Augmenting Chemosensitivity of Malignant Melanoma Tumors via Proteasome Inhibition: Implication for Bortezomib (VELCADE, PS-341) as a Therapeutic Agent for Malignant Melanoma

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ABSTRACT

Melanoma poses a great challenge to patients, oncologists, and biologists because of its nearly universal resistance to chemotherapy. Many studies have shown that nuclear factor κB is constitutively activated in melanoma, thereby promoting the proliferation of melanoma cells by inhibiting the apoptotic responses to chemotherapy. Nuclear factor κB activity is regulated by phosphorylation and subsequent degradation of inhibitor of nuclear factor κB by the ubiquitin-proteasome pathway. In this study, we show that the novel proteasome inhibitor, bortezomib, inhibited the growth of melanoma cells in vitro at a concentration range of 0.1–10 nM and in combination with the chemotherapeutic agent temozolomide. 0.1–10 nM and in combination with the chemotherapeutic agent temozolomide.

INTRODUCTION

Melanoma is the most aggressive form of skin cancer and has increased >6-fold in incidence over the past 50 years. Metastatic disease is estimated to have caused 7600 deaths in 2003 and is the second cause of lost productive years among cancers (1, 2). Melanoma is highly resistant to conventional chemotherapy with dacarbazine or its derivative temozolomide (TMZ) having the best single agent activity with a response rate of only 15–20% and a short 4-month median response duration. At this time, no randomized clinical trial has shown a survival advantage to any other more complex chemotherapy and/or biotherapy regimens over single agent dacarbazine (3, 4). Thus, it is imperative to investigate new therapeutic targets for the treatment of melanoma to improve the dismal prognosis for this disease. One such important target identified in melanoma tumor progression is the nuclear factor κB (NFκB) pathway (5, 6).

Constitutive activation of NFκB is an emerging hallmark of various types of tumors including breast, colon, pancreatic, ovarian, and melanoma (7–12). In the healthy human, NFκB regulates the expression of genes involved in normal immunological responses (e.g., generation of immunoregulatory molecules such as antibody light chains) in response to proinflammatory cytokines and byproducts of microbial and viral infections (13–15). However, increased activation of NFκB results in enhanced expression of proinflammatory mediators, leading to acute inflammatory injury to lungs and other organs and development of multiple organ dysfunctions. NFκB also modulates the expression of factors responsible for growth as well as inhibitors of apoptosis (13, 15, 16).

There are five known mammalian NFκB subunits, each characterized by ankyrin repeat elements: (a) Rel (c-Rel); (b) p65 (RelA); (c) RelB; (d) p50; and (e) p52. The NFκB protein is composed of two subunits, which may vary affecting the transcriptional activity of the protein. In the absence of activation, NFκB complexes (homo- and heterodimers composed of above the mentioned subunits) are sequestered in the cytoplasm because of their association with an inhibitor of κB protein (IκB). The IκB protein binds to the nuclear localization signal of NFκB Rel proteins, thereby inhibiting translocation of the complexes into the nucleus (13–15). When the cell is exposed to activating signals, such as tumor necrosis factor-α, the IκB protein is phosphorylated by IκB kinase, ubiquitinated, and then broken down in the 26 S proteasome (17). This frees the NFκB to translocate into the nucleus, where it binds to κB sites in the promoter/enhancer regions of specific genes, including the promoter/enhancer for IκB, to transactivate transcription (13, 15, 17).

Persistent activation of NFκB inhibits apoptosis and promotes proliferation leading to hyperplasia (13, 16, 18, 19). Previous studies in our laboratory have shown an elevated basal IκB kinase activity in Hs294T melanoma cells, which leads to an increased rate of IκB phosphorylation and degradation. This increase in IκB-α phosphorylation and degradation leads to an ~19-fold higher nuclear localization of NFκB (20). We have shown that this constitutive activation of NFκB facilitates the immortalization and proliferation of melanocytes and provides a means to escape apoptosis (20–23). These findings suggest that NFκB may represent an effective molecular target in melanoma tumorigenesis.

