A Phase I Trial of Bortezomib with Temozolomide in Patients with Advanced Melanoma: Toxicities, Antitumor Effects, and Modulation of Therapeutic Targets

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Abstract

Purpose: Preclinical studies show that bortezomib, a proteasome inhibitor, blocks NF-κB activation and, combined with temozolomide, enhances activity against human melanoma xenografts and modulates other critical tumor targets. We initiated a phase I trial of temozolomide plus bortezomib in advanced melanoma. Objectives included defining a maximum tolerated dose for the combination, characterizing biomarker changes reflecting inhibition of both proteasome and NF-κB activity in blood (if possible tumor), and characterizing antitumor activity.

Experimental Design: Cohorts were enrolled onto escalating dose levels of temozolomide (50-75 mg/m²) daily, orally, for 6 of 9 weeks and bortezomib (0.75-1.5 mg/m²) by i.v. push on days 1, 4, 8, and 11 every 21 days. Peripheral blood mononuclear cells were assayed at specified time points for proteasome inhibition and NF-κB biomarker activity.

Results: Bortezomib (1.3 mg/m²) and temozolomide (75 mg/m²) proved to be the maximum tolerated dose. Dose-limiting toxicities included neurotoxicity, fatigue, diarrhea, and rash. Nineteen melanoma patients were enrolled onto four dose levels. This melanoma population (17 M1c, 10 elevated lactate dehydrogenase, 12 performance status 1-2) showed only one partial response (8 months) and three with stable disease ≥4 months. A significant reduction in proteasome-specific activity was observed 1 hour after infusion at all bortezomib doses. Changes in NF-κB electrophoretic mobility shift assay and circulating chemokines in blood failed to correlate with the schedule/dose of bortezomib, inhibition of proteasome activity, or clinical outcome.

Conclusions: We have defined phase II doses for this schedule of temozolomide with bortezomib. Although proteasome activity was inhibited for a limited time in peripheral blood mononuclear cells, we were unable to show consistent effects on NF-κB activation. Clin Cancer Res; 16(1); 348–57. ©2010 AACR.

Melanoma is a significant health problem in the United States. More than 68,000 new cases and greater than 8,500 deaths have been projected for 2009 (1). Therapeutic options beyond surgical treatment have had little effect on outcome. Chemotherapy has limited efficacy due in part to the multiple mechanisms of resistance of melanoma to apoptosis (2–5). Dacarbazine, an alkylating agent approved for treatment of melanoma, offers only a limited benefit for a small percentage of patients (5-20%) and usually for short durations (6). Temozolo-

mide, an oral chemotherapy, is converted to the active alkylating metabolite (methyl-triazene-1-yl)-imidazole-4-carboxamide like dacarbazine (7). Unlike dacarbazine, however, this conversion is spontaneous, nonenzymatic occurs under physiologic conditions in all tissues to which the drug distributes, and temozolomide is able to cross the blood-brain barrier. Although not Food and Drug Administration approved for melanoma, temozolomide is widely used and has response rates similar to dacarbazine in melanoma (7, 8). Temozolomide can be given by various schedules with limited toxicities, including daily for 6 to 8 weeks with a 2- to 4-week break (9). Nevertheless, resistance to most chemotherapy occurs de novo or is rapidly selected for in melanomas. There are likely multiple mechanisms of chemotherapy resistance, including those specific to dacarbazine or temozolomide, such as DNA repair of the adduct formation (O⁶-guanine) with the O⁶-methylguanaine-DNA methyltransferase (10, 11). Other mechanisms of resistance include Bcl-2 overexpression, silencing of apoptotic protease activating factor-1 gene (a key mediator of apoptosis), and activation of proliferative and antiapoptotic
signaling pathways including the mitogen-activated protein kinase and phosphoinositide 3-kinase/Akt pathways (4, 5, 12–14). In addition, NF-κB activation of its downstream target genes (angiogenesis, adhesion, antiapoptosis, and chemokine growth factors) likely plays a central role in the proliferation and chemotherapy resistance observed in melanoma (15–20). It is well established that chemotherapy itself can induce NF-κB activation in cancer cells (16, 20). Any number of these or other biological processes are likely important to chemotherapy resistance in melanoma.

