Inhibition of Proteasome Activity by Bortezomib in Renal Cancer Cells Is p53 Dependent and VHL Independent

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Abstract. Background: Antiproliferative effects of proteasome inhibitors are suggested to be primarily due to effects on nuclear factor-κB (NF-κB)-dependent pathways and the induction of apoptosis. The objective of this study was to elucidate the mechanistic basis for the antiproliferative effects of the proteasome inhibitor, bortezomib, in human clear cell renal cell cancer cells (CCRCC). Materials and Methods: von Hippel Lindau (VHL) mutation/methylation status and cytotoxic response to bortezomib was determined in a panel of CCRCC cell lines. Effects on target protein/gene expression and the role of p53 in bortezomib-mediated cytotoxicity, inhibition of proteasome activity, survivin transcript and protein expression as well as induction of p21 expression was determined in CCRCC that differed in their intrinsic sensitivity to bortezomib. Results: VHL status was not associated with cytotoxic response to bortezomib treatment. Cytotoxicity in cell lines that differed in intrinsic sensitivity to bortezomib correlated with sustained inhibition of proteasome activity, survivin expression and induction of p21 expression. Stable down-regulation of p53 expression by siRNA led to attenuation of bortezomib effects, survivin down-regulation and p21 induction, suggesting that cellular effects are p53-dependent. Conclusion: These results demonstrate that the antiproliferative effects of bortezomib in CCRCC cells are VHL independent and dependent on pathways regulated by p53.

Kidney cancer is responsible for more than 12,000 deaths per year in the United States and it has been reported that the incidence is increasing (1). Although the prognosis for localized renal cell carcinoma (RCC) is excellent, there is significantly reduced survival with advanced disease.

Key Words: Bortezomib, proteasome, renal cancer, p53, VHL.

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Treatment with interferons and/or interleukin-2, as well as inhibitors of angiogenesis have clinical activity in metastatic RCC (2-4).

Clear cell renal cell carcinoma (CCRCC) comprises 75% of kidney cancer cases. We focused on CCRCC because it is the only histological subtype where the majority of hereditary and sporadic forms are associated with alterations in the von Hippel Lindau (VHL) tumor suppressor gene either through gene mutation or methylation of the promoter region (5-7). The product of the VHL gene forms a complex with a number of proteins, including elongin C, elongin B, Cul-2 and Rbx1. This heterodimeric complex targets the hypoxia-inducible factors (HIF1α and HIF2α) for ubiquitin-mediated degradation (5-7) by the 26S proteasome. Mutation of the gene in clear cell kidney cancer prevents the VHL complex from targeting HIFs for proteasome-mediated degradation, resulting in its accumulation. Increased levels of HIF result in increased transcription of downstream targets such as vascular endothelial growth factor (VEGF), glucose transporter-1 (GLUT1), platelet-derived growth factor (PDGF), and transforming growth factor (TGF-α) (5-7). Clear cell RCC with wild-type or mutant VHL have also been described to differ in their response to apoptosis and clinical treatment (8-10).

The 26S proteasome is integral to the ubiquitin-proteasome pathway for degradation of proteins. Following polyubiquitination, targeted proteins undergo proteolysis by the 26S proteasome, which is comprised of a 20S proteasome core, the 19S regulatory complex and an 11S activator unit (11). The 26S proteasome plays a major physiological role in cell cycle progression, angiogenesis and cell motility by regulating the level of a number of target proteins including HIF, p53, I-κB, the topoisomerases, Bid, Bad, cyclins A, B, D and E, c-myc and others (12-15).

Bortezomib (PS-341; Velcade®) is the first proteasome inhibitor approved for clinical use in the treatment of multiple myeloma. In vitro studies have shown that it is cytotoxic against a range of cancer types including colon, breast, lung and renal (16-18). Previous studies have
indicated that inhibition of proteasome activity by bortezomib is associated with enhanced apoptosis due to inhibition of nuclear factor (NF)-κB activity (19, 20). Interestingly, our studies in human colon carcinoma and non-small cell lung carcinoma have demonstrated that proteasome inactivation can sensitize DNA damage-induced apoptosis by p53-dependent and NF-κB independent mechanisms (21, 22). Clinical studies have shown efficacy of bortezomib against multiple myeloma (11, 23). More recently, clinical studies are showing encouraging results with respect to response rates with non-Hodgkin’s lymphoma (24, 25) and some solid tumors including of the lung (26) and prostate (27) in combination with other antineoplastic agents. Bortezomib has demonstrated limited efficacy against renal cancer in clinical trials when given alone (28, 29).

