The proteasome inhibitor bortezomib synergizes with gemcitabine to block the growth of human 253JB-V bladder tumors in vivo

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Abstract

Bortezomib (PS-341, Velcade) is a dipeptidyl boronic acid inhibitor of the 20S proteasome that was developed as a therapeutic agent for cancer. Here, we investigated the effects of bortezomib on the growth of human 253JB-V bladder cancer cells. Although the drug did not stimulate significant increases in levels of apoptosis, it inhibited cell growth in a concentration-dependent fashion and augmented the growth inhibitory effects of gemcitabine in vitro. These effects were associated with accumulation of p53 and p21 and suppression of cyclin-dependent kinase 2 activity. Bortezomib also inhibited secretion of the proangiogenic factors matrix metalloproteinase-9, interleukin-8 (IL-8), and vascular endothelial growth factor (VEGF). In vivo studies with 253JB-V tumors growing in nude mice demonstrated that bortezomib (1 mg/kg) did not inhibit tumor growth when it was delivered as a single agent, although it reduced tumor microvessel density and inhibited expression of VEGF and IL-8. However, combination therapy with bortezomib plus gemcitabine produced synergistic tumor growth inhibition associated with strong suppression of tumor cell proliferation. Together, our results demonstrate that bortezomib has significant antiproliferative activity in aggressive bladder cancer cells, which is best exploited within the context of combination chemotherapy. [Mol Cancer Ther. 2004;3(3):279–290]

Introduction

The American Cancer Society estimated that there were about 12,600 deaths from transitional cell carcinoma (TCC) of the bladder in the United States in 2002, most of which occurred as the result of metastatic disease. Although TCC is a chemosensitive malignancy, most patients with metastasis eventually succumb to the disease because of emerging resistance to conventional chemotherapy (1–5). For more than a decade, the standard treatment for metastatic urothelial cancer has been combination therapy with methotrexate-vinblastine-adriamycin-cisplatin (MVAC). This regimen is consistently reported to produce a median survival in the range of 13–15 months (1). In spite of considerable effort to dose escalate the components of MVAC, no improvement in survival has been observed. Recently, paclitaxel, gemcitabine, and ifosfamide have been recognized to be quite active against TCC, and many novel combination regimens have been reported (1–5). Unfortunately, although some of these newer regimens are less toxic than MVAC, there is as yet no compelling evidence that survival is improving. Indeed, there is a growing conviction that the cytotoxic paradigm, as we have known it, will not provide the means to qualitatively change the outcome for patients with metastatic bladder cancer. Therefore, the development of novel therapeutic approaches is imperative.

The proteasome is a multisubunit protease complex involved in the regulation of proliferation, transcription, and apoptosis (6–8). Substrates targeted for proteasomal degradation are conjugated to polymers of the 8-kDa polypeptide, ubiquitin, and are degraded via an ATP-dependent process (8). Although patterns of proteasomal substrate degradation in mammalian cells are complex, many proteasome-regulated proteins are familiar because they are deregulated during cancer progression, including p53, p21, p27, and nuclear factor-κB. Furthermore, in vitro studies have demonstrated that proteasome inhibitors induce apoptosis in tumor cells (9–11) while sparing or even preventing apoptosis in certain normal cells (12, 13).

Bortezomib (PS-341, Velcade) is a dipeptide boronic acid that was developed specifically for the therapy of human tumors (14, 15). Preclinical studies demonstrated that bortezomib inhibited proliferation at a mean of IC50 of 7 nM in the 60 cell lines included in National Cancer Institute’s panel, making it among the most potent compounds tested in the screen to date (16). Subsequent studies demonstrated that maximal tolerated doses of bortezomib (1 mg/kg) inhibited the growth of several human xenografts in nude mice (16–20). Because bortezomib and gemcitabine both activate the p53 pathway via distinct mechanisms (21, 22), we reasoned that combination therapy with both drugs might produce additive or synergistic effects on tumor growth inhibition and/or apoptosis in 253JB-V tumors because they retain wild-type p53. Furthermore, in preliminary studies, we observed that gemcitabine induced activation of the transcription factor, nuclear factor-κB, in several human bladder cancer cell
lines independently of p53, and this effect was blocked by bortezomib (23). Therefore, we undertook the present study to investigate the effects of bortezomib alone or in combination with gemcitabine on parameters of tumor growth and angiogenesis in 253JB-V TCC cells.

**Materials and Methods**

**Tumor Cell Lines**

The highly metastatic human TCC cell line 253JB-V was generated in our laboratory via orthotopic recycling of the 253Jp human TCC cell line (24). RT4 cells were purchased from American Type Culture Collection (Rockville, MD), and KU7, UM-UC3, and UM-UC14 cells were generously provided by Dr. Barton Grossman (Department of Urology, University of Texas MD Anderson Cancer Center). All of the cell lines were maintained as monolayers in modified Eagle’s MEM supplemented with 10% fetal bovine serum, vitamins, sodium pyruvate, l-glutamine, penicillin, streptomycin, and nonessential amino acids.