More recently, pharmaceutical agents have been developed that inhibit proteasome degradation and limit the breakdown of numerous short-lived proteins, which regulate key cellular events (21–23). The regulation of the half-lives of short-lived proteins, including the cyclin-dependent kinase inhibitors p16, p21, and p27, by the proteasome is critical to the proliferation and survival of the malignant cell (22, 23). Inhibitor of NF-κB (IκB) is a protein that binds p50/p65 NF-κB in the cytosol, preventing its translocation into the nucleus required to activate its genetic program (24, 25). Cytoplasmic IκB levels are regulated primarily by proteasome-mediated degradation (26, 27). Among many inhibitors of the proteasome, bortezomib is the first inhibitor in the clinic to show striking clinical benefit in multiple myeloma (28–30). In phase I testing of single-agent bortezomib, one schedule [i.v. push (IVP) on days 1, 4, 8, and 11 every 21 days] showed dose-limiting toxicity (DLT) from neuropathy, diarrhea, rash, and fatigue (28, 29). The maximum tolerated dose (MTD) was 1.56 mg/m^2 i.v. on days 1, 4, 8, and 11 every 21 days. Phase II studies in melanoma with bortezomib alone have shown no sign of clinical antitumor activity (31).

We have previously reported impressive results with temozolomide in combination with bortezomib in a human melanoma xenograft model (32). Whereas both temozolomide and bortezomib have some clinical activity in this model, the combination led to durable complete responses in many mice even with cessation of therapy. The clinical benefit seemed to be secondary to bortezomib inhibition of temozolomide-induced NF-κB activation. Others have reported that bortezomib cytotoxicity for melanoma cells was dependent on NOXA (33, 34). NOXA is a melanoma-associated (not normal melanocytes) proapoptotic Bcl-2 homologue. Additionally, bortezomib causes endoplasmic reticulum stress that rapidly induces components of the proapoptotic/terminal unfolded protein response.

The development of combination therapy with temozolomide and bortezomib may provide a means to improve the frequency and duration of temozolomide responses. We undertook a phase I clinical trial to develop a combination regimen of temozolomide and bortezomib for patients with advanced melanoma in hopes of recreating the benefit observed in melanoma xenografts. By defining the DLT and MTD for the combination, while assessing molecular changes in the blood (and tumor if feasible), we focused on showing that the combination of temozolomide and bortezomib can modulate proteasome activity and determine whether there are in vivo changes indicative of NF-κB inhibition.

Materials and Methods

Patient eligibility. Patients were required to have histologically proven, advanced-stage (III or IV) melanoma not curable by standard therapies, an Eastern Cooperative Oncology Group performance status of 0 to 1, good organ function, and no grade ≥2 peripheral neuropathy or ≥320 mg/m^2 cisplatin cumulatively, with no limit on prior treatments. Patients with controlled brain metastases off systemic steroids were also eligible. Patients were ages ≥18 y and competent to sign an Institutional Review Board–approved informed consent.

Tumor biopsy for research purposes. Tumor biopsies before and after treatment with bortezomib were taken from patients with either a 14-gauge core needle or incisional biopsy of at least 5 mm in diameter that was easily accessible, including skin, soft tissue, or lymph node metastases, who signed informed consent to allow removal of tissue for research purposes. Immediately after harvest of melanoma tissues, all samples were fixed in 4% paraformaldehyde for at least 24 h and embedded in paraffin. These biopsies, taken on day 0 before treatment and on days 21 to 35 after treatment, were analyzed for activation of RelA/p65 based on phosphorylation of RelA/p65 at Ser^29 by nuclear staining for RelA/p65.