To date, many different strategies have been used to inhibit NFκB activity in tumors with various degrees of success. We propose to use the target 26 S proteasome for inhibition of NFκB activity in melanomas. Among proteasome inhibitors, bortezomib (VELCADE), formerly known as PS-341, inhibits more specifically the chymotryptic enzyme activity of the proteasome. Bortezomib is a low molecular weight, water-soluble dipeptide that binds to the proteasome with very high affinity and dissociates slowly, imparting stable but reversible proteasome inhibition (24, 25). Bortezomib has shown great promise in the preclinical studies for cancers such as ovarian, lung, squamous cell carcinoma, prostate, and pancreatic (26–30), and many clinical trials for the treatment of these cancers have been initiated (31, 32).

More recently, bortezomib received accelerated approval from the United States Food and Drug Administration for the treatment of patients with refractory multiple myeloma who failed prior chemotherapy (33), highlighting the potential effectiveness of the drug in the treatment of cancer.

This is the first study to investigate the efficacy of bortezomib in melanoma cells and in a murine xenograft model of melanoma to inhibit NFκB and, in turn, melanoma tumor progression. In particular,
we were interested in the combination therapy involving bortezomib and TMZ. TMZ is currently one of the most prescribed chemotherapeutic treatments for metastatic melanoma despite its marginal effectiveness (3). We hypothesized that the combination of chemotherapy with proapoptotic therapy could result in a synergistic effect, providing a more effective strategy to eliminate melanoma tumors.

MATERIALS AND METHODS

Materials. TMZ was obtained from the Vanderbilt pharmacy. Bortezomib was provided by Millennium Pharmaceuticals (Cambridge, MA).

Cell Culture. The human melanoma cell line Hs294T was obtained from American Type Culture Collection (Manassas, VA), and normal retinal pigment epithelial cells, RPE-476, were generously provided by Glenn Jaffe at Duke University (Durham, NC). The cells were grown in 50% DMEM, 50% F-12 supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 100 mg/ml penicillin, and 100 mg/ml streptomycin. Cell cultures were maintained at 37°C.

Cell Growth Response. Melanoma cell lines SK-MEL-5, SK-MEL-28, WM 115, and Hs 294T (5 × 10^5 cells/well) were seeded in six-well plates. Cells were treated with increasing doses (0–25 nM) of bortezomib for 48 h, and the number of viable cells was scored after addition of trypan blue using a hemocytometer. The results are reported as sigmoidal dose-response curve depicting the mean sensitivity of the 4 melanoma cell lines using the software GraphPad Prism. The GI50 value was calculated by the same software. RPE 476 and H294T cell lines were seeded in six-well plates and treated with 1 nM bortezomib and/or TMZ at increasing doses of 10, 100, and 1000 μM 12 h after plating. Control groups were culture medium alone and 5% DMSO. Cell counts were performed after addition of trypan blue to the cells, using the hemocytometer on day 3 of treatment.

Tumor Growth Response. BALB/c-nu/nu female mice were assigned to each of the following groups with 5 mice/group: (a) Control; (b) bortezomib; (c) TMZ; (d) bortezomib and TMZ. One million Hs 294T cells were injected s.c. (day 0). Treatment began on day 8, when tumors were palpable. Each mouse received 1.25 mg/kg bortezomib peritumorally and/or 20 mg/kg TMZ peritumorally dissolved in 100 μl of saline on a twice-weekly schedule. The control group received the vehicle. Bidimensional tumor measurements were assessed three times weekly using microlongies. The Vanderbilt University Institutional Animal Care and Use Committee approved experimental protocols.

Immunohistology. Whole cell extracts were obtained according to our standard protocol using radioimmunoprecipitation assay buffer. Extracts from tumor tissue were made according to our standard protocol. Briefly, tumor tissues with fresh frozen in liquid nitrogen. The tissue was homogenized in tissue homogenizer containing TNN buffer (0.05 M NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Triton X-100, 0.1% SDS, and 1% sodium citrate buffer (pH 6.0)). The homogenates were centrifuged for 10 min at maximum speed, and the cleared supernatant was collected for analysis. The lysates were subjected to SDS-PAGE and probed with appropriate antibodies. Antibodies used were anti-p21, anti-p53, anti-MDR-1, and antiactin from Santa Cruz Biotechnology (Santa Cruz, CA). For secondary antibodies, horseradish peroxidase-conjugated antimouse, goat, or rabbit IgG were obtained from Chemicon International. The antibodies were visualized using an enhanced chemiluminescence kit obtained from Amersham Biosciences (Piscataway, NJ).

