170,000 and 40,000, respectively, in the United States (1). All conventional therapies eventually fail if the disease has progressed beyond the confines of the original organ (2). Thus, there is currently an aggressive search for new therapeutic strategies to combat advanced disease.

The 26S proteasome is responsible for ubiquitin-mediated protein degradation in eukaryotes, and it has recently gained attention as a therapeutic target in cancer (3). Bortezomib (Velcade; formerly called PS-341) is the first and currently only proteasome inhibitor to undergo clinical trials for several forms of cancer (4). The therapeutic potential of the drug is thought to lie in its ability to cause the stabilization of proteins that inhibit cell survival and cell cycle progression (5).

The tumor suppressor p53 controls genetic damage and signals responses to cellular stress (6). In quiescence, p53 has a very short half-life (7) that is mediated by the mdm2 gene product. Mdm2 binds to p53, exports it from the nucleus (8), and, by way of its ubiquitin ligase function (9), targets the tumor suppressor for degradation by the 26S proteasome. Cellular stress (most notably DNA damage) can lead to a series of posttranslational modifications of p53 that result in its activation (6). These changes, including a series of phosphorylation events, cause p53 to stabilize by disassociating from mdm2, tetramerize, localize to the nucleus, and function primarily as a regulator of transcription (10–12). The consequences of p53 activation include cell cycle arrest at the G1 and G2 checkpoints, activation of the DNA repair machinery, and, if repair is unsuccessful, the initiation of apoptosis (13). The loss of p53 has been implicated in advanced prostate cancer, but p53 remains wild type in a large subset of cases (14). p53 stabilization correlates with proteasome inhibitor-induced apoptosis, but previous studies differ with respect to whether or not p53 mediates the apoptotic and cell cycle-regulatory effects of proteasome inhibitors when they are used alone or in combination with other agents (15–23).

Given this controversy, we sought to better characterize the structure and function of proteasome inhibitor-stabilized p53 in LNCaP-Pro5 cells exposed to bortezomib. We designed the experiments to test several biochemical parameters normally associated with p53 activation, including serine phosphorylation, association with mdm2, and nuclear localization. We also performed direct analyses of the DNA binding and transcriptional transactivation functions of p53 in cells exposed to the drug. Together, our data demonstrate that bortezomib promotes p53 activation via a novel mechanism. The results challenge previous conclusions concerning the roles of p53 phosphorylation and dissociation from mdm2 in its transcriptional transactivation function.

MATERIALS AND METHODS

Cell Culture and Reagents. We used the LNCaP-derived cell line LNCaP-Pro5 (developed and kindly supplied by Dr. Curtis Pettaway), which has a faster growth rate (conducive to efficient experimentation) but retains many of the qualities of the parental LNCaP cell line (24, 25). Cells were maintained in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% FCS (Life Technologies, Inc.) and 1% each of MEM vitamin solution (Life Technologies, Inc.), sodium pyruvate (BioWhittaker), t-glutamine (BioWhittaker), penicillin/streptomycin solution (BioWhittaker), and nonessential amino acids (Life Technologies, Inc.) under an atmosphere of 5% CO2 in an incubator. Bortezomib was generously supplied by Millennium Pharmaceuticals, and etoposide (Bristol-Meyers Squibb) was obtained from the M. D. Anderson pharmacy.

Immunoblotting. Cells in 6-well plates were lysed by incubation for 1 h at 4˚C in 100 μl of Triton X-100, 150 mM NaCl, 25 mM Tris (pH 7.5), 1 mM glycerol phosphate, 1 mM sodium orthovanadate, 1 mM sodium fluoride, and 1 Complete Protease Inhibitor Cocktail tablet (Roche). Lysates were transferred to microcentrifuge tubes and centrifuged at 4˚C for 10 min at 12,000 × g. Supernatants were transferred to new tubes, and 40-μg samples were mixed with equal volumes of SDS-PAGE sample buffer (50 mM Tris-HCl, 2% SDS, 0.1% bromophenol blue, 10% glycerol, and 5% β-mercaptoethanol). Samples were boiled, centrifuged briefly, and resolved by 10% SDS-PAGE (100 V for 90 min). Polypeptides were transferred to nitrocellulose membranes for 90 min at 100 V in a buffer containing 39 mM glycine, 48 mM Tris base, and 20% methanol. Membranes were blocked in TBS-T and 5% skim milk for 2 h. Blots were washed briefly in TBS-T before incubation with primary antibodies specific for p53 [CM-1 polyclonal antibody (Novacastra), 1:5000 dilution] or mdm2 [SMP-14 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), 1:1000 dilution] diluted in TBS-T containing 1% milk. Blots were gently shaken overnight at 4°C and washed in TBS-T (2 × 5 min) before incubation with appropriate secondary antibodies [horse-radish peroxidase-conjugated sheep antimouse or donkey antirabbit antibody (Amersham), 1:1000 dilution] for 2 h at 4°C. Blots were washed in TBS-T (4 × 5 min) and developed by enhanced chemiluminescence (Renaissance; New England Nuclear, Boston, MA).