**Antibodies**

The monoclonal antibody to cyclin-dependent kinase (cdk) 2 (D-12) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal antibody to p21 was obtained from BD PharMingen (San Diego, CA). The anti-proliferating cell nuclear antigen (PCNA) monoclonal antibody was from DAKO Corp. (Carpinteria, CA), and the rabbit polyclonal anti-p53 antibody was from Novacasta (Newcastle-upon-Tyne, United Kingdom).

**Animals**

Male athymic nude mice were obtained from the animal production area of the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD) and their use in these experiments was approved by the Institutional Animal Care and Use Committee. The mice were housed and maintained in laminar flow cabinets under specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care in accordance with current regulations and standards of the U.S. Department of Agriculture, U.S. Department of Health and Human Services, and NIH. The mice were used when they were 8–10 weeks of age.

**Drugs Used**

Gemzar (gemcitabine hydrochloride) was purchased from Eli Lilly and Co. (Indianapolis, IN). Bortezomib [pyrazylCONH(CHPhe)CONH(CHisobutyl)B(OH)₂] was a kind gift from Millennium Pharmaceuticals, Inc. (Cambridge, MA). As recommended, bortezomib was dissolved in DMSO and stored at −20°C until use. For *in vitro* studies, the drug was reconstituted in DMSO at a stock concentration of 50 mM, and this stock was diluted in medium just prior to use so that the concentration of DMSO never exceeded 0.1%. For *in vivo* studies, clinical-grade vials of bortezomib were reconstituted in water according to the manufacturer’s instructions. Solutions were sterilized by filtration through a 0.22 μm syringe filter.

**Measurement of Cell Growth Inhibition**

We measured the effects of bortezomib on DNA synthesis by pulse labeling cells with [3H]-thymidinium. Cells (10,000) were plated in 96-well plates and exposed to various concentrations of bortezomib, gemcitabine, or both for 24 h. Medium was removed and replaced with fresh medium containing 10 μCi/ml [3H]-thymidinium (ICN Biochemicals, Costa Mesa, CA). Cells were pulsed with [3H]-thymidinium for 1 h and lysed by addition of 0.1 N KOH (100 μl). Cells were harvested onto fiberglass filters, and incorporated tritium was quantified in a beta counter. Cell growth inhibition was also measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction after 72 h of drug exposure as described previously (20).

**Immunoblotting**

Untreated control cells or cells incubated with drugs were harvested by trypsinization and lysed as described previously (20). Total cellular protein (20 μg) from each sample was subjected to SDS-PAGE, proteins were transferred to nitrocellulose membranes, and the membranes were blocked with 5% nonfat milk in a Tris-buffered saline solution containing 0.1% Tween 20 for 2 h at 4°C. The blots were then probed overnight with relevant antibodies, washed, and probed with species-specific secondary antibodies coupled to horseradish peroxidase. Immunoreactive material was detected by enhanced chemiluminescence (West Pico; Pierce Chemical Co., Rockville, IL). Equal protein loading was confirmed by reprobing blots with a rabbit polyclonal antibody specific for actin (Sigma Chemical Co., St. Louis, MD).

**Quantification of DNA Fragmentation**

Cells (100,000) were grown in six-well plates in the presence of 1% MEM. After reaching 70% confluence (by 24 h), the cells were exposed to various concentrations of bortezomib, gemcitabine, or both for 48 h. Cells were harvested by exposure to trypsin and pelleted by centrifugation. The pellets were then resuspended in PBS containing 50 μg/ml propidium iodide (PI), 0.1% Triton X-100, and 0.1% sodium citrate (25). PI fluorescence was measured by fluorescence-activated cell sorting (FACS) analysis (FACScan FL-3 channel, Becton Dickinson, Mountain View, CA). Cells displaying a hypodiploid content of DNA indicative of DNA fragmentation were scored as apoptotic (25).

**Measurement of Matrix Metalloproteinase-9, Interleukin-8, and Vascular Endothelial Growth Factor Production by ELISA**

253JB-V cells were grown in equal numbers in six-well plates in the presence of 10% MEM. Conditioned medium was removed after 24 h and cells were washed with PBS (2 ml) and then incubated with medium (2 ml) containing bortezomib (1 μM) and gemcitabine (100 μM), neither, or both for 24 h. The supernatants from each well were collected and debris was removed by high-speed centrifugation. Factor levels in cell-free culture supernatants were determined using commercial Quantikine ELISA kits (R&D Systems, Inc., Minneapolis, MN) following the manufacturer’s instructions. The protein concentrations of...
interleukin-8 (IL-8) and vascular endothelial growth factor (VEGF) were determined by comparison of absorbance with the standard curve. The effect on matrix metalloproteinase-9 (MMP-9) protein levels is shown as a ratio of absorbance relative to untreated control cells.