Under normal physiological conditions, p53 is maintained at low steady-state levels by 26S proteasome degradation following ubiquitination by MDM2, an E3 ubiquitin ligase. Following DNA damage, p53 undergoes stabilization via post-translational modification and activates a number of different signaling pathways involved in cell cycle arrest, DNA repair, or apoptosis. The mechanism for p53-mediated apoptosis is poorly defined. Induction of p21 is a key mechanism by which p53 arrests cells in the G1 and/or G2 phase of the cell cycle (30).

Survivin is a member of the inhibitor of apoptosis (IAP) family (31) which includes c-IAP1, c-IAP2, XIAP, NAIP and others. The IAPs contain one to three zinc baculovirus inhibitor of apoptosis repeat (BIR) domains and suppress caspases. Survivin is expressed at mitosis and is associated with the mitotic apparatus and is thus essential for proper cell division in addition to inhibition of caspase-9 processing (32). It is undetectable in most adult tissues; yet, it is overexpressed in most types of human cancer. Down-regulation of this protein has been shown to enhance apoptosis (33). Regulation of survivin is mediated by oncogenes including signal transducer and activator of transcription-3 (STAT3) (34) and loss of tumor suppressors genes including p53 (31).

Given the limited information from preclinical and clinical studies on molecular determinants in bortezomib-mediated cytotoxicity in CCRCC and the potential impact of the role of VHL as an E3 ligase in regulating HIF expression, we sought to identify the mechanism for the antiproliferative effects of bortezomib in in vitro models of renal cell carcinoma expressing either wild-type or mutant VHL.

**Materials and Methods**

**Cell lines and transfection.** The RCC cell lines were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, non-essential amino acids and sodium pyruvate. Transient transfection with siRNA-scrambled or siRNA-p53 was carried out with 10 μl of Lipofectamine 2000®/3×10^5 cells in 1 ml of medium for 4 h. Stable expression of si-RNAs were carried out by cloning into a modified pBabe-puromycin retroviral vector. This vector contains the RNA polymerase III-dependent H1 RNA promoter cloned into the NheI site of the U3 region of the 3’LTR, which is used to drive expression of the si-RNAs. si-RNA oligonucleotides were cloned into BglII and HindIII sites immediately downstream of the H1 RNA promoter. The sequences of the si-p53 oligonucleotides cloned into the vector and protocol for transduction have been described previously (35). Following transduction, the target cells were selected for viral integration with 1 μg/ml of puromycin for 1 week. Transfection of 786-0 cells with wild-type VHL cDNA-containing vector, pCR3 (Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford, UK), or empty vector was carried out using 4 μg DNA/5×10^5 cells and Lipofectamine 2000®. Stable transfecants were selected by culturing in 1 mg/ml G418.

**DNA isolation.** DNA was isolated from approximately 2×10^6 cells using a Qiagen DNeasy DNA Isolation Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s protocol.

**VHL mutation analysis.** PCR-based amplification of each of the 3 exons was performed as described in Janezic et al. (37) using the following VHL primer sets: exon 1 forward, 5’-TGGAGGATCTTTCTGCGAC-3’; exon 1 reverse, 5’-GGCTTCAGCCGTGC TATCG-3’; exon 2 forward, 5’-GCTCTTTAAACACTTGTC-3’; exon 2 reverse, 5’-TTGATACCCGTCCTGACATC-3’; exon 3 forward, 5’-ACAGGT-AGTTGTTGGCAAAGCC-3’; exon 3 reverse, 5’-GAAGGACACAGTCTCCTATGC-3’. The amplicons were sequenced using an ABI377 automated sequencer (Applied Biosystems, Foster City, CA, USA) at the Cleveland Clinic Foundation’s Genomics Core Facility. Sequences derived from the amplified samples were compared to the wild-type VHL sequence (GenBank Accession No. AF010238) using LaserGene software (DNAStar, Perkin Elmer, Foster City, CA, USA) to identify and characterize the presence of mutations.