Treatment schedule and dose level. Treatment was initiated with bortezomib delivered as an IVP over 3 to 5 min on days 1, 4, 8, and 11 of every 21-d cycle. Temozolomide was initiated orally after bortezomib IVP and blood sample collection on day 8 of the first cycle and continued daily for 6 wk (Fig. 1). This was followed by a 3-wk rest without temozolomide to keep on schedule with the 3-wk bortezomib cycles. A single cycle of combined therapy was a total of 9 wk.
**Definition of DLT and toxicity criteria.** DLTs were monitored and evaluated in a standard design with standard criteria based on the Common Terminology Criteria for Adverse Events version 3 from Cancer Therapeutics Evaluation Program. All grade 3 and 4 nonhematologic toxicities were considered dose limiting, whereas grade 4 hematologic and prolonged grade 3 hematologic toxicities were dose limiting. This excluded non-life-threatening grade 3 toxicities that were either inadequately treated or very transient (<24 h). Dose escalation continued until DLT allowed no further escalation. A minimum of three patients was enrolled onto each dose level; the dose level was declared above the MTD if two of three, or two or more of six patients had DLT due to therapy. Finally, six patients were added to the MTD dose level to more accurately assess toxicity before phase II studies.

**Schedule of correlative studies.** Blood was obtained at baseline before initiation of therapy on days 8 and 29 following bortezomib treatment for assays of NF-κB activation by electrophoretic mobility shift assay (EMSA) using nuclear extracts of isolated peripheral blood mononuclear cells (PBMC) and for assays of serum chemokine levels by ELISA (Fig. 1). We were predominantly interested in NF-κB activation biomarkers in the day 8 sample following bortezomib day 1 of 21-d cycle and then 3 wk later on day 29 (3-wk cycle 2, day 8) following bortezomib after having been treated with 21 d of temozolomide. The rationale for examining NF-κB activation after temozolomide treatment is based on the assumption that temozolomide would further induce NF-κB expression in vivo and bortezomib could block this effect. When feasible, fresh tumor biopsy was obtained for immediate fixation before treatment and after day 21 of bortezomib. On treatment days 1, 8, and 32, blood for inhibition of 20S proteasome enzyme activity (pharmacodynamic marker) was taken both before bortezomib and 1 h after infusion (only on days 8 and 32). These assays of proteasome activity before infusions of bortezomib provided us with a steady-state measurement. The 1-h postbortezomib assays were done only to show that 20S proteasome inhibition by bortezomib was equivalent with and without temozolomide treatment. All samples were processed and frozen at −80°C until testing.

**Melanoma tissue preparation and immunohistochemical analysis.** From melanoma tissue embedded in paraffin, 6-μm-thick sections (three sections each) were cut and placed on glass slides and deparaffinized with xylene. The antigen was unmasked by heating samples in 10 mmol/L sodium citrate buffer (pH 6.0) for 5 min, and then the reaction was quenched using hydrogen peroxide. Tissue sections were immunostained for phospho-NF-κB expression using anti-human phospho-p65 NF-κB antibodies (1:100; Cell Signaling) based on findings that Ser 529 phosphorylation is important for IKK1 activation of NF-κB during tumor growth (35). Isootypic IgG of the same class and/or a second antibody served as negative controls. The ABC biotin/avidin reagent kit was used to visually capture the immunolocalization of antigen using Novared substrate, and cell contents were counterstained with hematoxylin. All primary antibody incubations were done in a moisture chamber overnight at 4°C.

**EMSA of NF-κB DNA binding activity in patient PBMCs.** As nuclear translocation of p65/p50 heterodimer or p65 homodimers and the binding of these NF-κB elements to DNA consensus promoter sequence are the critical steps for gene transactivation, the major nuclear NF-κB elements, p65 and p50, were determined by supershift assay with anti-human p65 and anti-human p50 antibodies in...
the EMSA. Patient leukocyte nuclear extracts were prepared for EMSA, and the preparation protocol was adopted as previously reported and described in Supplementary Materials and Methods (36).

**Fluorometric 20S assay.** The 20S proteasome activity assay was reported previously and as described in Supplementary Materials and Methods (37).