Subcutaneous and Orthotopic Implantation of Tumor Cells

To produce tumors, 253JB-V cells were harvested from 70–80% confluent cultures by exposure to trypsin. Proteolysis was stopped with medium containing 10% fetal bovine serum, and the cells were washed once in serum-free medium and resuspended in HBSS (for orthotopic injection) or Matrigel (for s.c. injection). Only single cell suspensions with >90% viability were used for injections. For s.c. injections, 2 × 10⁶ cells in Matrigel (200 μl) were injected over the flanks of the mice. For the orthotopic injections, the mice were anesthetized with sodium pentobarbital, a small lower abdominal incision was made, and the bladder was exteriorized. Tumor cells (0.5 × 10⁶ cells in 40 μl HBSS) were injected into the bladder wall muscle using a 30 gauge needle and a calibrated push button-controlled device (Hamilton Syringe Company, Reno, NV). To prevent leakage, a cotton swab was held for 30 s over the site of injection. The incision was closed in one layer with wound clips. The s.c. tumors were measured twice a week and the volume of tumors was calculated using the formula: A × B × C / 6 (where A, B, and C represented the longest longitudinal and transverse diameter and depth). Final orthotopic tumor weights were obtained after the bladder was removed at necropsy.

Therapy of Established Tumors

Treatment was initiated when tumors were palpable. The s.c. tumors were palpable at 7 days after implantation when the average tumor size was 144 ± 35 mm³. Orthotopic tumors were palpable at 10 days. At these different time points, mice were randomly separated into four groups (12 tumors/group): bortezomib (1 mg/kg), gemcitabine (12.5 or 50 mg/kg), combination bortezomib plus gemcitabine, or 0.9% saline (control), all on an every Monday and Thursday schedule. The number of animals in each treatment group was selected in consultation with our biostatistician (Dr. Yu Shen). Animals bearing s.c. tumors received a total of six doses of therapy, whereas animals bearing orthotopic tumors received five doses. Relatively low doses of gemcitabine were selected to allow for detection of additive or synergistic effects in the combination therapy arm of the study; higher doses of gemcitabine (>100 mg/kg) led to marked tumor growth inhibition that was not significantly affected by combination therapy with bortezomib. All groups received treatment by i.p. injection. Treated mice were closely monitored for any signs of progressive disease. Mice in all groups were sacrificed 4 weeks after tumor cell implantation because pilot studies had demonstrated that this was the time period at which control animals became moribund.

Immunohistochemistry

For immunohistochemical (IHC) analysis, frozen tissue sections (8 μm thick) were fixed with cold acetone, chloroform/acetone, and acetone. Tissue sections (5 μm thick) of formalin-fixed, paraffin-embedded specimens were deparaffinized in xylene followed by treatment with a graded series of alcohol [100%, 95%, and 80% ethanol/double-distilled H₂O (v/v)] and rehydrated in PBS (pH 7.5). Antigen retrieval for paraffin-embedded tissues was performed with pepsin (Biomedica, Foster City, CA) for 15 min at 37°C. Endogenous peroxidase was blocked by the use of 3% hydrogen peroxide in PBS for 10 min. The samples were washed thrice with PBS and incubated for 20 min at room temperature with a protein blocking solution containing 5% normal horse serum and 1% normal goat serum in PBS (pH 7.5). Excess blocking solution was drained, and the samples were incubated for 18 h at 4°C with one of the following: a 1:50 dilution of rabbit polyclonal anti-VEGF/vascular permeability factor antibody (Santa Cruz Biotechnology), a 1:50 dilution of a rabbit polyclonal anti-IL-8 antibody (Biosource International, Camarillo, CA), a 1:100 dilution of mouse monoclonal anti-MMP-9 antibody (Oncogene Research Products, Cambridge, MA), or a 1:100 dilution of rat monoclonal anti-CD31 antibody (BD PharMingen). The samples were then rinsed thrice with PBS and incubated for 60 min at room temperature with the appropriate dilution of the secondary antibody: peroxidase-conjugated anti-rabbit IgG, F(ab')₂ fragment (Jackson ImmunoResearch Laboratory, West Grove, PA), anti-mouse IgG1 (BD PharMingen), or anti-mouse IgG (Jackson ImmunoResearch Laboratory). Positive reactions were visualized by incubating the slides with stable 3,3′-diaminobenzidine (Research Genetics, Huntsville, AL) for 5–15 min. The sections were then washed thrice with PBS, counterstained with Gill’s hematoxylin for 10 s (Biogenex, San Ramon, CA), washed thrice with distilled water, and treated with PBS for 1 min. The slides were mounted with a mounting medium (Research Genetics) prior to imaging.

