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Proteasome Inhibition by Bortezomib Increases IL-8 Expression in Androgen-Independent Prostate Cancer Cells: The Role of IKK\(\alpha\)

Subrata Manna,* Bipradeb Singha,* Sai Aung Phyo,* Himavanth Reddy Gatla,* Tzu-Pei Chang,* Shannon Sanacora,* Sitharam Ramaswami,*† and Ivana Vancurova*

Expression of the proinflammatory and proangiogenic chemokine IL-8, which is regulated at the transcriptional level by NF-\(\kappa\)B, is constitutively increased in androgen-independent metastatic prostate cancer and correlates with poor prognosis. Inhibition of NF-\(\kappa\)B–dependent transcription was used as an anticancer strategy for the development of the first clinically approved 26S proteasome inhibitor, bortezomib (BZ). Even though BZ has shown remarkable antitumor activity in hematological malignancies, it has been less effective in prostate cancer and other solid tumors; however, the mechanisms have not been fully understood. In this article, we report that proteasome inhibition by BZ unexpectedly increases IL-8 expression in androgen-independent prostate cancer cells. Unexpectedly, we found that proteasome inhibition by BZ increases IL-8 expression in androgen-independent prostate cancer PC3 and DU145 cells, whereas expression of other NF-\(\kappa\)B–regulated genes is inhibited or unchanged. The BZ-increased IL-8 expression is associated with increased in vitro p65 NF-\(\kappa\)B DNA binding activity and p65 recruitment to the endogenous IL-8 promoter. In addition, proteasome inhibition induces a nuclear accumulation of I\(\kappa\)B kinase (IKK\(\alpha\)), and inhibition of IKK\(\alpha\) enzymatic activity significantly attenuates the BZ-induced p65 recruitment to IL-8 promoter and IL-8 expression, demonstrating that the induced IL-8 expression is mediated, at least partly, by IKK\(\alpha\). Together, these data provide the first evidence to our knowledge, for the gene-specific increase of IL-8 expression by proteasome inhibition in prostate cancer cells and suggest that targeting both IKK\(\alpha\) and the proteasome may increase BZ effectiveness in treatment of androgen-independent prostate cancer.


Interleukin-8, originally discovered as the neutrophil chemotactant and inducer of leukocyte-mediated inflammation, contributes to cancer progression through its induction of tumor cell proliferation, survival, and migration (1, 2). In addition, tumor-derived IL-8 activates endothelial cells to promote angiogenesis, induces neutrophil recruitment, and activates neutrophils and the tumor-associated macrophages to release more IL-8, which further amplifies the prosurvival, proangiogenic, and metastatic effect. IL-8 expression is increased in many types of advanced cancers, including the metastatic androgen-independent prostate cancer, and correlates with poor prognosis (2–4).

Prostate cancer is the third most common cause of death from cancer in men. It proceeds from a localized, curable, androgen-dependent disease to an invasive, metastatic, and always fatal, androgen-independent prostate cancer. One of the critical factors regulating progression to the metastatic androgen-refractory prostate cancer is the increased activity of NF-\(\kappa\)B, which induces synthesis of antiapoptotic and proinflammatory genes, including IL-8 (5–8). NF-\(\kappa\)B activity and IL-8 levels are increased in metastatic prostate cancer cells and in patients with hormone-refractory prostate cancer, where they promote angiogenesis, tumor growth, and metastasis (9–15).

In the canonical NF-\(\kappa\)B pathway, NF-\(\kappa\)B p65/p50 dimers are retained in the cytoplasm in an inactive form through their interaction with I\(\kappa\)B\(\alpha\). Following cell stimulation with proinflammatory signals and other forms of cellular stress, I\(\kappa\)B\(\alpha\) is phosphorylated through a cascade of inducible protein kinases that involve the enzymes of I\(\kappa\)B kinase (IKK) complex, ubiquitinated, and selectively degraded by the 26S proteasome. The proteasomal degradation of I\(\kappa\)B\(\alpha\) releases NF-\(\kappa\)B dimers to translocate to the nucleus and stimulate transcription of NF-\(\kappa\)B–dependent proliferative and antiapoptotic genes (16, 17). The inhibition of NF-\(\kappa\)B–dependent transcription was used as an anticancer strategy for the development of the first clinically approved 26S proteasome inhibitor, bortezomib (BZ) (18–22). Even though BZ (Velcade, PS-341) has shown remarkable antitumor activity in multiple myeloma and other hematological malignancies (18–22), it has been less effective in solid tumors (23–30); however, the mechanisms have not been fully understood.