Immunohistochemistry. Paraffin-embedded tumor sections were deparaffinized with xylene. The antigen was unmasked by heating samples in 10 mM sodium citrate buffer (pH 6.0) for 5 min and quenching with 0.03% hydrogen peroxide. Samples were immunostained for activated RelA/p65 (1:25) or CD31 (1:400). The ABC biotin/avidin reagent kit was used to visualize the immunolocalization of the antigen using NovaRed from Amersham Biosciences (Piscataway, NJ).

RESULTS

The Cytotoxic Effect of Bortezomib on Normal and Melanoma Cells in Vitro. To determine the activity of bortezomib against the proliferation of human melanoma cells, the human melanoma cell lines SK-MEL-5, SK-MEL-28, WM 115, and Hs 294T were exposed to increasing concentrations (0–25 nM) of bortezomib for 48-h continuous incubation at 37°C (Fig. 1A). Treatment of cells with bortezomib inhibited cell growth in a dose-dependent response, and the average GI50 value for the cell lines was 6 nM. To assess whether bortezomib increases the sensitivity of melanoma cells to the chemotherapy agent TMZ, Hs 294T cells were exposed to 1 nM bortezomib ± 10–1000 μM of TMZ for 72 h (Fig. 1B). Results show that Hs 294T cells show an increased sensitivity toward TMZ with the addition of bortezomib, because the lower concentrations of TMZ, when used in combination with bortezomib, have the same effect on cell growth as high-toxic dose TMZ. More interestingly, the sensitivity to the drug was much greater in melanoma cell line Hs 294T than in the normal human cell line, retinal pigment epithelial RPE-476 cells (Fig. 1C). Altogether, the data indicate that melanoma cells are more sensitive to bortezomib than normal cells and that bortezomib reduces the resistance of melanoma cells to TMZ.

Bortezomib Inhibits Expression of NFκB Target Genes. As chemoresistance has been reported to be conferred through NFκB activation in many cancers, and because melanomas show constitutive activation of NFκB and are highly resistant to chemotherapy, we determined the effect of bortezomib on NFκB inhibition after treatment with TMZ in Hs 294T cells. TMZ (100 μM for 24 h) increased significantly the secretion of the NFκB-regulated chemokine, CXCL8, although the number of viable cells decreased by half that of control cells (Fig. 2A). However, when cells are treated with TMZ (100 μM) in combination with 10 nM bortezomib, the CXCL8 induction by TMZ is no longer detected. The data suggest that TMZ induces NFκB activation and subsequent CXCL8 production, and cotreatment
treatment with 1.25 mg/kg bortezomib and 20 mg/kg TMZ resulted in effective tumor growth inhibition (Fig. 3). In these experiments, control groups comprised cells treated with culture medium alone, DMSO, bortezomib alone, and TMZ alone. C. RPE 476 cells as in B. The results are expressed as triplicate experiments (n = 3); bars, ± SE. TMZ, temozolomide.

with bortezomib inhibits this induction. Similar results were obtained for the NFκB-regulated chemokine, CXCL1 (data not shown).

Given the role of NFκB in drug resistance and evidence that chemotherapeutic agents may induce multidrug resistance genes, the expression level of family members MDR-1 and MRP1 were also investigated (Fig. 2B). Treatment of melanoma cells with TMZ at a dose of 100 μM induces MDR-1 and MRP1 expression. However, the expression of both proteins is abrogated when cells are treated with TMZ in combination with bortezomib. Altogether, the in vitro data indicate that bortezomib may be an excellent drug to use in combination with other chemotherapeutics in the treatment of melanomas.