Slides were examined under the microscope at fields of ×100 and ×200. For all markers, except CD31 and PCNA, absorbance was quantified by image analysis using the Optimas software program (Bioscan, Edmonds, WA) and corrected for differences in cell numbers. A minimum of three fields was analyzed from each tumor and five different areas from each field were evaluated to yield an average measurement of intensity of immunostaining. The results were presented as a ratio between the expression by the control tumor (arbitrarily set at 1.0) and the treated tumors. Cell proliferation was determined by light microscopy after immunostaining of sections with anti-PCNA. For the analysis of CD31 and PCNA staining, five ×200 fields from each treatment arm were used and the number of CD31-positive microvessels or the percentage of tumor cells staining for PCNA was expressed as the average of five fields.

Quantification of Apoptosis in Tumor Sections

Frozen tissue sections fixed and treated as above were washed with PBS containing 0.1% Brij (v/v). Terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling (TUNEL) was performed using a commercial kit.
(Promega, Madison, WI) according to the manufacturer’s instructions with the following modifications. Samples were fixed with 4% paraformaldehyde (methanol-free) for 10 min at room temperature, washed with PBS, and permeabilized by incubating with 0.2% Triton X-100 in PBS (v/v) for 15 min. The samples were incubated with equilibration buffer (from the kit). Reaction buffer containing equilibration buffer (45 μl), nucleotide mix (5 μl), and Tdt (1 μl) was added to the sections and incubated in a humidified chamber for 1 h at 37°C protected from light. The reaction was terminated by immersing the samples in 2× SSC [30 mM NaCl, 3 mM sodium citrate (pH 7.2)] for 15 min followed by three washes to remove unincorporated fluorescein-dUTP. Background reactivity was determined by processing the slides in the absence of Tdt (negative control). Nuclei were stained with PI (1 μg/ml) for 10 min. Fluorescent bleaching was minimized with an enhancing reagent (Prolong; Molecular Probes, Eugene, OR). Immunofluorescence microscopy was performed using a Zeiss Plan-Neofluar lens on an epifluorescence microscope equipped with narrow bandpass excitation filters mounted in a filter wheel (Ludl Electronic Products, Hawthorne, NY) to individually select for green, red, and blue fluorescence. Images were captured using a cooled CCD camera (Photometrics, Tucson, AZ). DNA fragmentation was detected by localized green fluorescence within the nucleus of apoptotic cells. For total TUNEL expression, apoptotic events were quantified using the laser scanning cytometer to count at least 10,000 cells/tumor as described previously (20, 26, 27).

Statistical Analyses

The primary end point of this study was tumor size after treatment in this 2×2 factorial design. Based on prior data, the study with the sample size of 10 mice/treatment group were expected to have >90% power to detect a minimum difference of 24 mm³ in tumor size at a statistical significance level of 0.05%.

Tumor weights, expression intensities of IHC, TUNEL, CD31, and PCNA counts, and PI-FACS percentages were compared by unpaired Student’s t test. The treatment effects of gemcitabine and bortezomib on tumor volumes were studied simultaneously in this 2 × 2 factorial design. The interaction between gemcitabine and bortezomib was estimated and tested using the method described previously (28). Statistical significance for this study was set at two-sided P < 0.05. All statistical analyses were performed using commercial software (InStat, San Diego, CA).

Results

Effects of Bortezomib on Cell Growth and Death

In a recent study, we demonstrated that bortezomib activates p53 in a variant of the androgen-sensitive LNCaP human prostatic adenocarcinoma line in vitro and that this p53 activation contributes directly to cell death (29). Because the 253JB-V cells contain wild-type p53, we performed immunoblotting studies to determine the

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**Figure 1.** Effects of bortezomib and gemcitabine on p53 and p21 expression. In all cases, results of one experiment that were representative of three independent replicates are shown. A, concentration-dependent effects of bortezomib. Cells were exposed to the indicated concentrations of drug for 4 h, and levels of p53 and p21 were determined by immunoblotting as described in Materials and Methods. B, concentration-dependent effects of gemcitabine. Cells were incubated with the indicated concentrations of drug for 4 h and levels of p53 and p21 were quantified by immunoblotting as described in Materials and Methods. C, enhanced p53 accumulation in cells treated with bortezomib plus gemcitabine. Cells were incubated for 4 h with the indicated concentrations of bortezomib, gemcitabine, or both drugs, and p53 accumulation was measured by immunoblotting.
effects of bortezomib and gemcitabine on p53 in the cells. Bortezomib stimulated accumulation of p53 and its target, p21, in a concentration-dependent manner (Fig. 1A). Very similar effects were observed in cells exposed to gemcitabine, a nucleoside analogue that activates p53 via the DNA damage response (Fig. 1B; Refs. 22, 30). Exposure to suboptimal concentrations of bortezomib plus gemcitabine led to at least additive accumulation of p53 (Fig. 1C).