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The abbreviations used are: HPV, human papillomavirus; TBS-T, Tris-buffered saline containing 0.1% Tween 20, CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; RPA, RNase protection assay; FACS, fluorescence-activated cell-sorting.
**Fluorescent Immunocytochemistry.** Cells grown on chamber slides were exposed to bortezomib or etoposide for 4 h before incubation for 1 h in 100 nm Mitotracker Red (Molecular Probes). Cells were then fixed in PBS containing 2% paraformaldehyde for 15 min and washed with PBS three times. Blocking and membrane permeabilization were achieved with the use of 0.1% saponin in serum (5% normal horse serum and 1% normal goat serum) and PBS for 20 min. Slides were incubated overnight at 4°C with anti-p53 antibody (diluted 1:1000 in blocking solution). Slides were washed twice in PBS before incubation for 2 h with FITC-conjugated secondary antibody (diluted 1:500 in blocking solution) in the dark. Slides were washed twice in PBS, counterstained with 10 μM of nuclear dye To-Pro-3 in PBS for 10 min, and washed an additional three times in PBS (all in the dark). Slides were treated with Prolong antifade mounting agent (Molecular Probes). Images were captured with an Axioplan microscope, a Hamamatsu color chilled 3 charge-coupled device (3CCD) camera, and OptiVIEW software (Bioscan).

**Immunoprecipitation.** Cells were lysed in 500 μl of the Triton lysis buffer described above. Lysates were diluted to a concentration of 400 μg of protein in a total volume of 500 μl and were preclarified by rotation with 50 μl of protein A/G-agarose beads (Santa Cruz Biotechnology) for 1 h at 4°C. Beads were collected by centrifugation, and the supernatants were transferred to new tubes containing agarose-conjugated antibodies to either p53 or mdm2 (from Santa Cruz Biotechnology). Lysates were rotated overnight at 4°C, and protein-antibody-bead complexes were washed three times in 500 μl of lysis buffer. Polyethylene glycol was resolved by 10% SDS-PAGE and analyzed by immunoblotting as described above.

**CAT Reporter Gene Assay.** Cells were plated on 10-cm dishes at 40% confluence and incubated in normal serum for 48 h. Plasmids (10 μg) containing the CAT gene driven by two copies of the consensus p53-responsive element (5′-GAAACATGTCTAAGCATGCTG-3′) were mixed with 30 μl of TransFast (Promega, Madison, WI) and 3 ml of serum-free medium, and the mixture was allowed to stand for 10 min. The mixture was then added to cells and left for 1 h before addition of 4 ml of normal complete medium. Cells were incubated overnight at 37°C in a humidified incubator before exposure to 1 μM bortezomib or 20 μM etoposide for 8 h. Cells were lysed in 100 μl of CAT Lysis Buffer (Promega) for 10 min at room temperature. Lysate protein concentrations were normalized to yield maximum protein levels in a total volume of 50 μl of Reporter Lysis Buffer. Samples were then mixed with 10 μl of a [14C]chloramphenicol preparation and incubated at 37°C for 4 h. One ml of ethyl acetate was added to the reaction, and samples were vortexed vigorously and centrifuged. A total volume of 900 μl of the upper layer was transferred to another tube and dried under vacuum (DNA Speed Vac; Savant). Cold ethyl acetate (12 μl) was added to each tube before 10 μl were added dropwise to a silica gel sheet (J. T. Baker). The sheet was placed in a chamber containing the CAT gene driven by two copies of the consensus p53-responsive element (5′-GAAACATGTCTAAGCATGCTG-3′) (Santa Cruz Biotechnology) and DNA binding buffer (Promega) for 30 min at 10°C. Complexes were stabilized by incubation with 1 μl of anti-p53 antibody (C-11; Cell Signaling) and DNA for 30 min. Complexes were then loaded onto 5% acrylamide gels, resolved by electrophoresis (100 V for 4 h), and dried. Protein-DNA complexes were detected by autoradiography.