Enhancement of TMZ-Mediated Antitumor Activity by Bortezomib. To determine whether combining TMZ treatment with the administration of proteasome inhibitor bortezomib could enhance the chemosensitivity of melanoma tumors, a Hs 294T xenograft model was used (Fig. 3). In these experiments, s.c. administration of bortezomib at 1.25 mg/kg or TMZ at 20 mg/kg alone to growing melanoma tumors initially resulted in a significant decrease in tumor size (P < 0.0001 and P < 0.0002, respectively) when compared with the control group receiving saline alone (Fig. 3A). However, when single agent treatments were withdrawn by day 36 after tumor implantation, tumor growth was recommenced quickly in these groups, and subsequent treatment with either drug did not result in effective tumor growth inhibition (Fig. 3B). Combined treatment with 1.25 mg/kg bortezomib and 20 mg/kg TMZ resulted in complete remission of all of the animals within the group with an average tumor size of 3.51 mm² and average tumor growth time of 27.75 days. Interestingly, the treatment group that received combined administration of bortezomib and TMZ was the only group to undergo a true tumoricidal response, where a persistent regression of tumor growth was observed in all of the animals to the point that the animals were cured of their tumor burden by day 36. The complete remission persisted even after the withdrawal of both agents for >200 days.

The Antitumorigenic Action of Bortezomib Is Through Increased Apoptosis. To determine whether apoptosis was increased in the xenograft tumor model with bortezomib treatment, tumor sections were stained for the presence of terminal deoxynucleotidyl transferase-mediated nick end labeling-positive cells after treatment (Fig. 4, A–C). As illustrated by the images, the tumors in the control group as well as the TMZ group exhibit low levels of apoptosis. However, a marked increase in apoptosis is observed in tumors treated with bortezomib. The increase in apoptosis was typical for all of the tumors in the bortezomib group, and the terminal deoxynucleotidyl transferase-mediated nick end labeling-positive cells were detected within the entire area of the tumor in contrast to nontreated tumors or tumors treated with TMZ, where slight staining was seen in the periphery of tumors. Thus, these findings suggest that the tumoricidal response to treatment with bortezomib appears to result from markedly increased levels of apoptosis throughout the tumors.

Fig. 1. The cytotoxic effect of bortezomib (Btzmbl) on normal and melanoma cells in vitro. A, melanoma cell lines were treated with bortezomib at final concentrations of 0, 0.01, 0.05, 0.1, 0.5, 1.0, 5, 10, and 25 nM for 48 h, and the mean dose-response curve was plotted. B, Hs 294T cells were treated with 1 nM bortezomib and/or TMZ at increasing doses of 10, 100, and 1000 μM for 72 h. In all of the experiments, control groups comprised cells treated with culture medium alone, DMSO, bortezomib alone, and TMZ alone. C. RPE 476 cells as in B. The results are expressed as triplicate experiments (n = 3); bars, ± SE. TMZ, temozolomide.

Fig. 2. Bortezomib (Btzmbl) inhibits nuclear factor κB-mediated gene expression. A, Hs 294T cells (5 × 10⁵)/well in six-well plates in duplicates were seeded in serum-free culture medium and incubated at 37°C for 12 h. The monolayers were then incubated with 10 nM bortezomib and/or 100 μM temozolomide (TMZ) in serum-free medium for 48 h at 37°C, at which time the supernatant was collected and cleared by centrifugation. Aliquots were then subjected to ELISA assay for CXCL8. The results are reported as the percentage of inhibition, considering 100% as the relative expression level of the control cells; bars, ± SD. *, *P < 0.05. B, 80% confluent Hs 294T melanoma cells in 60-mm culture dish containing serum-free media were treated with 10 nM bortezomib, 100 μM TMZ, or 10 nM bortezomib and 100 μM TMZ for 12 h. The control cells were incubated in serum-free media alone. Cells were lysed, and the expression levels of MDR-1 and MRP1 were determined by immunoblotting. The same blot was reprobed with antiactin antibody for protein loading control. This figure is a representative of three separate experiments. bars, ±SD.
Bortezomib Inhibits p65 Nuclear Translocation in Tumors and Causes Accumulation of Important Regulatory Proteins in Tumors. To determine whether bortezomib treatment of melanoma tumors inhibited NFκB, we performed immunohistochemical analysis on sections of fixed, embedded tumors using antibody against the nuclear NFκB subunit, RelA/p65 (Fig. 5). The immunohistochemical analysis shows that p65 accumulates in the nucleus in the control tumors at high levels (Fig. 5, A and B) and that treatment with TMZ additionally increases p65 nuclear localization even more (Fig. 5, C and D), confirming that TMZ is an inducer of NFκB. However, tumors that were treated with bortezomib alone exhibited a much reduced nuclear p65 level (Fig. 5, E and F), indicating that bortezomib actively inhibits the translocation of NFκB into the nucleus and, thus, inhibits NFκB activity in these tumors.