Downstream effects of p53 activation include cell cycle arrest [at the G1-S (31) and G2-M (32) checkpoints] and apoptosis (33). We therefore investigated the effects of bortezomib and gemcitabine on the baseline activity of cdk2 (34), a kinase required for transition through the G1-S checkpoint (35), using in vitro substrate kinase assays (Fig. 2A). Levels of cdk2 activity were high in untreated 253JB-V cells, and these levels were decreased by 67% in cells exposed to bortezomib (10 nM) for 24 h in vitro (Fig. 2B). Relatively low concentrations of gemcitabine did not inhibit cdk2 activity, consistent with the fact that the direct effects of the drug tend to cause accumulation of cells in S phase. However, combined treatment with bortezomib plus gemcitabine led to strong additive inhibition of cdk2 activity (92%; Fig. 2B).

To directly measure the effects of bortezomib on cell proliferation, we exposed a panel of human TCC lines to increasing concentrations of the drug for 24 h and measured DNA synthesis by 3H-thymidine incorporation. By this time point, effects of bortezomib on S phase were maximal. Consistent with previous findings (16), low concentrations of bortezomib (<10 nM) strongly inhibited tumor cell proliferation as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide analysis (at 72 h; data not shown) and [3H]-thymidine incorporation in all of the cell lines tested (Fig. 3A). Furthermore, a low concentration of bortezomib (10 nM) produced an additive increase in growth inhibition in 253JB-V cells exposed to low concentrations of gemcitabine (Fig. 3B). In contrast, levels of apoptosis were highly variable in cells incubated for 48 h in concentrations of bortezomib up to 10 μM (Fig. 3C). Similarly, the levels of DNA fragmentation observed in 253JB-V cells treated with high concentrations of bortezomib plus gemcitabine were indistinguishable from the levels observed in cells treated with gemcitabine alone (Fig. 3D). Together, these results demonstrate that bortezomib acts predominantly as an inhibitor of proliferation at clinically achievable concentrations (<10 nM) in vitro. The drug also interacted with gemcitabine to produce at least additive effects on cell growth inhibition as well as on probable molecular mediators of the response (p53 activation and cdk2 inhibition) in the 253JB-V cells. Our results do not exclude the possibility that low concentrations of bortezomib induce loss of clonogenic survival in longer-term measurements of cell viability.

Effects of Bortezomib and Gemcitabine on Angiogenic Factor Production in Vitro

Recent studies from our laboratory and others have demonstrated that proteasome inhibitors interfere with angiogenesis in solid tumor xenografts (19–21). Therefore, we incubated 253JB-V cells with bortezomib, gemcitabine, or both and measured the production of the angiogenesis-
related factors IL-8 (Fig. 4A), MMP-9 (Fig. 4B), and VEGF (Fig. 4C) by ELISA. Consistent with previous findings, the 253JB-V cells constitutively produced significant levels of IL-8, MMP-9, and VEGF (Fig. 4). Gemcitabine did not inhibit the production of any of the factors, but it produced a significant increase in IL-8 production. However, exposure to bortezomib alone or in combination with gemcitabine resulted in significant inhibition of factor production in all cases, although its effects on VEGF were most dramatic (70% inhibition; Fig. 4C). Importantly, the effects of bortezomib on angiogenic factor production were not associated with apoptosis.

**Effects of Bortezomib on Tumor Growth in Vivo**

Tumor cells were inoculated into the subcutis of nude mice, and therapy was initiated 7 days later when tumors became palpable. Control animals received biweekly i.p. injections with normal saline. All animals were sacrificed at 4 weeks, when control animals and animals treated with gemcitabine or bortezomib alone became moribund. We did not continue to monitor the growth of tumors in animals treated with bortezomib plus gemcitabine to determine the extent of tumor growth delay, but we plan to do so in subsequent studies. Therapy with bortezomib alone failed to inhibit the growth of the tumors; rather, the bortezomib-treated tumors were consistently larger than saline controls (Fig. 5A). Single-agent gemcitabine (50 mg/kg) produced very modest inhibition of tumor growth, and the volumes of the gemcitabine-treated tumors were not significantly different from controls (Fig. 5A). Furthermore, all of the mice in the control, bortezomib, and gemcitabine arms of the experiment displayed signs of progressive disease and became moribund. However, therapy with the combination of bortezomib plus gemcitabine resulted in dramatic inhibition of tumor growth (Fig. 5A), and the tumors were significantly smaller than controls or tumors exposed to either agent alone (*P* < 0.05). Average tumor sizes at the end of 4 weeks of therapy were 355, 461, 299, and 132 mm³ for the control, bortezomib, gemcitabine, and combination therapy groups, respectively. The volumes of the tumors harvested from animals treated with bortezomib plus gemcitabine were very

![Graph A](image_url)

