A Phase II Trial with Pharmacodynamic Endpoints of the Proteasome Inhibitor Bortezomib in Patients with Metastatic Colorectal Cancer

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Abstract Purpose: To evaluate the effects of the proteasome inhibitor bortezomib on tumor growth in patients with advanced colorectal cancer, and to explore the relationship between correlative studies and clinical outcome.

Design: Bortezomib (1.3 mg/m²) was administered i.v. on days 1, 4, 8, and 11 of a 21-day cycle. Tumor response was assessed after every two cycles. Tumor biopsies were done prior to treatment and on day 9 of the first treatment cycle. Biopsies were examined for Ser32/36-IκB, Ser276, nuclear factor-κB (NF-κB), hypoxia-inducible factor-1α (HIF-1α), carbonic anhydrase IX (CAIX), p53, and microvessel density using immunohistochemistry.

Results: Nineteen patients received 42 cycles (range 1-4) of bortezomib. No objective response was seen; three patients had stable disease at cycle 2, two patients had progressive disease after cycle 1, and 11 patients had progressive disease at cycle 2. Of the three patients with stable disease, one had progressive disease after cycle 4, and two were withdrawn due to toxicity. The median time to progression was 5.1 weeks (95% confidence interval, 5.1-11.1 weeks). There was a significant increase in the expression of HIF-1α relative to its transcriptional target CAIX following bortezomib, and a similar effect was also observed in a companion study using a human tumor xenograft model. Expression of p53, Ser32/36-IκB, and Ser32/36-IκB was unchanged.

Conclusion: Single agent bortezomib is inactive in metastatic colorectal cancer. Using this regimen, there was no detectable effect on NF-κB, but a significant accumulation of HIF-1α was seen relative to CAIX. This suggests that proteasome inhibition alters the response to tumor hypoxia, and further investigation of this effect is indicated.

Despite the introduction of regimens incorporating the newer agents irinotecan and oxaliplatin, the median survival for patients with metastatic colorectal cancer remains <20 months. The poor outlook for these patients warrants the investigation of new agents with novel mechanisms of action (1).

The ubiquitin-proteasome proteolytic pathway regulates the metabolism of key proteins including cyclins, p21, and p27 required for cell cycle progression (2). In addition, the proteasome regulates major transcription factors, such as p53, nuclear factor-κB (NF-κB) and hypoxia-inducible factor-1α (HIF-1α). NF-κB is activated by proteasome-mediated degradation of the inhibitory protein IκB (3). Activated NF-κB promotes cell survival, angiogenesis, and metastasis by inducing expression of pro-survival proteins (bcl2), cell adhesion molecules, and growth factors (interleukin-6; refs. 4, 5). Inhibition of the proteasome results in stabilization of IκB, enhanced apoptosis and down-regulation of factors related to cancer progression and metastasis (5, 6). In contrast, HIF-1α, which plays a major role in promoting cell survival under conditions of tumor hypoxia, is inactivated under aerobic conditions by proteasomal degradation of the HIF-1α subunit. Therefore, proteasomal inhibition might favor survival by accumulation of HIF-1α, although in vitro data suggests that under these conditions, HIF-1α may be transcriptionally inactive (7).

Targeting the proteasome has recently emerged as a novel approach to cancer therapy (8). Bortezomib (formerly known as PS-341), a dipeptidyl boronic acid, is a specific and highly selective inhibitor of the 26S proteasome. In preclinical studies, bortezomib shows significant and wide-ranging antitumor activity (8). I.v. administration of bortezomib to tumor-bearing mice results in significant regression in two human colon (LoVo and HT-29) cancer xenograft models (8, 9). A phase II trial of bortezomib in patients with metastatic colorectal cancer is therefore appropriate. Investigation of the biological effects of novel agents within tumor tissue is vital to understanding mechanisms of drug action at the molecular level. Therefore, in addition to the primary aims of determining the efficacy and toxicity in patients with advanced colorectal cancer, we investigated the pharmacodynamic effects using core biopsies obtained from liver metastases pretreatment and after 9 days of treatment with bortezomib. Whereas we found no consistent

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effects on p53 or NF-κB expression, differential effects on HIF-1α and the HIF-1 transcriptional target carbonic anhydrase IX (CAIX) indicated a significant disruption of the tumor response to hypoxia following treatment with bortezomib. This effect was also seen in a companion study using a human tumor xenograft model of hypoxia, suggesting that proteasome inhibitors should be further tested for their ability to target hypoxic tumor tissue.