We have previously shown that proteasome inhibition induces nuclear accumulation of I\(\kappa\)B\(\alpha\) that has a gene-specific effect on the regulation of NF-\(\kappa\)B–dependent genes, depending on the subunit composition of NF-\(\kappa\)B dimers (31–33). In this study, we have investigated the mechanism of how proteasome inhibition by BZ regulates NF-\(\kappa\)B–dependent transcription in androgen-independent prostate cancer cells. Unexpectedly, we found that proteasome inhibition significantly increased expression of IL-8, whereas expression of other NF-\(\kappa\)B–regulated genes was inhibited or unchanged. The BZ-increased IL-8 expression was associated with increased nuclear accumulation of IKK\(\alpha\), and suppression of IKK\(\alpha\) attenuated the BZ-increased p65 recruitment and IL-8 expression.
demonstrating the IKKα requirement for BZ-increased IL-8 expression in metastatic prostate cancer cells. These data provide the first evidence, to our knowledge, for the gene-specific increase of IL-8 expression by proteasome inhibition in prostate cancer cells and suggest that BZ-increased IL-8 expression may represent one of the underlying mechanisms responsible for the decreased effectiveness of BZ in androgen-independent prostate cancer treatment.

Materials and Methods

Abs and reagents

Purified polyclonal Abs against human NF-kB p65 (sc-372), NF-κB p50 (sc-7178), IκBα (sc-371), IKKα (sc-7218), IKKβ (sc-8014), IKKγ/ε (sc-37614), phosphorylated p65 at S536 (sc-33020), and lamin B (sc-6216) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Purified polyclonal Ab against lactate dehydrogenase ([LDH]; 20-LG22) was from Fitzgerald Industries International (Concord, MA), and actin Ab was from Sigma-Aldrich (St. Louis, MO). HRP-conjugated anti-rabbit, anti-mouse, and anti-goat secondary Abs were from Santa Cruz Biotechnology (Santa Cruz, CA). BZ was from ChemieTek (Indianapolis, IN). The IKK inhibitors Bay-117082 and PS-1145 were purchased from Sigma-Aldrich. All other reagents were molecular biology grade and were from Sigma-Aldrich.

Cell culture

All cell lines were obtained from American Type Culture Collection (Rockville, MD). PC3 cells were cultured in Ham’s F12K medium (American Type Culture Collection) supplemented with 2 mM l-glutamine, 1% supermix and streptomycin, and 10% FBS (Invitrogen, Grand Island, NY), as described (31). DU145 cells were grown in Eagle’s MEM supplemented with FBS and antibiotics. Hut-78 cells, HeLa cells, and U-937 cells were grown in RPMI 1640 medium with FBS and antibiotics, supplemented with FBS and antibiotics. Hut-78 cells, HeLa cells, and U-937 cells were grown in RPMI 1640 medium with FBS and antibiotics, as described (31–34). Prior to cell treatment, cells were seeded (10^4 cells per milliliter) for 24 h in six-well plates and grown at 37°C with 5% CO2. Hut-78 cells and HeLa cells, and U-937 cells were grown in RPMI 1640 medium with FBS and antibiotics, and washed, and eluted with 1% SDS-0.1 M NaHCO3. The cross-linking was reversed by heating with 5 M NaCl at 65°C for 4 h. Proteins were digested with proteinase K, and the samples were extracted with phenol/chloroform, followed by precipitation with ethanol. The pellets were resuspended in nuclease-free water and subjected to real-time PCR. Immunoprecipitated DNA was analyzed by real-time PCR (25 μl reaction mixture) using iQ SYBR Green Supermix (Bio-Rad) and the Bio-Rad MyIQ Single Color Real-Time PCR Detection System as described (34). The occupancy was calculated using the ChIP-qPCR Human IGX1A Negative Control Assay (GPH100001C) as a negative control and corrected for the efficiency of the primers, which detect specific genomic DNA sequences within open reading frame–free intergenic regions or “promoter deserts” lacking any known or predicted structural genes. The primers used for real-time PCR were the following: cIAP-1: forward, 5'-TGATCGTG-CAAGGCAGAATGTA-3'; and reverse, 5'-TTTGGGCCCCGTATCACTGCTGAT-3'; cIAP-2: forward, 5'-TCTGATGAAATGGCCGGAGAATG-3'; and reverse, 5'-TTGTTTGGCTGACTGCTGTTG-3'; Bcl-2: forward, 5'-CTCAGTCTGTCGGCAAGGG-3'; and reverse, 5'-CCCGAGAAGAAGAAGAAGG-3'; Bcl-3: forward, 5'-TTGCGCGAGGAGAATACCTA-3'; and reverse, 5'-CGCTCTCTCGCTCTGTCTT-3'; and IL-8: forward, 5'-GGGGCTATCAGTGTTGACCACT-3'; and reverse, 5'-GTCCTGGTGGTCTGTGTCTC-3'. The NF-κB promoter sequences of the above genes are shown in Table I.