**Figure 3.** Effects of bortezomib and gemcitabine on apoptosis and proliferation *in vitro*. A, concentration-dependent effects of bortezomib on DNA synthesis. Cells were incubated with the indicated concentrations of drug for 24 h, pulsed with [3H]-thymidine for 1 h, and harvested for scintillation counting as described in Materials and Methods. Points, mean; bars, SD (n = 3). B, effects of bortezomib plus gemcitabine on DNA synthesis. Cells were incubated with the indicated concentrations of gemcitabine in the absence or presence of bortezomib (10 nM), and DNA synthesis was measured as described in Materials and Methods. Columns, mean; bars, SD (n = 3). C, concentration-dependent effects of bortezomib on DNA fragmentation associated with apoptosis. Cells were treated with the indicated concentrations of drug in medium containing 1% serum for 48 h, and DNA fragmentation was measured by PI-FACS analysis as described in Materials and Methods. Points, mean; bars, SD (n = 3). D, effects of bortezomib on gemcitabine-induced apoptosis. Cells were incubated with gemcitabine (10 or 50 μM) with or without bortezomib (1 μM) for 48 h and DNA fragmentation was measured by PI-FACS. Columns, mean; bars, SD (n = 3).
similar to those observed at the time therapy was initiated (144 mm³). In the absence of bortezomib, the effect of gemcitabine on the tumor volume was estimated to be 56 mm³. In contrast, the effect of gemcitabine, when given concurrently with bortezomib, was 329 mm³. The estimated interaction effect of bortezomib and gemcitabine on tumor volume is 272 with a SD of 98, which is statistically significant ($P = 0.005$).

We also investigated the effects of bortezomib and gemcitabine on the growth of orthotopic 253JB-V tumors (Fig. 5B). Consistent with the results obtained with the s.c. tumors, orthotopic tumors exposed to bortezomib alone appeared somewhat larger than controls, and single-agent gemcitabine failed to affect tumor growth. However, tumors exposed to bortezomib plus gemcitabine were significantly smaller than those treated with either agent alone. Mean tumor weight was 186 ± 52 mg in mice treated with the drug combination, which was significantly smaller than the mean tumor weights observed in mice treated with bortezomib (327 ± 74 mg) or gemcitabine (277 ± 48 mg) alone ($P < 0.05$).

To investigate the mechanisms of combination therapy-induced tumor growth inhibition, we quantified levels of apoptosis and proliferation in sections prepared from the orthotopic tumors by immunofluorescent TUNEL and IHC PCNA staining, respectively (Table 1). Tumors obtained from mice in all treatment groups displayed low levels of apoptosis (Table 1). However, these data should be interpreted with caution because they were obtained at a single time point, and previous studies have shown that levels of apoptosis can vary markedly with time in xenografts treated with other therapeutic modalities (36, 37). Combination therapy did produce a significant (2-fold) increase in apoptosis, but the absolute levels of cell death were
Effects of therapy on the growth of 253JB-V tumors in vivo.

A, effects on s.c. tumors. Therapy was initiated when tumors became palpable (7 days). Tumor volumes were then measured at the time points indicated as described in Materials and Methods. Animals were treated with bortezomib (1 mg/kg), gemcitabine (50 mg/kg), or both drugs for 3 weeks. Points, mean; bars, SE (n = 12). Tumors obtained from mice treated with bortezomib alone appeared to be larger than control tumors, and therapy with gemcitabine alone appeared to slightly decrease tumor volumes, although these differences were not statistically significant. Tumor size decreased from a mean of 355 mm³ in controls to a mean of 132 mm³ in the combination therapy group (P < 0.01).

B, effects of therapy on the growth of orthotopic tumors. Established (10-day) tumors were treated for 18 days (5 treatments) with bortezomib (1 mg/kg), gemcitabine (12.5 mg/kg), or both drugs. The animals were then sacrificed, and tumor weights and volumes were determined as described in Materials and Methods. Columns, mean; bars, SE (n = 12). Similar to the effects of bortezomib on the s.c. tumors, therapy with bortezomib alone appeared to slightly increase tumor volumes, although the effects were not statistically significant. Tumors exposed to combination therapy were significantly smaller than those treated with either agent alone (P < 0.01). Tumor weight was 186 ± 52 mg in the combination therapy mice, which was significantly less than for tumors treated with bortezomib alone (327 ± 74 mg) or gemcitabine alone (277 ± 48 mg).