Materials and Methods

This was an open-label, nonrandomized multicenter phase II study by the Princess Margaret Hospital Phase II Consortium. The study was approved by the Local Research Ethics Committee at each of the participating institutions.

Patients. All patients entered into this study had histologically or cytologically confirmed recurrent or metastatic colorectal adenocarcinoma. Eligibility criteria included: life expectancy ≥12 weeks; Eastern Cooperative Oncology Group performance status ≤1, adequate hematologic (absolute neutrophil count ≥2 × 10^9/L; platelets ≥100 × 10^9/L), hepatic (bilirubin ≤1.25× upper limit of normal; aspartate aminotransferase/alanine aminotransferase ≤5× upper limit of normal) and renal function (serum creatinine ≤1.25× upper limit of normal) with at least one site of measurable disease (≥1 cm in at least one dimension with spiral computed tomography scan). Patients had received ≤1 prior line of chemotherapy for metastatic disease. This could include 5-fluorouracil, irinotecan and/or oxaliplatin (or 5-fluorouracil followed by irinotecan or oxaliplatin). Irradiation was allowed provided it was completed ≥4 weeks before study entry. Patients were excluded if they had ≥grade 1 neuropathy. In cases where there was a medical contraindication to tumor biopsy, study entry was at the discretion of the principal investigator. Written informed consent was obtained from all patients prior to starting treatment.

Treatment. Bortezomib (1.3 mg/m^2) was administered as an i.v. bolus over 3 to 5 seconds on days 1, 4, 8, and 11 of a 21-day cycle. Vital signs were recorded prior to treatment and every 15 minutes for 30 minutes after treatment. Routine antiemetic or anti diarrheal premedication was not required. Patients experiencing ≥grade 1 nausea or emesis received antiemetics, according to local practice, in subsequent cycles. Following a first loose stool, patients were advised to start loperamide (2 mg every 2 hours during the day and every 4 hours at night) and continue until free from diarrhea for 12 hours. During therapy, patients underwent three weekly (more frequently if clinically indicated) toxicity assessments, Eastern Cooperative Oncology Group performance status evaluation, and full physical examination. Complete blood count and biochemical profiles were done on days 1, 4, 8, and 11 for the first two cycles and on days 1 and 8 in subsequent cycles. Dose reductions were made on the basis of toxicity: ≥grade 3 hematologic toxicity, two dose reductions; ≥grade 3 diarrhea or grade 1/2 neurotoxicity, one dose reduction (dose level −1.1 mg/m^2; −2, 0.9 mg/m^2). Grade 3 neurotoxicity, treatment delay for ≥2 weeks, or requirement for a third dose reduction resulted in withdrawal from study. Treatment was discontinued for unacceptable toxicity, disease progression, or withdrawal of consent.

Evaluation of tumor response and toxicity. Response was evaluated, according to the Response Evaluation Criteria in Solid Tumors guidelines, after every 6 weeks (two cycles) of chemotherapy. Toxicity was assessed according to the expanded National Cancer Institute of Canada-Common Toxicity Criteria version 2.

Correlative studies. Tru-cut core biopsies of liver metastases were taken prior to, and on day 9 (or 10) of the first cycle of treatment, immediately placed in 10% neutral buffered formalin overnight, then processed for paraffin embedding. Four-micron-thick tissue sections were cut, de waxed, and rehydrated, then subjected to microwave antigen retrieval. Sections were labeled with the following primary antibodies: phospho-IκB (Ser^22/23) 1:200 dilution overnight, and phospho-NF-κB (Ser^276) 1:50 dilution overnight, both from Cell Signaling Technology (Beverly MA); p53 (Dako D07, Glostrup, Denmark) 1:100 dilution overnight; HIF-1α (BD Transduction Labs, Lexington, KY) 1:50 dilution overnight; CAIX (a gift from Dr. Adrian Harris, University of Oxford, United Kingdom) 1:200 dilution overnight; and CD34 (Novoceastra QBend10, Newcastle-upon-Tyne, United Kingdom) 1:80 dilution for 1 hour. The slides were then developed for immunohistochemistry using appropriate secondary antibodies and a standard streptavidin/biotin-immunoperoxidase method, with NovaRed (Vector Laboratories, Burlingham, CA) as the chromogen and hematoxylin as the counterstain.