To determine whether the bortezomib therapy effects are mediated through changes in important cell cycle regulatory proteins, we examined the effects of bortezomib on the protein level of the tumor suppressor proteins p53 and p21 in the tumors (Fig. 5G). Treatment with bortezomib resulted in stabilization of both p53 and p21, suggesting that one potential mechanism of action of the drug may be through the stabilization of p53 and p21. Because altered expression levels of MDR-1 and MRP1 were observed in the Hs 294T cell line treated with bortezomib and TMZ, we also evaluated the expression of these ATP-binding cassette transporters in the tumors. Both MDR-1 and MRP1 were up-regulated in tumors treated with TMZ, whereas in tumors treated with bortezomib, MDR-1 was undetectable, and MRP1 was detected at very low level. These data suggest that bortezomib has the potential for enhancing chemosensitivity through regulation of ATP-binding cassette transporters as well as induction of apoptosis.

Bortezomib Inhibits Angiogenesis in Tumors. To determine whether the significant decrease in the tumor size that resulted from bortezomib treatment was because of decrease in the microvasculature within the tumor, tumor sections were stained for endothelial cells using antibody against the endothelial cell marker CD31 (Fig. 6, A–C). Quantitative analysis of the tumor sections shows a significant decrease in tumor vessel density for tumors treated with bortezomib (P < 0.001) compared with treatment with TMZ or control (Fig. 6D). Interestingly, all of the TMZ-treated tumors exhibited slightly higher levels of microvasculature than the control tumors.

Considering that NFκB has been reported to induce the expression of the potent angiogenic factor vascular endothelial growth factor, which is required for tumor growth and metastasis, we next asked whether the significant decrease in tumor microvasculature after bortezomib treatment was due to decreased vascular endothelial growth factor production by the tumor tissue. ELISA analysis revealed that tumors in the bortezomib group exhibit a decrease in the level of vascular endothelial growth factor, whereas the tumors in control and TMZ treatment groups show high production of this growth factor (Fig. 6E). This result is consistent with the CD31 immunostaining of the tumors. Thus, the significant inhibition of tumor growth response...
reduced in tumors treated with bortezomib (Et al). Nuclear localization of RelA/p65 is greatly attenuated in melanoma tumors. Extracts from melanoma tumor homogenates were subjected to immunoblot analysis for expression of RelA/p65. The control tumors stain strongly for nuclear RelA/p65 (A, and Bortezomib-treated tumors (C, and D). Nuclear localization of RelA/p65 is greatly reduced in tumors treated with bortezomib (E and F). The density is expressed as counts per 10 fields per tumor for a total of 5 tumors from each treatment group; * * , P < 0.001. The density is expressed as counts from 10 fields per tumor for a total of 5 tumors from each treatment group, bars, ±SE. F, bortezomib inhibits vascular endothelial growth factor production in melanoma tumors. Tumor extracts were subjected to human vascular endothelial growth factor ELISA assay, and the values were normalized to total protein level in the samples. The experiments were done in triplicate (n = 3). TMZ, temozolomide.

of mice treated with bortezomib may be attributed partly to decreased angiogenesis in these tumors.

DISCUSSION

Melanoma presents a great challenge because of its resistance to systemic therapy and aggressive nature after dissemination (3). Patients at high risk for recurrence (stage III) are frequently treated adjuvantly with IFN-α. Its effectiveness is widely debated, but even supporters acknowledge its benefit as small, accompanied by a large cost in toxicity (34). Patients with metastatic disease (stage IV) have a median survival of 6–10 months with a 5-year survival of <5% (4). Effective treatment options are limited at best. Although both active and passive immunotherapy has been pursued vigorously over the past few decades, no melanoma vaccine has proven effective, and only interleukin 2 therapy has led to durable remission in only 5–8% of patients treated (35). The expectation is that novel treatment agents that target signaling pathways important to melanoma may offer hope for an otherwise dismal disease.