**RPAs.** Cells were split to 10-cm dishes at 1 × 106 cells/plate and left for 48 h. Cells were then treated with 1 μM bortezomib or 20 μM etoposide for 12 h before harvest by trypsinization, and total RNA was isolated with RNeasy kits (Qiagen). Twenty μg of RNA were subjected to analysis with an [α-32P]UTP-labeled, custom-designed RPA template set (BD Biosciences) and the Riboquant multiprobe RPA system (BD Biosciences). Hybridized and RNase-treated samples were loaded onto 5% acrylamide gels and run at 50 W for about 1.5 h. Gels were dried, and protected probe-RNA complexes were detected by autoradiography.

**Propidium Iodide and FACS Analysis of Apoptosis.** Cells were split to 6-well plates and left to recover for 48 h. Cells were then treated with 1 μM bortezomib or 20 μM etoposide for 48 h before harvest by trypsinization. Growth and wash media were saved along with trypsinized cells, and samples were centrifuged at 1500 × g for 5 min. Supernatants were removed, and pellets were resuspended in 400 μl of a propidium iodide solution (50 μg/ml propidium iodide, 0.1% Triton X-100, and 0.1% sodium citrate in PBS). Samples were stored at 4°C for 4 h before analysis by flow cytometry. The cells with subdiploid DNA content were quantified to determine the percent-ages of cells containing fragmented DNA.

**RESULTS**

**Proteasome Inhibitor Bortezomib Stabilizes p53.** To determine the effect of bortezomib on p53 accumulation, we treated LNCaP-Pro5 cells with bortezomib or etoposide and measured p53 accumulation by immunoblotting. Probesome inhibition resulted in concentration- and time-dependent stabilization of p53 (Fig. 1), consistent with previous observations in other systems (19, 26). When the...
LNCap-Pro5 cells were treated with bortezomib for 4 h, concentrations as low as 10 nM stabilized p53 to levels above those observed in untreated cells (Fig. 1A). To measure the kinetics of p53 stabilization, the LNCap-Pro5 cells were treated with 1/10000 M bortezomib or 20/10000 M etoposide (VP-16), and lysates were collected at various time points for immunoblot analysis. In both cases, stabilization above control levels was apparent by 1 h and maximal by 4 h (Fig. 1B).

**p53 Is Not Phosphorylated on Serines 15 or 20 When Stabilized by Bortezomib Treatment.** Phosphorylation of p53 on serines 15 and 20 is associated with the release of p53 from mdm2 and transcriptional activation. To assess the effects of bortezomib on p53 phosphorylation, we prepared whole cell lysates from cells treated with 1/10000 M bortezomib or 20/10000 M etoposide for 4 h and measured the phosphorylation states of serines 15 and 20 by immunoblotting with phospho-specific antibodies (Fig. 2). Although bortezomib and etoposide induced comparable p53 stabilization (Fig. 1B), etoposide also stimulated strong phosphorylation of serines 15 and 20, whereas bortezomib did not (Fig. 2).

**Cellular Distribution of Bortezomib-stabilized p53.** Because p53 is a transcription factor, it must be localized to the nucleus to interact with its DNA target. To investigate whether bortezomib stimulated nuclear p53 accumulation, we treated LNCaP-Pro5 cells for 4 h with 1 μM bortezomib or 20 μM etoposide before staining the cells with an anti-p53 antibody and analyzing subcellular p53 localization by immunofluorescence confocal microscopy. As shown in Fig. 3, bortezomib increased total cellular p53 levels above the levels observed in untreated cells (green staining). Proteasome inhibitor-stabilized p53 was predominately nuclear and displayed a distribution pattern comparable with that observed with etoposide (third column, overlays). Recent work also suggests that p53 can translocate to mitochondria in cells undergoing apoptosis (27–32). However, neither bortezomib nor etoposide promoted mitochondrial translocation of p53, as assessed by counterstaining cells with the mitochondrial probe Mitotracker Red (fourth column, overlays).

**Mdm2 Remains Associated with Proteasome Inhibitor-Stabilized p53.** The lack of phosphorylation on sites associated with interactions with mdm2 prompted us to investigate the interaction between p53 and mdm2 in cells exposed to bortezomib. Fig. 4 shows the results of a coimmunoprecipitation study on LNCap-Pro5 cells that were treated with 1 μM bortezomib or 20 μM etoposide for 4 h. Whole cell lysates were subjected to immunoprecipitation with agarose-bound antibodies to either p53 (A) or mdm2 (B), and Western blots were performed for the reciprocal protein. In both cases, we found that mdm2 remained bound to p53 after bortezomib treatment (Lanes B), whereas p53-mdm2 complexes were much less prominent in cells exposed to etoposide (Lanes VP).