The immunohistochemically stained sections, and an additional section stained with H&E, were first examined by the study pathologist (M-S. Tsao). Areas of viable tumor within the cores were identified, the extent of antibody staining assessed descriptively, and in consultation with D. Hedley and T. Nicklee, appropriate protocols for digital image capture and analysis were determined. Serial sections labeled for HIF-1α, CAIX, and CD34 were imaged at 10× objective, and composite, tiled-field images of the entire section obtained using a scanning autocstage as previously described (10). The images of phospho-NF-κB staining were obtained by blue laser light absorption, using a laser scanning system (TISSELeScope, Biomedical Photometrics, Waterloo, ON). The laser spot size used gave a pixel resolution of ~2 μm. Image analysis was done using MCID Elite software (Imaging Research, St. Catharines, ON).

Statistical methods. Summary statistics, such as the mean, median, range and, proportion were used to describe the patient sample. Overall and progression-free survival estimates were computed using the Kaplan-Meier method. Survival times were taken from the day the patient first received therapy until death or last known follow-up date for overall survival, and until death, date of progression, or last known follow-up date for progression-free survival.

HIF-1/CAIX ratio was calculated by summing the HIF-1 value for all sections of tissue imaged and dividing this value by sum of CAIX values for all tissue sections. This was done for each patient pre- and posttreatment. The percentage of increase in HIF-1/CAIX ratio was calculated for each patient and a Wilcoxon rank sum test was used to test for differences in pre- versus posttreatment ratio. As a secondary check of the corre lative data, generalized estimating equations were used to check for differences in the ratio (HIF-1/CAIX ratio computed for each section). NF-κB values for all sections of tissue imaged and Wilcoxon rank sum tests and generalized estimating equations used to investigate for changes from pretreatment to posttreatment. All tests were two-sided and a P value of ≤0.05 was considered statistically significant.

Results

Nineteen patients (9 men and 10 women) were enrolled in this study. All the patients had received chemotherapy for metastatic disease. Additional patient characteristics are listed in Table 1. A total of 42 cycles of chemotherapy were initiated and 38 cycles completed with a median of two cycles per patient (range 1-4). A fourth cycle was initiated in three patients but was completed by only one patient.

Response and survival. Three patients were invaluable for response. Two failed to complete two cycles of treatment due to toxicity and one patient completed two cycles but withdrew consent prior to radiological evaluation. No objective response to bortezomib was seen in this study. Stable disease was seen in three patients at first assessment. Two patients had symptomatic progressive disease after cycle 1 and the remaining 11 patients had progressive disease at first assessment. Of the patients with stable disease, two patients were removed from study during cycle 4 with toxicity and one patient had
progressive disease after cycle 4. The study design required 21 patients to be entered in the first stage, but as no responses were seen in the first 19 patients, the study was closed. The median time to progression was 5.1 weeks; 95% confidence interval, 5.1 to 11.1 weeks (Fig. 1). The 6-month progression-free survival was 11.4%; 95% confidence interval, 3.1-41.9%. All patients but one (who withdrew consent) had progressed at the time of last follow-up. Median overall survival was 28.3 weeks; 95% confidence interval, 19.4 weeks. Thirteen patients died. The median follow-up for the six patients who remained alive was 14.7 weeks (range, 4.9-72.4 weeks).

Toxicity. All patients were evaluable for toxicity in all cycles. The main nonhematologic toxicities were elevation of alkaline phosphatase (11 patients across 20 cycles), constipation (13 across 22 cycles), fatigue (14 across 32 cycles), and nausea (11 across 23 cycles). Four patients (21%) developed sensory neuropathy (any grade) and one patient a grade 2 motor neuropathy. Two patients had received oxaliplatin and neither patient developed neuropathy. There was no grade 4 toxicity, all grade 3 toxicities per cycle are summarized in Table 2. Four patients discontinued treatment due to toxicity: two patients developed grade 3 sensory neuropathy (cycles 1 and 4), one developed grade 3 abdominal pain (cycle 4) and one developed rashes, dyspnea, hypotension, diarrhea, myalgia, and elevated partial thromboplastin time (cycle 1). There were three hospitalizations (two for abdominal pain and one due to a cerebrovascular accident). Only two patients required antidiarrheal medication in two cycles. There were no treatment-related deaths.