**ELISA of serum chemokine concentrations.** Serum levels of chemokines [MGS/CXCL1, interleukin (IL)-8/CXCL8, MIP-1α/CCL3, RANTES/CCL5, and vascular endothelial growth factor (VEGF)] were determined by ELISA. All ELISA kits were purchased from R&D Systems, and ELISA was done according to the manufacturer's instructions in triplicate for each patient serum sample for the determination of serum levels of each chemokine/cytokine. In many instances, the samples were analyzed at several dilutions to ensure that the readings were within the linear portion of the standard curve for accurate determinations of chemokine/cytokine levels.

**Table 1. Patient characteristics**

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>No. patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
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<tr>
<td>Male/female</td>
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<tr>
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</tr>
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</tr>
<tr>
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</tr>
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<td>11</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>M1 staging</td>
<td></td>
</tr>
<tr>
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<td>0</td>
</tr>
<tr>
<td>M1b</td>
<td>2</td>
</tr>
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<tr>
<td>Serum lactate dehydrogenase</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>9</td>
</tr>
<tr>
<td>Elevated</td>
<td>10</td>
</tr>
<tr>
<td>Disease sites involved</td>
<td></td>
</tr>
<tr>
<td>Subcutaneous</td>
<td>9</td>
</tr>
<tr>
<td>Lymph node</td>
<td>8</td>
</tr>
<tr>
<td>Lung</td>
<td>13</td>
</tr>
<tr>
<td>Liver</td>
<td>7</td>
</tr>
<tr>
<td>Brain</td>
<td>3</td>
</tr>
<tr>
<td>Other viscera</td>
<td>10</td>
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<tr>
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<tr>
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<td>Yes/no</td>
<td>11/8</td>
</tr>
<tr>
<td>Prior chemotherapy (metastatic disease)</td>
<td></td>
</tr>
<tr>
<td>Yes/no</td>
<td>6/13</td>
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</table>

**Statistical methods.** The primary objective of this study was to evaluate the safety of bortezomib + temozolomide and identify the MTD of these two agents among patients with advanced-stage (III or IV) melanoma. The study design was a standard 3+3 algorithm. Categorical variables were summarized using frequencies, whereas continuous variables were reported using the median (range). To show proof of principle, we assessed serum levels of proteasome activity, NF-κB activity, and cytokines by dose across time using a linear model with normal errors that included a repeated-measures component for multiple observations on the same patient. The natural logarithm transformation was used as necessary to meet Gaussian model assumptions. Separate models were built for data from baseline to predosing measurements on day 8 and then day 8 to day 32. P values comparing pretreatment and posttreatment values on the same day are from the Wilcoxon signed rank test. The degrees of proteasome inhibition increased from baseline by 14.6% on day 8 before bortezomib (P = 0.008) and 26.8% on day 32 before bortezomib (P = 0.0004). The 1-h postbortezomib values for proteasome inhibition compared with prebortezomib on days 8 and 32 were 56.8% and 63.8%, respectively.

**Fig. 2.** Effect of bortezomib on proteasome activity short term (1 h after) and at steady state. The natural logarithm of proteasome activity data points overlay box plots, depicting the interquartile range (bottom line and top line of boxes) and median (thick solid bar). Box whiskers extend to the minimum and maximum data values but no further than 1.5 times the interquartile range. P values across days are from linear regression models with repeated measures comparing pretreatment values from baseline to day 8 and then day 8 to day 32. P values comparing pretreatment and posttreatment values on the same day are from the Wilcoxon signed rank test. The degrees of proteasome inhibition increased from baseline by 14.6% on day 8 before bortezomib (P = 0.008) and 26.8% on day 32 before bortezomib (P = 0.0004). The 1-h postbortezomib values for proteasome inhibition compared with prebortezomib on days 8 and 32 were 56.8% and 63.8%, respectively.
Table 2. Dose levels of temozolomide and bortezomib evaluated in phase I trial