### Table 1. Effects of therapy on apoptosis and proliferation in vivo

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Apoptotic index (%)</th>
<th>Proliferative index (%)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>0.61 ± 0.0011</td>
<td>71.32 ± 5.96</td>
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<tr>
<td>Bortezomib</td>
<td>2.19 ± 0.0123 (P = 0.08)</td>
<td>67.31 ± 5.68 (P = 0.307)</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>3.57 ± 0.0288 (P = 0.125)</td>
<td>62.05 ± 6.95 (P = 0.0535)</td>
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<tr>
<td>Combination</td>
<td>1.36 ± 0.0054 (P = 0.040)</td>
<td>29.67 ± 10.96 (P = 0.0001)</td>
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*Percentages of apoptosis were quantified by TUNEL staining and laser scanning cytometer analysis in sections prepared from orthotopic tumors treated with vehicle, bortezomib (1 mg/kg), gemcitabine (12.5 mg/kg), or both drugs twice weekly for 4 weeks. A minimum of 10,000 cells/slide was measured in slides obtained from three independent tumors. Values are means ± SD.

*Percentages of PCNA-positive cells were determined by IHC in five high-power fields in three independent tumors. Mean ± SD per ×200 field.
low (1.36%) compared with the levels observed in responsive tumors previously (>10%; Refs. 20, 21). Similarly, rates of proliferation were very similar in control tumors and tumors exposed to bortezomib or gemcitabine alone (Table 1). However, tumors isolated from mice treated with bortezomib plus gemcitabine displayed dramatic decreases in proliferation (from 71% to 30%; Table 1). Together, the results indicate that tumor growth inhibition was primarily due to inhibition of proliferation rather than induction of apoptosis.

**Effects of Bortezomib on Angiogenesis**

In a final series of experiments, we investigated the effects of proteasome inhibitor, gemcitabine, or both on tumor microvessel density (MVD) and expression of angiogenic factors by IHC. Tumors isolated from mice treated with bortezomib alone displayed significant decreases in MVD (Fig. 6) and expression of IL-8 and VEGF (Fig. 7; Table 2), although they grew larger on therapy. Single-agent bortezomib did not significantly inhibit tumor expression of MMP-9 (Table 2). Tumors isolated from mice treated with gemcitabine alone displayed modest but significant increases in MVD and modest decreases in IL-8 production, but gemcitabine had no effect on MMP-9 or VEGF expression (Table 2). The discrepancy between the effects of gemcitabine on IL-8 production *in vitro* (increased) versus *in vivo* (decreased) may be explained by

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**Figure 6.** Effects of therapy on tumor vascularity. Sections from orthotopic tumors treated with vehicle, bortezomib (1 mg/kg), gemcitabine (12.5 mg/kg), or both were stained with an antibody to CD31. **A**, representative images of high-power fields chosen at random from regions in the tumor periphery. **B**, quantitative analysis. Microvessel numbers were determined in five separate high-power fields for each of three tumors. *Columns*, mean; *bars*, SE.
the involvement of effects of gemcitabine on host cells (i.e., endothelial cells and other stromal elements) in the latter. MVD in tumors harvested from mice with both agents were similar to those observed in tumors exposed to bortezomib alone, although combination therapy did lead to reductions in IL-8, VEGF, and MMP-9 (Figs. 6 and 7; Table 2).

Discussion

TCC of the bladder initially responds well to aggressive, multidrug cytotoxic chemotherapeutic regimens, but eventually, most patients succumb to chemoresistant metastasis. There is currently considerable interest in chemotherapy regimens based on gemcitabine in this patient cohort, because there is evidence that some of these newer regimens are less toxic than MVAC. Furthermore, preclinical studies employing human TCC xenografts demonstrated that combined therapy with conventional agents and angiogenesis inhibitors results in strong tumor growth inhibition that is greater than is observed with therapy with either agent alone, without significant increases in toxicity (38, 39). Because the proteasome inhibitor bortezomib displayed strong antiangiogenic activity in other model

Table 2. Effects of therapy on angiogenesis

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>MVD</th>
<th>IL-8</th>
<th>MMP-9</th>
<th>VEGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>42.4± 6.3</td>
<td>1 ± 0.035</td>
<td>1 ± 0.039</td>
<td>1 ± 0.100</td>
</tr>
<tr>
<td>Bortezomib</td>
<td>23.8± 6*</td>
<td>0.72 ± 0.037*</td>
<td>0.90 ± 0.034</td>
<td>0.64 ± 0.049*</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>54.8± 4.8*</td>
<td>0.70 ± 0.027*</td>
<td>0.82 ± 0.053</td>
<td>0.90 ± 0.092</td>
</tr>
<tr>
<td>Combination</td>
<td>30.0± 6.9*</td>
<td>0.57 ± 0.025*</td>
<td>0.61 ± 0.028*</td>
<td>0.51 ± 0.059*</td>
</tr>
</tbody>
</table>

Note: Animals bearing orthotopic 253JB-V tumors were treated with bortezomib (1 mg/kg), gemcitabine (12.5 mg/kg), or both drugs twice weekly for 4 weeks. MVDs were determined by counting CD31-positive blood vessels in five high-power fields selected at random within the peripheries of five independent tumors. Absorbance values were measured as outlined in Materials and Methods using five high-power fields/tumor and at least five tumors/treatment group. Absorbance was normalized to control tumors using 1 as an arbitrary value. Values are means ± SE.