**VHL methylation analysis.** Methylation status was determined using VHL methylation-specific PCR primers after DNA bisulfite modification. Genomic DNA was modified using a CpGenome™ DNA modification kit according to the manufacturer’s protocol (Chemicon International, Temecula, CA, USA). The product then underwent PCR-based amplification using methylation-specific primers described in Herman et al. (38). Methylation status was determined by gel electrophoresis of the PCR products as described in Herman et al. (38).

**Cell lysis and Western blotting.** For immunoblot analysis, cell lysates were prepared in radio-immunoprecipitation assay buffer (RIPA), containing 20 mM Tris-HCl, pH 8.0, 0.425 M NaCl, 0.5% deoxycholate, 0.1% SDS, 1% NP-40, 2 mM EDTA, 0.5 mM EGTA and β-mercaptoethanol plus protease and phosphatase inhibitors (36).
An aliquot of the cell lysate was electrophoresed on a 10% Bis-Tris or 12% Tris-HCl gels. The separated proteins were electroblotted onto PVDF membranes and blocked in 5% non-fat dry milk (NFDM) in 20 mM Tris-HCl, pH 8.0, 150 mM NaCl and 0.05% Tween-20 (TBST) at room temperature for 1 h. The membrane was incubated with the primary antibody in 3% NFDM/TBST at 4°C (usually overnight), followed by a peroxidase labeled secondary antibody, diluted 40,000-fold, for 1 h at room temperature. The primary antibodies were survivin (polyclonal; Santa Cruz Biotech., Santa Cruz, CA, USA), actin (polyclonal), p21 (monoclonal), p53 (D01, monoclonal), VHL (11E12, mouse monoclonal, RB), and HIF1α (mouse monoclonal; BD Biosciences, San Jose, CA, USA). The relative intensity of the signal was determined for the protein band of interest and normalized with the relative intensity of actin.

Proteasome activity assay. Cell extracts were prepared in lysis buffer (10 mM Tris, 300 mM NaCl, 5 mM EDTA, 2 mM DTT, 1 mg/ml CHAPS and 1 mM PMSF) from control and treated cells retrieved at 6-12 h intervals, over a 48-h period, following treatment with different concentrations of bortezomib. Proteasome activity was determined using the substrate succinyl-Leu-Leu-Val-Tyr-7 amino methyl coumarin in a 20S Proteasome Assay Kit (Boston Biochem, Cambridge, MA, USA) according to the manufacturer’s protocol. Fluorescence was determined in a Wallac Victor 2 multilabel counter (Perkin Elmer, Gaithersburg, MD, USA) and activity was expressed as a percentage that of the control normalized for protein content.

Survivin transcript expression. RNA was isolated from approximately 2×10^6 cells using the Qiagen RNeasy RNA isolation kit (Qiagen) according to the manufacturer’s protocol. Single-stranded cDNA was synthesized from 1 µg of RNA using the SuperScript first-strand synthesis system (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. Following cDNA synthesis, relative levels of expression of survivin transcript in the renal cancer cell lines were determined in triplicate PCR reactions. The primers for survivin were forward: 5'-GGACCACCCGATCTCTCATCCTCAAA-3' and reverse: 5'-CGTTCCTCAGTGCGGCAGTGGAT-3'. The primers for β2-microglobulin were forward: 5'-CTTGTCTTCAAGCAAGGCTGG-3' and reverse: 5'-CATGA TGCTGTCATTATACTGCTTC-3'. The PCR reaction was performed on a ABI7700 sequence detection system (ABI, Foster City, CA, USA) according to the manufacturer’s protocol. Relative survivin expression was determined by comparison of the critical amplification threshold values in the treatment group against those of the untreated control group after normalization with the relative intensity of the signal.

Microarray analyses. Expression profiles were determined using a custom cancer cDNA array containing probes to 9240 cDNA clones corresponding to 4900 Unigenes or unclustered expressed sequence tags (ESTs) relevant to cancer or kidney development (39). The expression profiles of si-GFP and si-p53 transfected RC26B cells 24 hours after bortezomib treatment were simultaneously compared on the same slide and processed as described by Frevel et al. (39).