ELISA

The IL-8 release was measured in cell culture supernatants by a commercially available ELISA kit (R&D Systems, Minneapolis, MN), as previously described (32).

Statistical analysis

The results represent at least three independent experiments. Numerical results are presented as means ± SE. Data were analyzed using an InStat software package (GraphPad, San Diego, CA). Statistical significance was evaluated using the Mann–Whitney U test with Bonferroni correction for multiple comparisons, and p < 0.05 was considered significant.

Results

Proteasome inhibition by BZ increases NF-κB p65 nuclear accumulation and DNA binding activity in androgen-independent prostate cancer PC3 cells

The androgen-independent prostate cancer cells are characterized by high levels of constitutive NF-κB p65/p50 DNA binding and proteasome activity (35–38). To investigate the mechanism of how proteasome inhibition regulates NF-κB–dependent transcription in prostate cancer cells, we first analyzed the nuclear–cytoplasmic
distribution of NF-κB p65 and p50 subunits in PC3 cells cultured 24 h in the presence of increasing concentrations of BZ. Unexpectedly, we found that BZ increased the nuclear levels of p65; the increased nuclear accumulation of p65 was most pronounced at 0.1- and 1-μM BZ concentrations (Fig. 1A). In contrast, BZ did not significantly increase the nuclear levels of p50. The highest BZ concentration (10 μM) was associated with a decreased p50 expression, both in the cytoplasm and in the nucleus (Fig. 1A). In addition, BZ induced IκBα translocation from the cytoplasm to the nucleus; this was consistent with our previous studies showing that proteasome inhibition induces IκBα nuclear translocation and accumulation in prostate cancer and leukemia cells (31, 33). BZ at concentrations up to 10 μM did not have any effect on the actin levels used as a loading control, or LDH and lamin B, which were used as specific cytoplasmic and nuclear markers, respectively.

To confirm that the 0.1 μM BZ concentration, which induced the maximal nuclear accumulation of p65 (Fig. 1A), also inhibits proteasome activity in PC3 cells, we measured activity of the 26S proteasome in whole-cell extracts prepared from cells treated for 24 h with increasing BZ concentrations. As shown in Fig. 1B, 0.1 μM BZ, which approximately corresponds to the clinically used BZ concentrations in cancer patients (39), inhibited ~90% of the original proteasome activity. In contrast, the 0.1 μM BZ concentration reduced the PC3 cell viability and total cell count only by ~5% and 15%, respectively (Fig. 1C), which is consistent with the relative resistance of metastatic prostate cancer cells to BZ (26).

To determine whether the increased nuclear p65 levels resulted in an increased p65 NF-κB DNA binding activity, we analyzed the in vitro p65 DNA binding activity in nuclear extracts prepared from PC3 cells incubated 24 h with increasing concentrations of BZ. As shown in Fig. 2A, BZ significantly increased the p65 DNA binding activity measured by TransAM assay, which measures the amount of p65 NF-κB bound to the NF-κB consensus 5′-GGGACTTTCC-3′ oligonucleotide. Compared with untreated cells, those treated with 0.1 and 1 μM BZ exhibited three times

**FIGURE 1.** Proteasome inhibition by BZ induces nuclear accumulation of p65 NF-κB in prostate cancer PC3 cells. (A) Western blotting of cytoplasmic (CE) and nuclear extracts (NE) prepared from PC3 cells treated with increasing concentrations of BZ for 24 h, and analyzed using p65, p50, and IκBα Abs. The presence of cytoplasmic proteins in nuclear fraction was evaluated by reprobing the membrane with LDH Ab. Nuclear contamination in the cytoplasmic fraction was assessed using lamin B-specific Ab. To confirm equal protein loading, the membranes were stripped and reprobed with actin Ab. Each lane corresponds to ~5×10⁴ cells. (B) The proteasome activity was measured in whole-cell extracts prepared from PC3 cells treated with increasing BZ concentrations for 24 h. The activity is expressed as relative fluorescence units (RFU) of BZ-treated cells compared with untreated (UT) cells. (C) Cell viability and total cell count of PC3 cells incubated 24 h with increasing BZ concentrations were measured using trypan blue exclusion. The data are expressed as the percentage compared with UT cells. The values represent the mean ± SE of four experiments; *p < 0.05, statistically significant inhibition compared with control UT cells.