<table>
<thead>
<tr>
<th>Dose levels</th>
<th>Temozolomide (orally, daily, 6 of 9 wk)</th>
<th>Bortezomib (i.v., on days 1, 4, 8, and 11 every 21 d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50 mg/m²</td>
<td>1.00 mg/m²</td>
</tr>
<tr>
<td>2</td>
<td>75 mg/m²</td>
<td>1.00 mg/m²</td>
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<td>3</td>
<td>75 mg/m²</td>
<td>1.00 mg/m²</td>
</tr>
<tr>
<td>4</td>
<td>75 mg/m²</td>
<td>1.00 mg/m²</td>
</tr>
</tbody>
</table>

Results

Clinical characteristics. Nineteen metastatic melanoma patients were enrolled onto this phase I trial (Table 1). Enrollment included a preponderance of male patients (15:4, male/female), 3 patients with ocular primary, 17 with M1c disease, and 10 with elevated serum lactate dehydrogenase. Disease sites included liver metastases in seven patients and three with brain metastases. Most patients (13) had prior therapy for metastatic disease, including six with prior dacarbazine or temozolomide. Eight patients had prior adjuvant IFN therapy.

Toxicities and clinical outcome. Patients enrolled onto the trial at the initial two dose levels [1.0 mg/m² bortezomib/50 mg/m² temozolomide and 1.0 mg/m² bortezomib/75 mg/m² temozolomide] had minimal grade 1 or 2 toxicity. There were only two patients with grade 2 fatigue or anorexia. On the other hand, dose level 4 (1.5 mg/m² bortezomib/75 mg/m² temozolomide) was associated with DLTs in two of the initial three patients enrolled. Toxicities included grade 3 fatigue, nausea/vomiting, diarrhea, neuropathy, and rash in patients 10 and 11 (Supplementary Table S1). Dose level 3 (1.3 mg/m² bortezomib/75 mg/m² temozolomide) ultimately enrolled a total of 10 patients with 9 evaluable. Toxicities of significance at this dose level were observed in three patients (13, 17, and 18), including fatigue, nausea/vomiting, and diarrhea. These toxicities were all grade 3 but transient and rapidly reversible during planned rest periods off drugs (Supplementary Table S1). Therefore, this dose (1.3 mg/m² bortezomib/75 mg/m² temozolomide) was declared the MTD. There were no grade 3 or 4 hematologic toxicities at any of the dose levels during the initial 9 weeks of treatment.

There was a single objective response (partial response for 8-month duration) at dose level 3, and three other patients at dose level 1 (two patients) and level 3 (one patient) had stable disease for ≥4 months (4, 4, and 30 months). There were four patients with rapid disease progression before the 9-week evaluation, but the duration on treatment for three of these patients allowed assessment of toxicity. Median (95% confidence interval) progression-free and overall survival were 2.1 months (2.00-2.23) and 6.3 months (4.14-12.68), respectively. At this time, all patients have progressed and only one patient remains alive, being censored at last follow-up.

Inhibition of proteasome activity. For each patient, one blood sample was collected on day 0, and both before and 1 hour after bortezomib administration on days 8 and 32 for 20S proteasome activity in isolated PBMC. The data provide information on the sustained or nadir level of proteasome inhibition (when comparing the values in day 0, day 8 before bortezomib infusion, and day 32 before bortezomib infusion). In addition, we were able to assess the inhibition of proteasome activity by bortezomib from the 1-hour postinfusion (by comparing the values of presamples and postsamples on days 8 and 32). As shown in Fig. 2, the median values of 20S proteasome activity at day 0, before treatment, and before bortezomib at days 8 and 32 (nadir values) were 0.41 ± 0.11, 0.35 ± 0.07, and 0.30 ± 0.06 pmol/s/mg protein. The proteasome activity achieved at the nadir before bortezomib treatment at days 8 and 32 was decreased by 14.6% and 26.8%, respectively, from baseline (day 0). These decreases were statistically significant compared with baseline (P = 0.006 and 0.0004). To assess the influence of temozolomide therapy on bortezomib pharmacodynamics, we compared bortezomib-induced inhibition of proteasome activity at 1 hour after its IVI before temozolomide treatment (on day 8) and during (on day 32) temozolomide treatment course. The results showed similarly significant decrease of proteasome activity in both day 8 (56.8%) and day 32 (63.8%) at 1 hour after bortezomib, with values suggesting minimal interference of temozolomide on bortezomib-induced proteasome inhibition. In addition, no significant dose-dependent difference in the bortezomib IVI-induced inhibition of proteasome activity was observed among the four dose levels of the bortezomib/temozolomide combination.