Three patients required dose reductions. Two patients had one dose reduction (rash peripheral neuropathy) and one patient had two dose reductions (nausea and vomiting). Four patients missed doses of bortezomib. Two patients missed two consecutive doses (vomiting and abdominal cramps) and one patient missed one dose in two consecutive cycles (patient request and sensory neuropathy).

Correlative studies. Pre- and posttreatment biopsies were obtained from 11 patients. Histologic examination identified adequate tumor tissue for analysis in nine patients. Pretreatment samples were obtained from a further three patients.

p53. p53-stained slides were scored independently by two trained observers for the estimated intensity and percentage of tumor cells that showed nuclear and/or cytoplasmic staining. Nuclear staining for p53 was detected in 30% to 90% of the tumor cells in seven of nine cases, with cytoplasmic staining seen in one of these tumors. No significant or consistent changes between pre- and posttreatment biopsies were detected in these scores (data not shown).

NFkB. Our initial assessment was based on Ser32/36-phosphorylated IκB, because this is targeted for proteasomal degradation. However, only weak staining was seen in the samples and there was no discernable increase following drug treatment (Fig. 2A). In contrast, strong nuclear staining for Ser78-phosphorylated NFκB was present in all samples

### Table 1. Patient characteristics (n = 19)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (range)</td>
<td>62 (46-81)</td>
</tr>
<tr>
<td>Gender</td>
<td>Male: Female 10:9</td>
</tr>
<tr>
<td>Primary malignancy</td>
<td>Colon 17</td>
</tr>
<tr>
<td>No. of lines prior chemotherapy</td>
<td>1:2:3, 12:5:2</td>
</tr>
<tr>
<td>Prior chemotherapy</td>
<td>Adjuvant 5</td>
</tr>
<tr>
<td>Prior radiotherapy</td>
<td>Metastatic 19</td>
</tr>
<tr>
<td>Median target/nontarget lesions</td>
<td>Lung 7 of 9</td>
</tr>
<tr>
<td>Target/nontarget lesions</td>
<td>Liver 15 of 12</td>
</tr>
<tr>
<td>Abdomen</td>
<td>Nodes 6 of 2</td>
</tr>
<tr>
<td>Ascites</td>
<td>Pelvis 3 of 0</td>
</tr>
<tr>
<td>Other</td>
<td>5 of 2</td>
</tr>
</tbody>
</table>

### Table 2. Grade 3 toxicity all cycles (n = 42)

<table>
<thead>
<tr>
<th>Toxicity</th>
<th>No. of cycles of grade 3 toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphopenia</td>
<td>5</td>
</tr>
<tr>
<td>Abdominal pain/cramping</td>
<td>3</td>
</tr>
<tr>
<td>Vomiting</td>
<td>2</td>
</tr>
<tr>
<td>Sensory neuropathy</td>
<td>2</td>
</tr>
<tr>
<td>Myalgia</td>
<td>1</td>
</tr>
<tr>
<td>Pain (other)</td>
<td>1</td>
</tr>
<tr>
<td>Dyspnea</td>
<td>1</td>
</tr>
<tr>
<td>Rash</td>
<td>1</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>1</td>
</tr>
<tr>
<td>Hyponatremia</td>
<td>1</td>
</tr>
<tr>
<td>Elevation of partial thromboplastin time</td>
<td>1</td>
</tr>
</tbody>
</table>
Examination of the cores showed considerable intratumoral heterogeneity in the extent of staining, with individual strongly staining nuclei interspersed among weakly or negatively stained cells. In order to address the problem of intratumoral heterogeneity, we obtained digital images of the entire tissue section and determined the number of positively stained nuclei per 100,000 $\mu$m$^2$ of viable tumor tissue. As shown in Fig. 3, there was no consistent effect of treatment on the levels of Ser$^{276}$-NF$\kappa$B.