Results

Cytotoxic response to bortezomib is independent of VHL in renal cancer cell lines. Five renal cancer cell lines were treated with bortezomib, demonstrating a range of sensitivities to treatment (Table I). The VHL mutation/methylation status of these lines is presented in Table II. CAKI-1 is wild-type (WT) for VHL; all the remaining lines harbored VHL mutations, with the exception of RC13, where the promoter of VHL was methylated. The IC50 for cells wt for VHL were within the range of the VHL-deficient lines suggesting that VHL does not play a critical role in bortezomib-mediated apoptosis. Two lines, RC13 and RC26B, both VHL-deficient due to promoter methylation and sequence mutation, respectively, were selected for further evaluation because they had demonstrated the largest differential response to bortezomib treatment in our panel. VHL inactivity in these lines is demonstrated with the lack of pVHL expression and elevated pHIF1α expression under normoxic conditions (Figure 1).

Differential response to bortezomib is associated with a persistent down-regulation of proteasome activity. The differential response to bortezomib was associated with a persistent inhibition of proteasome activity to 25% that of the untreated control in RC26B cells beginning at 4 hours post treatment and continuing up to 48 hours. In contrast, while RC-13 cells demonstrated an initial similar decrease in proteasome activity at 4 hours post treatment, a bortezomib dose-dependent recovery in activity was
observed at 24 and 48 hours (Table III).

**Differential response to bortezomib is associated with down-regulation of survivin and induction of p21 protein.** Our ongoing studies in human non-small cell lung carcinoma have suggested that down-regulation of the anti-apoptotic protein survivin, which is regulated by p53 [31], is linked to bortezomib-induced apoptosis [21]. Thus, we determined protein levels of survivin and p21 in RC-13 and RC-26B cells treated for 30 min with 100 or 250 nM bortezomib and re-incubated in drug-free medium for 24 and 48 h. The data in Figure 2 demonstrate that unlike RC-13 cells, significant down-regulation of survivin and up-regulation of p21 protein occurs in RC-26B cells treated with bortezomib. The induction of p21 protein is also consistent with the markedly higher accumulation of cells in the G2+M fraction in RC-26B (40%) compared to RC-13 (9%) at 24 h following treatment with 250 nM bortezomib (Figure 3). Using primers specific for survivin and β2-microglobulin as the internal control, the effect of bortezomib treatment on survivin transcript levels by real-time RT-PCR (Figure 4) demonstrate that following bortezomib treatment there is persistent and significant down-regulation of survivin transcript levels in RC-26B compared to RC-13 cells.

**Down-regulation of p53 protein in RC-26B attenuates the apoptotic response to bortezomib.** Survivin is possibly regulated by p53 [40]; thus, we sought to test the effect of manipulating p53 expression on cellular effects of bortezomib. To test the functional role of p53 in mediating the antiproliferative effects of bortezomib, RC-26B and RC-13 cells were selected for stable expression of si-RNA directed to p53 [35]. Si-RNA directed to green fluorescent protein (GFP) was used as the control. The data in Figure 5 demonstrate significant down-regulation of p53 protein in the RC-26B (26B-si-p53) and RC-13 (13 si-p53) compared to the respective control RC26B-siGFP and RC13-siGFP lines. Using these cells and the treatment protocol described earlier for data in Table I, we tested the cytotoxic effects of bortezomib. The data in Table IVA clearly demonstrate that down-regulation of p53 protein in the RC-26B (si-p53) cells led to a 2- to 12-fold decrease in the growth inhibitory effects of bortezomib compared to the 26B-siGFP line, suggesting a functional role for p53. In contrast, the cytotoxic effects did not significantly differ in the RC-13 si-p53 lines compared to the RC-13 si-GFP cells (Table IVB).

**Differential gene expression in RC-26B si-P53 and RC-26B-siGFP following bortezomib treatment.** Microarray analysis was performed to determine the effects of bortezomib treatment on expression of p53-associated genes in the RC-26B si-p53 and RC-26B si-GFP lines. Data in Table V demonstrates that waf1/p21 and p21 were significantly down regulated in the si-p53 lines compared to the si-GFP lines.
following bortezomib treatment. In contrast, the IAP and cell proliferation-associated genes, survivin and topo IIα, respectively, which are p53 regulated were up-regulated in si-p53 cells.