**FIGURE 2.** Proteasome inhibition by BZ increases p65 NF-κB DNA binding activity in PC3 cells. (A) NF-κB p65 DNA binding activity was measured in nuclear extract prepared from PC3 cells treated with increasing concentrations of BZ for 24 h. (B) Specificity analysis of the constitutive p65 NF-κB DNA binding activity in PC3 cells, measured in nuclear extracts of untreated (UT) cells in the absence and presence of mutant (mut.) or wild-type (WT) oligonucleotides. The values represent the mean ± SE of four experiments; *p < 0.05, statistically significant inhibition compared with control UT cells.
higher p65 DNA binding activity. Fig. 2B demonstrates specificity of p65 DNA binding for the NF-κB binding site, because the mutated oligonucleotide did not exhibit any p65 binding. Even though the increased p65 DNA binding activity induced by proteasome inhibition was surprising, as proteasome inhibition suppresses NF-κB activity in most tumor cells (19–21), it correlated well with the BZ-increased p65 nuclear levels in PC3 cells (Fig. 1A).

### Proteasome inhibition by BZ significantly increases IL-8 expression in metastatic prostate cancer cells, whereas it decreases or does not affect expression of other NF-κB–dependent genes

To determine whether increased p65 nuclear levels and DNA binding activity correlate with the expression of NF-κB–dependent genes, we analyzed mRNA levels of the regulatory gene belonging to the IκB family, Bcl-3; the antiapoptotic genes Bcl-2, cIAP-1, and cIAP-2; and IL-8 in PC3 cells treated with increasing concentrations of BZ. As shown in Fig. 3A, expression of Bcl-3, cIAP-1, and cIAP-2 was suppressed, and Bcl-2 was unchanged. This finding is in agreement with previous studies demonstrating that proteasome inhibition suppresses most NF-κB–dependent genes, whereas it does not affect Bcl-2 expression (31, 33). Remarkably, however, proteasome inhibition significantly increased the IL-8 expression and protein release in PC3 cells (Figs. 3B–D). In cells incubated 24 h with 0.1 and 1 μM BZ, the IL-8 mRNA levels increased almost 30- and 50-fold, respectively, compared with levels in untreated PC3 cells (Fig. 3B). However, it is important to note that even in the absence of BZ, PC3 cells expressed a considerable amount of IL-8 mRNA, which is consistent with IL-8 release from untreated cells (Fig. 3D). To better demonstrate the level of IL-8 mRNA expression in PC3 cells, we compared IL-8 mRNA levels in different untreated cell lines: HeLa cells, used as a reference; two metastatic prostate cancer cell lines, PC3 and DU145 cells; and two leukemia cell lines, U937 and Hut-78 cells (Fig. 3E). Compared with HeLa cells, untreated PC3 and DU145 cells expressed significantly more IL-8 mRNA; the highly metastatic prostate cancer PC3 cells had ~ 25-fold higher IL-8 mRNA levels than did HeLa cells; and the moderately metastatic prostate cancer DU145 cells had ~ 3 times more IL-8 mRNA (Fig. 3E). In contrast, untreated leukemia U937 and Hut-78 cells had very low IL-8 mRNA levels. However, the control Bcl-2 mRNA levels were comparable in all cell lines tested, and cIAP-1 mRNA levels were comparable between PC3 and Hut-78 cells, and DU145 and HeLa cells (Fig. 3E). These results demonstrate that the metastatic prostate cancer cells express significantly more IL-8 than do other cancer and leukemia cells, and that the IL-8 mRNA levels in prostate cancer cells correlate with their metastatic potential, as was previously reported (3, 4).

To determine whether proteasome inhibition increases IL-8 expression also in moderately metastatic, androgen-independent
prostate cancer DU145 cells, we analyzed IL-8 mRNA expression and protein release in DU145 cells treated for 24 h with increasing BZ concentrations. As shown in Fig. 4, both IL-8 mRNA levels (Fig. 4A) and protein release (Fig. 4B) were significantly increased in BZ-treated DU145 cells; 0.1 μM BZ increased IL-8 mRNA expression ~20-fold compared with that in untreated cells, and protein release ~5-fold. These data demonstrate that the increased IL-8 expression induced by proteasome inhibition is not unique to highly metastatic PC3 cells but is induced in other metastatic prostate cancer cells as well.