*HIF-1$\alpha$. We anticipated that treatment with bortezomib would result in the accumulation of HIF-1$\alpha$ in nonhypoxic tumor tissue. However, our group and others have shown that the measurement of hypoxia markers such as HIF-1$\alpha$ in cancer biopsies is subject to sampling error due to intratumoral heterogeneity (11). Our initial plan was therefore to measure HIF-1$\alpha$ as a function of distance from the nearest blood vessel using a dual color immunofluorescence technique, because we have previously shown in human tumor xenografts that this

![Fig. 2. Representative immunohistochemical staining of liver biopsies, illustrating: Ser$^{32/36}$-phosphorylated I$\kappa$B (A), and Ser$^{276}$-phosphorylated NF$\kappa$B (B). C and D, individual 10 x fields of the same tumoral area in serial sections, showing HIF-1$\alpha$ and CAIX staining pretreatment. E and F, individual 10 x fields of the posttreatment biopsy obtained from same patient. G and H, composites, consisting of ~100 individual 10 x fields of a single core obtained from patient 012-013 pretreatment, stained for HIF-1$\alpha$ (G) and CAIX (H). I and J, two individual cores, embedded side by side, obtained from patient 012-013 posttreatment.](Image)
value is less subject to intratumoral heterogeneity (10). Because of high background autofluorescence in some patient samples, we also examined the distribution of the HIF-1-dependent surface membrane-associated protein CAIX, which gives a much stronger immunofluorescence signal than HIF-1α. Unexpectedly, we found that although HIF-1α and CAIX were strongly colocalized in the pretreatment biopsies, there was an obvious decrease in the relative expression levels of CAIX in the posttreatment samples (Fig. 2C-F). To investigate this further, we used immunohistochemistry to label HIF-1α and CAIX in two serial sections, and imaged the entire length of the core biopsies (Fig. 2G-J). We next determined the percentage of the total viable tumoral area for each sample that was positively labeled for each marker. As shown in Fig. 4A, there was a trend for HIF-1α labeling to increase following treatment with bortezomib, whereas CAIX decreased (Fig. 4B). Neither of these trends was statistically significant, but we considered that an effect might be obscured by sampling error due to intratumoral heterogeneity in the distribution of hypoxia. To address this, we took the ratio of the percentage of HIF-1α and CAIX labeling areas for each biopsy, and then compared the results for the pre- and posttreatment samples. As seen in Fig. 4C, an increase in HIF-1α relative to CAIX was seen in eight of nine cases. As an alternative approach to image analysis, we manually outlined all of the hotspots of tumor tissue that were positively labeled for HIF-1α, and then copied these regions to the CAIX image. We then measured the numbers of positive pixels labeled for each marker within the tumoral areas defined by HIF-1α staining, and took the ratio of the two values (Fig. 4D). These two image analysis techniques gave similar results, with a significant decrease in the amount of CAIX relative to HIF-1α in the posttreatment biopsies (P < 0.015 for each of the two analyses; Wilcoxon rank sum test).

**Microvessel density.** On histologic examination, the greatest density of CD34-positive blood vessels occurred in the fibrovascular stroma surrounding tumor deposits. These areas were manually outlined on the CD34 digital images, following which a threshold was set to outline positively stained areas. We then calculated the number of CD34-positive objects per 100,000 mm² of stroma area, their size distribution (<100, 100-200, or >200 mm²), as well as the total area of all CD34-positive blood vessels divided by the total stroma area. As shown in Fig. 4E and F, when we applied defined image analysis criteria to the entire tissue sections, we obtained close concordance between the pre- and posttreatment values for the total number of blood vessels and the percentage of tumoral area stained for CD34-positive objects, with no evidence for a significant antiangiogenic effect.

**Xenograft studies.** Analysis of the core biopsies showed a significant decrease in CAIX labeling relative to HIF-1α, but did not identify if this was the result of an overall increase in HIF-1α due to the inhibition of proteasomal degradation, or a decrease in CAIX expression. To examine this further, we (D. Birle and D. Hedley) tested the effects of bortezomib in severe combined immunodeficiency mice bearing S2Hα cervical carcinoma xenografts: a well-characterized model of tumor hypoxia (12, 10). Two groups of quadruplicates were treated with a single i.p. dose of 2 mg/kg bortezomib, and after 24 hours, the tumors were excised and immediately snap-frozen in liquid nitrogen. Three hours prior to sacrifice, the animals were injected with the nitroimidazole hypoxia probe EF5 (13). Cryostat sections were cut, and a triple immunofluorescence staining protocol used to label HIF-1α, CAIX, and EF5. The slides were then imaged at 20× objective using a fluorescence microscope and scanning autostage to generate tiled-field images, as previously described (10). As illustrated in Fig. 5A-H, these showed that relative to the extrinsic hypoxia marker EF5, there was an increase in labeling with HIF-1α following treatment with bortezomib, and a decrease in CAIX. The images were analyzed to measure the relative amounts of HIF-1α and CAIX in the EF5-positive versus EF5-negative tumoral areas. As shown in Fig. 5I, following treatment with bortezomib, there was an ~2-fold increase in the amount of HIF-1α in the EF5-negative tumor tissue, which was statistically significant (P < 0.05). In contrast, no significant effect was seen in the EF5-positive tissue, likely because all the nuclei were positively labeled in the untreated controls, as can be seen in Fig. 5A and B. Relative to the effects on HIF-1α, there was a much greater decrease in CAIX in EF5-positive tissue (Fig. 5I), indicating that bortezomib disrupts the activation of CAIX that normally occurs in response to tumor hypoxia.