Systemic inhibition of NF-κB in PBMC. Systemic inhibition of NF-κB (RelA/p50) activation level was determined by EMSA on patient PBMC isolated from blood samples collected on day 0, day 8 after bortezomib infusion, and day 32 after bortezomib infusion (Fig. 1; Table 2). Overall, the majority of patients accrued at dose level 3 (MTD) were included in the assay. The data showed variable levels of NF-κB activation in PBMC at baseline (day 0) as well as following treatment at days 8 and 29. Presumably, the degree of bortezomib inhibition of proteasome activity at day 29 would be equivalent to day 32 because we saw no change in the inhibition of proteasome activity comparing day 8 with day 32. Some patients did have declines in NF-κB activation level at day 8 following bortezomib, before temozolomide, but this was still quite variable (Fig. 3).
Chemokine expression profile during the clinical trial. As indirect biomarkers, we hypothesized that peripheral blood cytokines and chemokines may be a sensitive indicator of NF-κB activity (38–40). Therefore, we assayed several factors, including MGSA/GROα/CXCL1, IL-8/CXCL8, MIP-1α/CCL3, RANTES/CCL5, and VEGF levels. All of the cytokines/chemokines were analyzed on days 0, 8, and 29; identical to NF-κB EMSA (Fig. 4). Higher baseline CXCL1 \((P = 0.021)\) correlated with improved progression-free survival. However, these associations were not large enough to reach statistical significance in relation to overall survival. The changes seem to differ among doses and time points but for the most part showed no definite trend. Many of the chemokines seemed to trend upward by the day 29 time point.

Discussion

The ability of bortezomib to block NF-κB activation through proteasome inhibition of IκB degradation, along with the antitumor activity of temozolomide plus bortezomib against human melanoma xenografts, provided a rationale to examine the toxicity, pharmacodynamics, and clinical response of patients with advanced malignant melanoma treated on a phase I study of bortezomib in combination with temozolomide (32, 41). We hypothesized that the inhibition of proteasome activity leading to inhibition of NF-κB may enhance the antitumor effects of the DNA alkylating agent temozolomide.

![Fig. 3. Nuclear NF-κB DNA binding changes with therapy of bortezomib and temozolomide. The natural logarithm of EMSA data points overlay box plots, depicting the interquartile range (bottom line and top line of boxes) and median (thick solid bar). Box whiskers extend to the minimum and maximum data values but no further than 1.5 times the interquartile range. \(P\) values across days are from mixed-models ANOVA for repeated measures comparing pretreatment values from baseline to day 8 and then day 8 to day 29.](image)

The primary end point of the trial was the definition of a MTD for bortezomib in combination with temozolomide and secondarily to show that bortezomib is able to exert sufficient pharmacologic effect on the proteasome activity to impair NF-κB activation. DLT was reached at the 1.5 mg/m\(^2\) bortezomib/75 mg/m\(^2\) temozolomide dose level with toxicities including grade 3 and 4 fatigue, nausea and vomiting, diarrhea, neuropathy, and rash. The MTD or recommended phase II dose was determined to be at 1.3 mg/m\(^2\) bortezomib/75 mg/m\(^2\) temozolomide, where a total of 10 patients (9 evaluable) were enrolled. A significant reduction in the specific activity of the proteasome enzyme activity was observed at all dose levels. However, proteasome activity returned to within 15% to 30% of baseline before the subsequent dosing of bortezomib. The degree and short-lived nature of proteasome inhibition was not accompanied by consistent decreases in NF-κB activity within PBMC or the target genes of NF-κB as reflected by serum chemokine levels.