Proteasome inhibition increases p65, but not p50, recruitment to the endogenous IL-8 promoter

Because proteasome inhibition increased the in vitro p65 DNA binding activity (Fig. 2), we analyzed whether it also increases p65 recruitment to the endogenous IL-8 promoter. Cells were incubated 24 h with 0, 0.1, or 1 μM BZ; cross-linked with formaldehyde and lysed; and chromatin was sheared by sonication. NF-κB p65 and p50 recruitment to IL-8, cIAP-1, cIAP-2, Bcl-2, and Bcl-3 promoters was analyzed by ChIP using p65 and p50 Abs and quantified by real-time PCR. The NF-κB binding sites of the above genes are shown in Table I. Fig. 5A illustrates the proximal NF-κB binding site in human IL-8 promoter that was shown to be required for the IL-8 expression (40–42).

Proteasome inhibition by BZ significantly increased p65 recruitment to IL-8 promoter (Fig. 5B). In PC3 cells treated 24 h with 0.1 and 1 μM BZ, p65 occupancy at the IL-8 promoter increased ~6- and 9-fold, respectively, compared with that in untreated cells. In contrast to p65, p50 recruitment to IL-8 promoter was not changed (Fig. 5C). Proteasome inhibition also increased p65 recruitment to cIAP-1 and cIAP-2 promoters, even though the p65 occupancy at these promoters was significantly lower than on the IL-8 promoter (Fig. 5B). However, in contrast to IL-8, BZ increased p50 recruitment to Bcl-3, cIAP-1, and cIAP-2 promoters (Fig. 5C). Of interest, the high p50 occupancy on Bcl-3 and cIAP-2 promoters (Fig. 5C) was associated with the maximum inhibition by BZ (Fig. 3A).

The BZ-increased IL-8 expression in PC3 cells is mediated by IKKα

On the basis of previous studies indicating that the constitutively increased NF-κB activity in metastatic prostate cancer cells is mediated by the increased IKK activity (9, 11, 43–46), we hypothesized that proteasome inhibition increases IL-8 expression by an IKK-dependent mechanism. To this end, we first analyzed whether proteasome inhibition increased the intracellular levels of IKKα, IKKβ, or IKKε. PC3 cells were treated for 24 h with 0, 0.1, or 1 μM BZ, and the cytoplasmic and nuclear extracts were analyzed by Western blotting followed by densitometry. As shown in Fig. 6A and B, IKKα and IKKβ were localized both in the cytoplasm and in the nucleus, and BZ further increased their nuclear accumulation. The increased nuclear accumulation of IKKα and IKKβ in response to proteasome inhibition is likely caused by inhibited proteasomal degradation of IKKα and IKKβ, because BZ did not increase the IKKα or IKKβ mRNA levels in PC3 cells (data not shown). This idea is supported by a previous study indicating that IKKα and IKKβ are subject to proteasomal degradation (47). In contrast to IKKα and IKKβ, IKKε was localized mainly in the nucleus in PC3 cells, and its levels did not change after proteasome inhibition (Fig. 6A, B).

To determine whether IKK activity is required for the BZ-induced IL-8 expression in metastatic prostate cancer cells, we analyzed IL-8 expression in PC3 and DU145 cells treated with the IKK inhibitors Bay-117082 and PS-1145. Cells were pretreated for 12 h either with 5 μM Bay-117082, a broad-spectrum IKK inhibitor (48, 49), or with 20 μM PS-1145, an IKKβ-specific inhibitor (43), before 24-h incubation with 0, 0.1, or 1 μM BZ. Both in PC3 cells (Fig. 6C) and in DU145 cells (Fig. 6D), the IKK inhibition by Bay-117082 resulted in a significantly reduced IL-8 expression in BZ-treated cells, whereas PS-1145 did not have any effect. These data indicated that the increased IL-8 expression induced by proteasome inhibition in metastatic prostate cancer cells is mediated by IKKα, but not IKKβ.