**Discussion**

Despite the introduction of regimens combining fluoropyrimidines with oxaliplatin and/or irinotecan, the survival of patients with advanced colorectal cancer remains poor (1, 14). Laboratory and preclinical studies have identified the proteasome as a novel drug target (8). Bortezomib has emerged as the first member of this new class of agents to enter clinical trials. We have conducted the first phase II trial of bortezomib in patients with advanced colorectal malignancy. In addition, we report the effect of bortezomib on proteasome-regulated proteins in tumor tissue.

We observed no objective response to bortezomib in this study. Three (16%) patients had stable disease after cycle 2, but no patient was treated beyond cycle 4, and the study was closed due to lack of efficacy. Bortezomib results in an impressive 35% objective response rate in refractory myeloma (15), and in a recent (weekly) phase I study, showed efficacy against androgen-independent prostate cancer (16). No significant
activity, however, was observed in patients with metastatic renal cell cancer (17). Data from the weekly phase I study suggests that response may be related to the degree of 20S proteasome inhibition observed in whole blood. Most of the clinical activity seen in patients with androgen-independent prostate cancer occurred at doses of bortezomib resulting in average 1 hour posttreatment 20S proteasome inhibition of \(70\%\) (16). Data from the phase I study using the twice weekly schedule show a mean percentage proteasome inhibition of 65% with 1.3 mg/m\(^2\) bortezomib (18). It is possible that the doses and schedule employed in our study produces suboptimal inhibition of the proteasome resulting in lack of efficacy. Alternatively, this may simply reflect differences in tumor biology.

The toxicity reported here was comparable to that reported in other studies with bortezomib, with the most common adverse events being elevation of alkaline phosphatase (11 patients), fatigue (14), nausea (11) and diarrhea (8). The incidence of neuropathy (all grades) was 26% (five patients) with two patients developing a grade 3 sensory neuropathy. This compares with 35% and 47%, respectively, in myeloma and metastatic renal cell cancer (15, 17). Grade 3 abdominal pain (three patients) was more common in our study but we saw less diarrhea (42% of patients) with only one patient experiencing grade 3 toxicity. This probably reflects the dose and tumor type as loperamide was only required by two patients.

Many proteins involved in cancer cell growth and survival are regulated by proteasomal degradation (8). There has been particular interest in the activation of the NF\(\kappa\)B transcription factor, which seems to play an important role in drug and radiation resistance, as well as tumor angiogenesis. The inhibitory subunit I\(\kappa\)B sequesters NF\(\kappa\)B by masking the nuclear localizing sequence. Following serine phosphorylation by I\(\kappa\)B kinases, I\(\kappa\)B undergoes ubiquitination and proteasomal degradation, releasing NF\(\kappa\)B that translocates to the nucleus. Serine phosphorylation at the 276 site of the p65 subunit of NF\(\kappa\)B seems to play a critical role in binding to DNA and to the transcriptional coactivator p300/CBP (19), and the detection of nuclear staining using phosphospecific antibodies to that site has been shown to correlate with NF\(\kappa\)B activation (20). A large body of experimental and preclinical data suggests that the disruption of NF\(\kappa\)B activity is an important anticancer effect of proteasome inhibitors including bortezomib (8). However, in our series of nine paired tumor samples no significant change was seen in the relative amounts of phosphorylated I\(\kappa\)B and active NF\(\kappa\)B following treatment with bortezomib.

Recently, there has been considerable interest in the role that the HIF-1 transcription factor plays in solid tumor growth (21). Under aerobic conditions, the HIF-1\(\alpha\) subunit undergoes oxygen-dependent proline hydroxylation, followed by VHL-dependent ubiquitination and proteasomal degradation, whereas under hypoxic conditions, it is able to accumulate...
and bind to the H552IF-1β subunit, forming the HIF-1α transcription factor. This can then activate a range of genes coding for proteins that support tumor growth under hypoxic conditions, including enzymes involved in anaerobic metabolism such as CAIX, as well as vascular endothelial growth factor.