A growing body of evidence suggests that melanomas acquire the ability to attenuate signals that would normally lead to apoptosis by using major transcriptional regulators such as p53 and NFκB. The tumor suppressor protein p53 plays an important role in the regulation of the mitochondrial apoptotic pathway by transcriptional activation of proapoptotic Bcl-2 family members (such as Bax, Bam, Puma, and Noxa; reviewed in Refs. 36, 37) and by repression of antiapoptotic Bcl-2 family proteins (38, 39). Although p53 is not mutated in the majority of human melanomas, altered or impaired transcriptional activities of p53 have been reported (40, 41). NFκB, on the other hand, attenuates tumor necrosis factor-α-induced apoptosis by up-regulating expression of c-IAP1, c-IAP2 (42, 43), TRAF-1, TRAF-2 (43, 44), and c-FLIP (45). Advanced melanomas often exhibit a high level of TRAF-2 expression (46), which results in constitutively active stress kinases and constitutive activation of the IκB kinase pathway, resulting in the elevated levels of activated NFκB, thus feeding back into the circuit (23, 46–48). In addition to its role in protection against apoptosis, NFκB may also play an important role in resistance to conventional chemotherapy (49) by inducing expression of ATP-binding cassette transporters (50, 51). Thus, targeting these pathways
may prove to be advantageous in the treatment of malignant melanoma.

The aim of this study was to explore the potential use of the proteasome inhibitor bortezomib in the treatment of melanoma. Recent studies have shown that proteasome inhibitors represent novel anticancer therapeutic agents by inhibiting degradation of cell cycle regulatory proteins such as cyclins, cyclin-dependent kinase inhibitors, as well as other important regulatory proteins such as IκB. Our report highlights the capacity of bortezomib to overcome chemoresistance to conventional melanoma therapy and induce apoptosis in malignant melanoma tumors.

We first showed that bortezomib acts directly to inhibit the growth of melanoma cancer cell lines, more so than normal cell lines, and that this antimelanoma activity is enhanced when cells are treated with bortezomib in combination with the chemotherapeutic agent TMZ. Our data confirm additionally that the inhibitory effects of bortezomib on cell growth *in vitro* are potentially because of down-regulation of NFκB and, in turn, NFκB regulated genes such as cytokines CXCL8 and CXCLI that play an important role in promoting growth and metastasis of melanomas. Bortezomib also reduces expression of the ATP-binding cassette drug transporter family members MDR-1 and MRPI, rendering it a noteworthy candidate to be used in combination with many antineoplastic therapies, such as doxorubicin, that are inactivated through this pathway. Furthermore, bortezomib results in increased accumulation of important cell cycle regulatory proteins such as p53 and p21. Our study demonstrates that the *in vitro* data were comparable with *in vivo* studies in mice, which is evidenced by substantial tumor growth inhibition. Additional analysis of the tumor tissues revealed decreased NFκB activation as well as decreased vascular endothelial growth factor production, leading to decreased tumor microvasculature and, hence, an increased level of apoptosis in tumors. Although NFκB is a key factor in bortezomib-induced selective toxicity against melanoma, bortezomib acts also through multiple pathways to block cell proliferation and induce apoptosis in melanoma. One might assume that bortezomib may also act on other important survival signaling pathways in melanoma such as phosphatidylinositol 3'-kinase/ Akt and Raf/mitogen-activated protein kinase pathways, both of which have been shown to be disregulated in melanoma and to impinge on the NFκB pathway (52), therefore, warranting the use of bortezomib in combination with inhibitors of these pathways for future studies.

Our studies demonstrate that the proteasome inhibitor bortezomib both induces apoptosis and abrogates angiogenesis in human melanoma tumors. Moreover, bortezomib sensitizes these tumors to conventional TMZ chemotherapy. Given the impressive result from combination therapy of bortezomib with TMZ of human tumors in mice, these studies provide the framework for ongoing clinical trials of bortezomib in melanoma in the hope of improving the outcome for patients with advanced melanoma, who have a dismal prognosis with few therapeutic options available.

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4917
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