Biopsy samples were obtained from the nine patients with accessible tumor before treatment, but only three patients had both pretreatment and posttreatment tumor tissue. The few paired biopsies were due to several factors, including decline in patients' performance status, patient refusal, inaccessibility of tumors remaining, and inability to coordinate further biopsies with the surgeon. Tissue sections were immunostained for phospho–NF-κB expression using anti-human phospho-p65 NF-κB Ser\(^{529}\). We observed staining for p65 on Ser\(^{529}\) in a subpopulation of cells within the tumor. This was done based on findings that Ser\(^{529}\) phosphorylation is important for tumor necrosis factor-α–induced activation of NF-κB during tumor growth (35). As shown in Supplementary Fig. S1, patient 5 did have an apparent reduction of Ser\(^{529}\) p65 staining following therapy. Unfortunately, due to the few pre- and posttreatment paired tumor biopsies, there was an inadequate number of patients to determine if immunohistochemical staining of Ser\(^{529}\) p65 could be a useful biomarker for bortezomib therapy. These data suggest that in phase II studies, this analysis should be prioritized to evaluate the effects of treatment on RelA/p65 phosphorylation status and that we should extend the analysis to include p65 phosphorylation on Ser\(^{365}\), which is very important for NF-κB transcriptionsal activation.

The inability to show target (NF-κB) inhibition in peripheral blood could be attributed to several potential issues with the study. Possible reasons for our inability to show NF-κB inhibition include the following:

1. The time points when the assays were done were scheduled in a way that missed the important degree of NF-κB inhibition. Other time points may have shown more effective NF-κB inhibition.
2. The dose and/or schedule of bortezomib administered were inadequate to effectively block the proteasome to the degree and duration that would allow levels of IκB to increase, thereby preventing NF-κB activation.
Fig. 4. Changes in chemokine levels in peripheral blood with therapy of bortezomib and temozolomide. The natural logarithm of chemokine data points overlay box plots, depicting the interquartile range (bottom line and top line of boxes) and median (thick solid bar). Box whiskers extend to the minimum and maximum data values but no further than 1.5 times the interquartile range. P values across days are from mixed-models ANOVA for repeated measures comparing pretreatment values from baseline to day 8 and then day 8 to day 29.
3. At tumor sites, NF-κB inhibition may have been superior to that shown in blood. This is unlikely because it is difficult to believe that drug delivery to tumor would be better than delivery to circulating cells. Furthermore, the relative lack of promising clinical activity suggests that this is not the case. However, it will remain essential to obtain paired biopsies before and during therapy to address this question. In the early stage of the phase II trial, we have already obtained 10 paired biopsies.

4. It is entirely possible that bortezomib inhibition of proteasome activity does not effectively inhibit NF-κB even at the optimal dose and schedule of proteasome inhibition. The association between proteasome inhibition and NF-κB inhibition in patients has been assumed based on preclinical data but never convincingly established in cancer patients. Demonstration of this is even lacking for multiple myeloma or mantle cell lymphoma where bortezomib is a very active single agent (28–30, 34).

5. Finally, bortezomib may result in melanoma cell death through other mechanisms such as c-myc–dependent increase in NOXA, a proapoptotic protein, leading to apoptosis (33, 34). Bortezomib causes endoplasmic reticulum stress that rapidly induces components of the proapoptotic/terminal unfolded protein response, including PERK, elf-2α kinase, ATF4 (an endoplasmic reticulum stress–induced transcription factor), and its proapoptotic target (CHOP/GADD153). Ultimately, apoptosis occurs via c-Jun NH₂-terminal kinase–dependent mechanisms and caspase-4. In addition, bortezomib will interfere in the breakdown of cell cycle regulatory proteins (p16, p21, and p27) causing cell cycle arrest, stabilize other proapoptotic proteins (p53 and Bax), and reduce levels of other antiapoptotic proteins (Bcl-2). These effects may not be inducible in vivo, and subsequent apoptosis may not occur as expected. We are exploring this further in the phase II trial already in progress, where more matched (pretreatment and posttreatment) specimens will be obtained.