**Discussion**

Our results demonstrate that bortezomib-mediated proteasome inhibition is p53 dependent in renal cancer cell lines. In addition, we found that VHL status does not impact on bortezomib-mediated cytotoxicity in our panel of renal cancer cell lines. Notably, transfection of the 786-0 line with wt VHL did not affect cytotoxic response to bortezomib (Table I). Interestingly, it was reported that VHL expression sensitized renal cancer cells to bortezomib with continuous exposure for 48 hours and that these effects are NF-κB dependent at concentrations of 1 micromolar or higher (41). In contrast, our treatment protocol was significantly different and likely addresses why pathways other than VHL and NF-κB may be critical in bortezomib-mediated toxicity. As previously outlined in this report, cells were treated for 30 minutes with bortezomib with concentrations at or below 1 micromolar, which are usually encountered in patients on bortezomib treatment. The differential effects on proteasome activity, survivin expression and p21 induction that were observed up to 48 hours after bortezomib was removed were also correlated with cytotoxicity determination 7 days post treatment. This protocol allows for the cells to recover post treatment over several population doublings and thus to assess the biological effects of the drug, in the absence of bortezomib. Our results further indicate that 30 minutes’ exposure to bortezomib is sufficient for cells to either recover or commit to cell death and is possibly more relevant to treatment schedules used clinically.

Proteasome activity was maximally suppressed 4 hours
post bortezomib treatment across several concentrations, with no significant differences in the RC-13 (resistant) and RC-26B (sensitive) lines. However, proteasome activity recovered significantly in the RC-13 line at 24 and 48 hours post treatment, while there was no significant recovery in RC-26B. This demonstrates that while bortezomib down-regulates proteasome activity to a similar extent in both lines, the sensitivity to the bortezomib lies in the cell’s ability to rebound or recover proteasome activity post bortezomib treatment. This suggests that differences in cell response among the lines may correlate to the duration of inhibition of proteasome activity.

Our previous work in lung cancer and colon cancer cell lines demonstrated a p53-dependent and NF-κB-independent pathway by which bortezomib mediates its cytotoxic effects (21, 22), since apoptosis was demonstrated only in the wt p53 lines. Furthermore, manipulating p53 expression or down-regulating survivin in these lines inhibited apoptosis. In this study, we found that RC-26B which responded to bortezomib,

Figure 3. Cell cycle traverse analysis of RC-13 and RC-26B cells treated for 30 minutes in the presence or absence of 250 nM bortezomib. Following incubation of the cells in drug-free medium for 24 hours, cells were washed and stained in propidium iodide for flow cytometric analysis. Results from a representative experiment show the cell cycle phase distribution profile and the relative percent of cells in the G_1, S and G_2+M fractions.

Figure 4. Quantitative analysis of survivin transcript levels in RC-13 and RC-26B cells after 30 minutes incubation in bortezomib and reincubation in drug-free media. Real-time PCR was carried out on cDNA synthesized from total RNA from cells collected at 4, 8, 24, 32 and 48 hours after treatment. Values are mean survivin levels and SD bars expressed as a percentage of untreated control after normalization to β2-microglobulin.
displayed prolonged down-regulation of survivin transcript and protein expression as well as p21 induction, while RC-13, had only a transient decrease in survivin transcript expression, no decrease in survivin protein or induction of p21 protein expression. This correlated with a significant increase in the G2+M phase fraction in the RC-26B line compared to the RC-3 line 24 hours after treatment. These data coupled with results following targeted down-regulation of p53 suggest that p53 regulates bortezomib-mediated cytotoxicity. This also supported results of Ling et al. (17), who demonstrated that p53 may be involved in bortezomib-induced G2-M phase arrest and apoptosis in lung cancer cell lines.

Recent clinical trials have demonstrated that bortezomib alone is not an effective agent in renal cell carcinoma treatment (28, 29). One reason for this may be that bortezomib mediates its cytotoxic effects through p53. The expression of WT p53 is suppressed in renal cell carcinomas cells (42). Thus, in renal cell carcinoma, bortezomib may be useful when used in combination with agents that activate p53, since the mechanism of p53 repression in renal cancer cells has been reported to be reversible with activating agents (42).

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