**DNA Binding Activity of Proteasome Inhibitor-Stabilized p53.** Active p53 displays increased in vitro DNA binding activity in EMSAs. To test the effect of bortezomib on p53’s DNA binding...
activity, we prepared nuclear extracts from LNCaP-Pro5 cells treated for 4 h with either bortezomib (1 μM) or etoposide (20 μM) and incubated them with a 32P-labeled oligonucleotide containing the consensus p53 response element found in the GADD45 promoter. An antibody (clone 421) specific to p53 was used to stabilize any protein-DNA complexes that were formed as reported previously (33, 34). As shown in Fig. 5, EMSA analyses confirmed that bortezomib stimulated increases in DNA binding activity (Fig. 5, Bort.) that were comparable with those observed in cells exposed to etoposide (VP16).

RPA of p53 Target Genes. To further characterize the effects of bortezomib on p53 function, we measured the effects of bortezomib on p53 target mRNA accumulation by multiprobe RPA analysis. For these studies, we generated stable transfectants of LNCaP-Pro5 expressing the p53 inhibitor HPV-E6 (35) to confirm the p53 dependency of any changes detected. LNCaP-Pro5 cells, LNCaP-Pro5-E6 transfectants, and the p53-null human PC-3 prostate cancer line were treated for 8 h with bortezomib (1 μM) or etoposide (20 μM). We then prepared total RNA and hybridized it with a custom probe set containing probes for the p53 target genes Fas (36), GADD45 (37), DR5 (38), p21 (39), Bax (40), and Mdm2 (41), with L32 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the loading controls. Fig. 6 shows that when the band intensities were averaged, bortezomib and etoposide induced comparable accumulation of p53 target transcripts in the parental LNCaP-Pro5 cells and vector controls, and these levels were significantly higher than those observed in PC-3 cells or in any of the three independent HPV-E6-expressing clones.

Proteasome Inhibitor-Driven Activation of a p53-Responsive CAT Construct. To obtain more direct information about the effects of bortezomib on p53 activity, we measured p53-dependent promoter activation in LNCaP-Pro5 cells transfected with a CAT construct containing two copies of the p53 consensus-binding site in a minimal promoter backbone (Fig. 7). Although previous studies used luciferase-based reporters to investigate the transcriptional activation modulated by proteasome inhibition (42), proteasome inhibition interferes with luciferase posttranscriptionally (43), an artifact that can be avoided by using a CAT-based system. Cells were treated for 8 h with bortezomib (1 μM) or etoposide (20 μM) before analysis of promoter activity. The results confirmed that bortezomib increased reporter activity to levels beyond those observed in either the untreated or the etoposide-treated controls (Fig. 7). This result directly demonstrates that proteasome inhibition stabilizes a transcriptionally active form of p53.

HPV-E6 Reduces Sensitivity to Bortezomib-Induced Apoptosis. In a final series of experiments, we treated LNCaP-Pro5 cells and the E6 clones with 1 μM bortezomib for 48 h and quantified apoptosis-associated DNA fragmentation by propidium iodide staining and...
FACS analysis (Fig. 8). The E6 clones displayed 40–50% lower levels of proteasome inhibitor-induced death compared with the levels observed in either the parental LNCaP-Pro5 cells or vector controls. These results strongly suggest that p53 activation contributes directly to bortezomib-induced cell death.

DISCUSSION

The loss of functional p53 is considered a late event in the progression of prostate cancer that occurs in approximately 30–50% of tumors (44, 45). This characteristic of the disease might be exploited in patients whose tumors retain wild-type p53 through the use of DNA-damaging agents such as the anthracyclines, etoposide, or ionizing radiation, although it is currently thought that conventional chemotherapy is not very effective in advanced prostatic adenocarcinoma (46, 47). Proteasome inhibition has demonstrated antitumor effects on various cancer cell lines in vitro and in vivo (48–52). These observations led to the development of bortezomib, which is currently being evaluated in Phase I and II clinical trials in patients with hematological and solid tumors, including prostate cancer. The drug has been well tolerated and has shown particular promise in treating multiple myeloma (53). Furthermore, at target doses leading to >70% proteasome inhibition in surrogate cells (lymphocytes), bortezomib decreased serum prostate-specific antigen and interleukin 6 levels in patients with androgen-independent prostate cancer.4 Currently, there are plans to combine bortezomib with conventional therapy. Several signaling pathways have been implicated in the antitumoral effects of the drug including inhibition of nuclear factor κB (54, 55), steroid receptors (56–58), and receptor tyrosine kinase phosphorylation cascades (59–62), although our understanding of how proteasome inhibition preferentially causes apoptosis in malignant cells is incomplete.