*P < 0.001, significant with respect to control.
systems (19, 20), we suspected that its effects on angiogenesis coupled with direct effects on cell proliferation and survival would promote the effects of gemcitabine in TCC. To test this hypothesis, we characterized the effects of the drug with or without gemcitabine on growth and angiogenesis in 253JB-V cells, a variant of the human 253J TCC line selected for aggressive growth via orthotopic “recycling” in vivo (24). Tumors derived from these cells secrete high levels of VEGF, IL-8, and active MMP-9 (38) and they are refractory to gemcitabine-induced apoptosis. These features mirror the properties of advanced TCC in patients.

The results presented here confirm that bortezomib inhibits angiogenesis in the 253JB-V model, but this inhibition is incomplete. In vitro experiments demonstrated that it inhibited the production of VEGF, IL-8, and MMP-9 (Fig. 4), and in vivo studies demonstrated that it reduced tumor production of VEGF and IL-8 (Table 2). Nonetheless, the observed reductions in MVD were not dramatic, and there was no difference between the effects of bortezomib alone and bortezomib plus gemcitabine on MVD. Furthermore, single-agent bortezomib not only failed to inhibit tumor growth but also appeared to slightly enhance tumor growth compared with untreated controls. To our knowledge, this is the first example of a situation where an agent promotes modest tumor growth when given on its own but synergistically promotes the antitumoral effects of another agent (in this case, gemcitabine) when given in combination. While we did not identify the mechanism(s) underlying bortezomib-mediated enhancement of tumor growth, proteasome inhibition apparently results in elimination of smaller, poorly functional microvessels that actually interfere with perfusion. Such a phenomenon has been observed recently in other tumor models, where antiangiogenic factors cause “pruning” of vascular tumorigenicity leading to increased blood flow (40, 41). Because the 253JB-V tumors are highly angiogenic, the incomplete inhibition of angiogenic factor production (maximal 35% inhibition for VEGF) likely did not reach a critical threshold required to affect overall tumor growth. Although low concentrations of bortezomib blocked cell proliferation in vitro, the dose of the drug used in the animal studies clearly was not sufficient to produce comparable effects on PCNA staining in bortezomib-treated tumors (Table 1), which probably also allowed the drug-treated tumors to grow on therapy. Regardless of the mechanisms involved in tumor growth promotion, our data raise a cautionary note about using single-agent bortezomib in this disease, especially in light of the fact that there is now extensive phase I toxicity information available from several different trials conducted in patients with solid and hematological tumors (15). We intend to evaluate this drug combination ourselves in patients with refractory TCC tumors as part of a Specialized Program of Research Excellence-sponsored phase II clinical trial.

Our results strongly suggest that inhibition of tumor cell proliferation played a central role in the effects of bortezomib plus gemcitabine on the 253JB-V tumor cells. In vitro, concentrations of bortezomib (≤10 nM) produced strong inhibition of DNA synthesis as measured by 3[H]-thymidine incorporation, whereas much higher concentrations failed to induce significant levels of tumor cell apoptosis. Importantly, the most striking effect of combination therapy with bortezomib plus gemcitabine in vivo was inhibition of tumor cell proliferation, assessed by PCNA IHC, and in the s.c. model, tumor volumes at the end of therapy were almost identical to those observed at the start of therapy. The effects of bortezomib plus gemcitabine on PCNA staining more closely paralleled overall effects of therapy on tumor growth than any of the other surrogates (TUNEL, MVD, or angiogenic factor production). Together, our data indicate that the bortezomib/gemcitabine combination exerts cytostatic rather than cytoreductive effects in this model of aggressive disease. Thus, we would assume that this approach would be most effective in the setting of minimal residual disease, and long-term maintenance may be required.

Consistent with our findings in other systems (29), the effects of bortezomib on cell cycle arrest in 253JB-V cells appeared to involve p53. Growth inhibition was associated with accumulation of p53 and its transcriptional target, p21, as well as inhibition of cdk activity. Low concentrations of gemcitabine also caused p53 and p21 accumulation, and combined treatment with both drugs produced at least additive effects on all measures of cell growth arrest. Although bortezomib clearly affects p53-dependent processes, it is important to emphasize that its effects on cell proliferation are not completely dependent on p53, because cell lines that lack p53 entirely (i.e., UM-UC3) are still strongly growth inhibited by the drug (Fig. 3A). Whether these effects occur via direct stabilization of cdk inhibitors (p21, p27, etc.) will require additional investigation.

References


The proteasome inhibitor bortezomib synergizes with gemcitabine to block the growth of human 253JB-V bladder tumors in vivo

Ashish M. Kamat, Takashi Karashima, Darren W. Davis, et al.


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