Although frustrated by the inability to show NF-κB inhibition in blood and the lack of antitumor efficacy, we have been able to establish a tolerable dose and schedule for this combination for future phase II trials. We have undertaken a phase II trial at these doses in chemotherapy-naïve and chemotherapy-refractory melanoma patients. A major effort has been made to do pharmacodynamic studies at tumor sites through paired biopsies as part of this phase II trial. We still believe that NF-κB provides a good target for melanoma therapy but may require a less toxic proteasome inhibitor that allows for continuous administration (orally or s.c.) or a more targeted approach to NF-κB inhibition, such as a specific IKKα/β inhibitor (3). Five recent publications describe trials combining bortezomib either with temozolomide and irradiation in brain tumors, with pegylated liposomal doxorubicin or doxorubicin in solid tumors, or with reirradiation in squamous cell carcinoma of the head and neck (42–45). The Kubicek and Werner-Wasik trial combining bortezomib with temozolomide and irradiation in brain tumors did not reach DLT but was able to administer the same doses (1.3 mg/m² bortezomib and 75 mg/m² temozolomide) as we found safe but with concurrent brain radiotherapy. The investigators did not evaluate any pharmacodynamic markers of proteasome or NF-κB inhibition but did describe some promising antitumor results (42). LoConte et al., combining doxorubicin with bortezomib, showed MTD as 1.3 mg/m² bortezomib on days 1, 4, 8, and 11 and doxorubicin at 20 mg/m² on days 1 and 8. Toxicities included neutropenia, thrombocytopenia, neuropathy, and diarrhea. There were some inconsistent increases in ubiquitination protein complexes in the blood of patients (43). The Dees et al. trial defined the MTD as the same dose and schedule of bortezomib at 1.3 mg/m² on days 1, 4, 8, and 11 and pegylated liposomal doxorubicin as 30 mg/m². The DLT included cytopenias, nausea, vomiting, and diarrhea. The trial included pharmacodynamics of proteasome inhibition showing no influence of concurrent chemotherapy (44). Finally, Van Waes et al. (45) treated only nine patients at two dose levels, 0.6 and 0.9 of bortezomib, with reirradiation to squamous cell cancer of the head and neck cancer. In all patients, the MTD was exceeded at both doses and DLT included hypotension and hyponatremia. Proteasome inhibition was monitored in blood at 1, 24, and 48 hours following bortezomib. Only two patients had tumor biopsies obtained and these revealed genes activated by NF-κB activation, colocalization into nucleus of p65 and apoptosis, and reduction in cyclin D1. In a single patient, there was a drop in serum IL-8 and IL-6, consistent with NF-κB inhibition. Recently, a follow-up study on patients treated with 0.6 mg/m² bortezomib before irradiation was done on matched biopsies. For three of the four patient biopsies obtained and analyzed for bortezomib inhibition of NF-κB, there was a reduction in nuclear REL, but other NF-κB subunits (extracellular signal-regulated kinase 1/2 and signal transducer and activator of transcription 3) were variably affected or not affected (46). None of these trials were successful at showing consistent pharmacodynamic effects on NF-κB or other secondary markers. Proteasome activity in blood was consistently inhibited by bortezomib and unaffected by chemotherapy or irradiation when examined.

Finally, others have found enhanced effects of bortezomib in combination with mitogen-activated protein kinase pathway inhibitors (mitogen-activated protein/extracellular signal-regulated kinase kinase inhibitors) on apoptotic pathways in melanoma xenografts, providing a rationale for combining the two agents in melanoma therapy (47–49). The melanoma research community must remain committed to the development of new and novel drug therapy based on strong preclinical rationale.
Furthermore, regardless of clinical efficacy, performance of corollary studies that attempt to show effective target inhibition are critical. The effort should be aimed at testing changes at tumor sites whenever possible. These studies provide insight into a complex and resistant disease such as melanoma and offer potential for better avenues of treatment.

Disclosure of Potential Conflicts of Interest

J.A. Sosman received a commercial research grant from Millenium to assist in the performance of the clinical trial. The other authors disclosed no potential conflicts of interest.

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