Many groups have demonstrated that p53 stabilization correlates with proteasome inhibitor-induced apoptosis (18, 19). There is dis-

C. Papandreou and C. Logothetis, personal communication.
agreement, however, as to whether or not the increased levels of p53 activate target genes and mediate cell death. Of the groups implicating p53 activation, the supporting evidence has ranged from correlation (19, 26, 59), through comparisons between nonisogenic cell lines (63), to the implied removal of the functional protein by viral inhibitors (15, 17) or the expression of a dominant negative p53 mutant construct (18). Evidence countering this proposition has been of a similar mold (16, 22, 64–68). All previous reports have been limited in the scope of their structural and functional analyses. Here we show that bortezomib stimulates increased p53 DNA binding, target gene mRNA accumulation, and transcriptional activity. The results of experiments with HPV-E6 strongly suggest that p53 mediates the transcriptional activation and cell death observed in this system.

The phosphorylation of p53 on several sites is considered among the necessary posttranslational modifications for initiating transcriptional modulation in response to stress, particularly DNA damage. Serines 15 and 20 are phosphorylated by DNA damage-responsive kinases such as ATM and DNA-activated protein kinase, and phosphorylation is necessary for transactivation after the release of mdm2 from p53 (6, 69, 70). Mdm2 inhibits p53 in several ways. On binding to the inactive tumor suppressor, mdm2 exports p53 from the nucleus (71). Degradation of p53 by the 26S proteasome is mediated by the E3 ubiquitin ligase function of mdm2 (9, 72). We show here that bortezomib-stabilized p53 localizes primarily to the nucleus in LNCaP-Pro5 cells. However, the proteasome inhibitor failed to stimulate p53 phosphorylation on serines 15 and 20, and the stabilized p53 remained bound to mdm2. Very few precedents for phosphorylation-independent p53 activation exist in the published literature (73, 74), and it appears that the mutational studies indicate that these sites may be required for p53 activation in cells exposed to DNA-damaging agents. In ongoing studies, we are directly comparing the effects of bortezomib on p53 activation in cells expressing wild-type versus mutant forms of p53, including phosphorylation-defective mutants. It should also be noted that whereas serines 15 and 20 are the most widely reported sites of phosphorylation-induced activity, there are many other sites of phosphorylation and other posttranslational modification that have been associated with p53 function (13). It may be that, within the context of bortezomib treatment, these other modifications are more important. Our observation that bortezomib-stabilized p53 remains bound to mdm2 is consistent with the reported requirement for phosphorylation on serines 15 and 20 to separate the two proteins. It also suggests that proteasome inhibition does not activate p53-targeting kinases such as ATM and DNA-PK. Others have indicated that mdm2 can form a low affinity DNA-binding complex with p53 (75). This may be of significance during proteasome inhibition because our DNA binding assay shows the formation of a complex, and this may include mdm2.

Our observations indicate that p53 can function as a proapoptotic transcription factor in cells exposed to bortezomib, even though it displays biochemical traits implicated in inhibition of function. Atypical functions of p53 have been shown, including recent reports implicating mitochondrial translocation of p53 in cell death (28–32). Our immunofluorescence studies failed to demonstrate significant mitochondrial translocation of p53 in cells exposed to either bortezomib or etoposide, suggesting that this mechanism may be highly cell type dependent. Finally, our demonstration that proteasome inhibitor causes accumulation of a functional, nonphosphorylated, mdm2-bound p53 induced by proteasome inhibition points to another potential role for this class of agent. We may find that proteasome inhibition therapy can reactivate certain p53 mutations. This hypothesis is being explored by other agents (76–78) and is worth investigating to help explain some of the success of bortezomib in the clinic.

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Fig. 8. HPV-E6 overexpression confers resistance to bortezomib-induced apoptosis. Cells stably expressing the E6 gene were untreated (□) or treated with bortezomib (1 μM; ■) for 48 h. Apoptosis was determined by quantifying the sub-G0-G1 population of cells stained with propidium iodide and subjected to FACScan analysis. Mean ± SE, n = 3. Paired t test of bortezomib-treated conditions between parental LNCaP-Pro5 cells and others.
BORTezomib STABILIZES A NOVEL ACTIVE FORM OF p53


The Proteasome Inhibitor Bortezomib Stabilizes a Novel Active Form of p53 in Human LNCaP-Pro5 Prostate Cancer Cells

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