To confirm the above data and determine which IKK isoform is responsible for the BZ-increased IL-8 expression, we used IKK suppression by siRNAs. PC3 cells were transfected with IKKα, IKKβ, IKKε, or control nonsilencing siRNA before 24-h treatment with 0 or 0.1 μM BZ. In addition, to determine whether the BZ-induced nuclear IκBα regulates IL-8 expression in PC3 cells, cells were transfected also with IκBα siRNA. As shown in Fig. 6E, transfection using IKKα siRNA significantly suppressed the BZ-increased IL-8 expression. In contrast, transfection with IKKβ siRNAs did not have any effect on the BZ-increased IL-8 expression, compared with transfection with control siRNA. Even though transfection using IKKε siRNA somewhat decreased the BZ-induced IL-8 expression in PC3 cells, it was not statistically significant. Transfection using IκBα-specific siRNA did not have any effect on the BZ-increased IL-8 expression, suggesting that the nuclear IκBα induced by proteasome inhibition does not regulate IL-8 expression in prostate cancer cells. Together, these results demonstrated that the IL-8 expression induced by proteasome inhibition in prostate cancer cells is mediated, at least partly, by IKKα.

IKKα mediates the BZ-increased p65 recruitment to IL-8 promoter in PC3 cells

To investigate the function of IKKα in BZ-induced IL-8 expression, we analyzed by ChIP whether enzymatic IKKα activity is
required for BZ-increased p65 recruitment to IL-8 promoter. As illustrated in Fig. 7A, 12-h preincubation of PC3 cells with 5 μM Bay-117082 significantly attenuated the BZ-induced p65 recruitment to IL-8 promoter. Considering that IKKβ is not involved in IL-8 regulation (Fig. 6), and that suppression of IKKα has only an insignificant effect on IL-8 expression in PC3 cells (Fig. 6E), these results strongly indicated that IKKα enzymatic activity is required for BZ-increased p65 recruitment to IL-8 promoter.

Because previous studies showed that IKKα can phosphorylate p65 at S536, resulting in its increased transcriptional activity (50, 51), we investigated whether BZ increases S536 p65 phosphorylation and recruitment to the IL-8 promoter. Western analysis using S536P-p65-specific Ab demonstrated that similarly to p65, S536P-p65 is localized mainly in the nucleus of PC3 cells (Fig. 7B). However, in contrast to p65, BZ decreased the nuclear levels of S536P-p65 (Fig. 7B, 7C), suggesting that proteasome inhibition reduces p65 phosphorylation at S536. Importantly, we did not detect any S536P-p65 recruitment to the IL-8 promoter in untreated or BZ-treated PC3 cells (data not shown), indicating that S536P-p65 is not recruited to IL-8 promoter in prostate cancer cells. Together, these results show that proteasome inhibition induces IL-8 expression in prostate cancer cells through the increased p65 recruitment that is facilitated by IKKα, independently of p65 phosphorylation at S536 (Fig. 8).

**Discussion**

The present data show that proteasome inhibition by BZ unexpectedly increases expression of the proangiogenic and proinflammatory chemokine IL-8 in androgen-independent metastatic prostate cancer PC3 and DU145 cells, whereas expression of other NF-κB–dependent genes is inhibited or unchanged. The increased IL-8 expression is associated with increased p65 nuclear accumulation and recruitment to the IL-8 promoter. Importantly, proteasome inhibition also increases nuclear accumulation of IKKα, and suppression of IKKα protein levels and enzymatic activity significantly decreases the BZ-induced p65 recruitment and IL-8 expression. These data provide the first evidence, to our knowledge, for gene-specific increase of IL-8 expression by proteasome inhibition in prostate cancer cells and indicate that BZ-increased IL-8 expression is mediated by the IKKα-dependent enhanced p65 recruitment to the IL-8 promoter.

BZ is the first clinically approved proteasome inhibitor that has been very effective in the treatment of multiple myeloma and other hematological malignancies (18–21). One of the main mechanisms of BZ function is the suppressed proteosomal degradation of IκBα in the cytoplasm, resulting in the inhibition of inducible NF-κB activity and expression of NF-κB–dependent proinflammatory and antiapoptotic genes (19–21). NF-κB activity is constitutively increased in metastatic prostate cancer cells through the increased activation of IKK, resulting in increased cell survival and resistance to chemotherapy (9–12). BZ has so far failed to exhibit a significant clinical activity in prostate cancer patients (23–30); however, the mechanisms underlying prostate cancer resistance to BZ are largely unknown.