Along with other groups, we have identified intratumoral heterogeneity as a significant problem in the assessment of tumor hypoxia based on intrinsic markers such as HIF-1α or CAIX (11, 22). However, when we assessed the expression of HIF-1α in relation to that of its transcriptional target CAIX, there was a decrease in CAIX relative to HIF-1α in the posttreatment samples in eight of nine patients. Furthermore, when we measured the overall staining for HIF-1α and CAIX across the entire tumoral areas, we found that the two exceptions had the lowest values, suggesting that these patients had intrinsically low levels of tumor hypoxia. It should also be noted that although we use the ratios of HIF-1α and CAIX staining to illustrate effects in Fig. 4C and D, these effects could be inflated by the occurrence of relatively small changes in marker expression in tumor with low levels of hypoxia. To address that concern, the statistical analysis used the Wilcoxon rank sum test that is nonparametric, and only uses the rank, not the actual ratio values, to test for significance.

To further explore the possibility that bortezomib might be acting to suppress the activation of CAIX in regions of tumor hypoxia, we examined effects in a xenograft model where we

Fig. 5. Effects of 24-hour treatment with 2 mg/kg bortezomib on hypoxia markers in SiHa xenografts. Triple immunofluorescence staining protocol combining HIF-1α (green) and CAIX (blue) with the extrinsic nitroimidazole hypoxia probe EF5 (red). A-C, 2 × 2 composites of 20× fields obtained from an untreated control. D-F, effects of bortezomib. G and H, overlays of (A-C) and (D-F), respectively. Bar, 500 μm (H). Note that similar to the patient samples shown in Fig. 2, HIF-1α and CAIX show colocalization in the control, whereas posttreatment there is a marked decrease in CAIX relative to HIF-1α. As shown in the bar graphs, which relate HIF-1α and CAIX to the extrinsic hypoxia probe EF5, bortezomib treatment results in both an increase in HIF-1α in nonhypoxic tissue, and a decrease in CAIX. Bars, SE; based on tiled field imaging of the entire viable tumoral area in three tissue sections, obtained from four individual tumors.
were able to use the extrinsic nitroimidazole probe EF5 to map the distribution of hypoxia independent of HIF-1α activation. After 24 hours of treatment with a single dose of bortezomib, the level of HIF-1α increased in the better oxygenated regions, defined by negative EF5 staining. This is the expected finding based on our original hypothesis. However, we also saw a large decrease in CAIX in the EF5-positive regions, indicating that bortezomib disrupts the tumor response to hypoxia. When our clinical data are also taken into consideration, these findings suggest that proteasome inhibitors might show particular activity against hypoxic tumor cells. Indeed, preclinical testing has shown synergy between bortezomib and CPT11 (23) and ionizing radiation (24). Our results also predict that plasma vascular endothelial growth factor might be a useful biomarker for bortezomib effects in cancer patients, but we did not obtain samples in the present trial to test this. Further experimental work to understand the mechanisms and consequences of bortezomib effects in relation to tumor hypoxia is ongoing, and will be reported separately.

Given that like CAIX, vascular endothelial growth factor is driven by HIF-1, we considered the possibility that bortezomib might be exerting an antiangiogenic effect in colon cancer patients, as has been reported to occur in human tumor xenografts (25). However, when we measured the microvessel density of the pre- and posttreatment biopsies using objective image analysis criteria, we found no consistent change in the number or size distribution of small blood vessels to suggest that bortezomib was having an antiangiogenic effect in the patients. However, such an effect cannot be excluded due to the small number of cases, and the relatively short time interval between the two biopsies.

In summary, bortezomib (1.3 mg/m²) administered twice weekly every 21 days does not have clinical activity in patients with metastatic colorectal cancer and should not be investigated further in clinical trials as a single agent. However, although our correlative markers failed to show evidence for an effect on NFκB activation or p53 accumulation, they suggest a novel and potentially important effect on the tumoral response to hypoxia that is further supported by our ongoing human tumor xenograft studies. It will be important to confirm our biomarker results in other clinical trials involving bortezomib, and also to test the potential for an antihypoxia effect to sensitize to other cancer treatment modalities including radiotherapy.

Acknowledgments

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References

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