We have found that BZ unexpectedly increases the nuclear levels of p65 in prostate cancer cells; the highest p65 nuclear accumulation was achieved by 0.1 μM BZ (Fig. 1A), which approximately corresponds to the clinically used BZ concentrations (39). Because BZ did not have any effect on p65 mRNA levels in PC3 cells (data not shown), it seems likely that BZ prevents the proteasomal degradation of nuclear p65 in prostate cancer cells. This idea is supported by previous studies demonstrating that p65 is a target of proteasomal degradation both in canonical and in atypical pathways of NF-κB activation (52–54). The increased p65 nuclear accumulation was associated with increased in vitro

### Table I. NF-κB binding sites in the NF-κB–regulated promoters

<table>
<thead>
<tr>
<th>Gene</th>
<th>NF-κB Site Location</th>
<th>NF-κB Site Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-κB consensus oligonucleotide used in ELISA assay</td>
<td>N/A</td>
<td>5'-GGGACTTTCCC-3'</td>
</tr>
<tr>
<td>IL-8</td>
<td>-82</td>
<td>5'-GAAATTTCCT-3'</td>
</tr>
<tr>
<td>Bcl-3</td>
<td>-293</td>
<td>5'-GCGGGAAGGACC-3'</td>
</tr>
<tr>
<td>cIAP-1</td>
<td>-1153</td>
<td>5'-GAGATTCCCCC-3'</td>
</tr>
<tr>
<td>cIAP-2</td>
<td>-174</td>
<td>5'-GGAAATCCCCC-3'</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>-161</td>
<td>5'-GGGAACACCC-3'</td>
</tr>
</tbody>
</table>
then incubated for 24 h with 0 or 0.1 mM BZ. The values represent the mean ± SE of four experiments. *p < 0.05, statistically significant inhibition, compared with cells pretreated with control DMSO or transfected with control non-specific (NS) siRNA.

IL-8 mRNA levels in PC3 cells transfected with specific (NS) siRNA. control DMSO or transfected with control non-inhibition, compared with cells pretreated with 0, 0.1, or 1 mM BZ for 24 h with DMSO, Bay-117082 (5 μM), or PS-1145 (20 μM) before 24-h incubation with 0, 0.1, or 1 mM BZ. (B) Densitometric evaluation of IKKα, IKKβ, and IKKε levels in nuclear extracts of BZ-treated PC3 cells. The nuclear IKKα, IKKβ, and IKKε bands were scanned, and their densities were normalized to the densities of actin used as a loading control. The values for NE of untreated cells were arbitrarily set to 1, and the other values are presented relative to these values. The data represent the means of three experiments ± SE. (C) Real-time RT-PCR analysis of IL-8 mRNA levels in PC3 cells pretreated for 12 h with control DMSO or the IKK inhibitors Bay-117082 (5 μM) and PS-1145 (20 μM) before 24-h incubation with 0, 0.1, or 1 mM BZ. (D) Real-time RT-PCR of IL-8 mRNA levels in DU145 cells pretreated for 12 h with DMSO, Bay-117082 (5 μM), or PS-1145 (20 μM) before 24-h incubation with 0, 0.1, or 1 mM BZ. (E) Real-time RT-PCR analysis of IL-8 mRNA levels in PC3 cells transfected with control, IKKα, IKKβ, IKKε, or IκBα siRNA and then incubated for 24 h with 0 or 0.1 μM BZ. The values represent the mean ± SE of four experiments. *p < 0.05, statistically significant inhibition, compared with cells pretreated with control DMSO or transfected with control non-specific (NS) siRNA.

p65 DNA binding activity (Fig. 2A) and with the increased p65 recruitment to promoters of NF-κB–regulated genes, especially to the IL-8 promoter (Fig. 5B).

In contrast to p65, proteasome inhibition did not increase the nuclear levels of p50 NF-κB. On the contrary, PC3 cells treated with 10 μM BZ exhibited somewhat decreased p50 levels both in the cytoplasm and in the nucleus. One possible mechanism that may be responsible for the decreased cellular levels of p50 in BZ-treated cells is the previously described p50 cleavage by the proteasome-regulated, calcium-dependent protease calpain (55, 56). However, even though BZ did not increase the nuclear levels of p50 NF-κB in PC3 cells, it increased the p50 recruitment to Bcl-3 and cIAP-2 promoters. Of interest, the increased occupancy of p50 at Bcl-3 and cIAP-2 promoters was associated with the highest gene suppression by BZ, suggesting that the p50 promoter binding inhibits transcription of these genes in prostate cancer cells. The suppressor role of p50 promoter binding in regulating Bcl-3 and cIAP-2 expression in prostate cancer cells is supported by earlier studies demonstrating that p50 homodimers inhibit transcription of a subset of NF-κB–regulated genes (57, 58). In contrast to p65, p50 recruitment to IL-8 promoter was not increased by BZ (Fig. 5C), indicating that in prostate cancer cells, the IL-8 promoter is regulated predominantly by p65 homodimers.

In addition to p65, BZ induced the nuclear accumulation of IκBα in PC3 cells (Fig. 1A). This finding was consistent with our previous studies showing that proteasome inhibition suppresses NF-κB activity by an additional mechanism that consists of inducing the translocation of IκBα from the cytoplasm to the nucleus, resulting in the gene-specific inhibition of NF-κB–dependent transcription (31, 33). However, the BZ-increased IL-8 expression in PC3 cells is not regulated by IκBα because suppression of IκBα levels by siRNA did not have any effect on IL-8 expression (Fig. 6E). Together, these results indicate that in prostate cancer cells, the IL-8 promoter is regulated by p65, but not by p50, NF-κB or IκBα. This observation is consistent with our previous study demonstrating that in human leukocytes, the IL-8 promoter is regulated by p65/65 homodimers, independently of the nuclear IκBα (32). In addition, these data suggest that proteasome inhibition induces IL-8 expression in prostate cancer cells by increasing p65 nuclear accumulation and recruitment to the IL-8 promoter; when proteasome is inhibited, p65 is persistently bound to the IL-8 promoter, resulting in increased transcriptional activity.

Previous studies have shown that proteasome inhibitors block the inducible NF-κB activity in prostate cancer cells (44, 59, 60). Moreover, the proteasome inhibitor MG132 reduced the in vitro binding of p65/50 heterodimers to NF-κB consensus
oligonucleotides measured by EMSA in nuclear extracts from unstimulated PC3 cells (11, 31). However, the in vitro binding of transcription factors to consensus oligonucleotides measured by EMSA does not necessarily predict the in vivo binding to endogenous promoters. Our data indicate that proteasome inhibition by BZ has a promoter-specific effect on NF-κB-dependent transcription. Although most genes are inhibited, or not affected, the IL-8 expression is greatly increased, and this is associated with increased p65 recruitment. Even though the BZ-increased p65 promoter recruitment and IL-8 expression in prostate cancer cells are surprising, they are supported by recent studies revealing that BZ increases constitutive NF-κB activity in endometrial carcinoma cells (61) and multiple myeloma cells (62).

In addition, previous studies indicated that BZ induces the proteasome-independent NF-κB activation in intestinal epithelial cancer cells, lung cancer cells, and bone marrow stromal cells (53, 55, 63).

BZ increased the nuclear accumulation of IKKα and IKKβ in PC3 cells (Fig. 6A, 6B), without increasing the IKKα/β mRNA levels (data not shown). Thus, it appears that the nuclear IKKα and IKKβ in prostate cancer cells are subject to proteasomal degradation, as was previously suggested (47). However, only IKKα seems to be required for BZ-induced p65 recruitment and IL-8 expression in metastatic prostate cancer cells, because siRNA suppression of IKKα, but not IKKβ, decreased the BZ-induced IL-8 expression (Fig. 6E). A previous study has demonstrated that the nuclear levels of IKKα correlate with prostate cancer progression (64). Although no activated IKKα was detected in nuclear fractions of normal human prostate or benign prostate hyperplasia, stage 4 tumors exhibited the highest nuclear levels and activity of IKKα (64). Furthermore, a recent study showed that IKKα regulates expression of androgen receptor in prostate cancer cells (65).

The enzymatic activity of IKKα is required for BZ-induced p65 recruitment and IL-8 expression in prostate cancer cells, because inhibition of IKK activity significantly attenuates the BZ-induced p65 recruitment (Fig. 7A) and IL-8 mRNA levels in PC3 and DU145 cells (Fig. 6C, 6D). However, our data indicate that IKKα does not phosphorylate the nuclear p65 at S536 in prostate cancer cells and that S536-P-p65 is not recruited to the IL-8 promoter. Recent studies have shown that the kinase-dependent nuclear functions of IKKα include the transcriptional activation by histone H3 phosphorylation; phosphorylation of CREB-binding protein, resulting in p65 transcriptional activation; and regulation of the metastatic suppressor Maspin (64–70). In addition, the nuclear IKKα can remove the inhibitory HDAC3 from certain promoters, allowing increased p65 recruitment and transcriptional activity (51, 71).

IL-8 contributes to prostate cancer progression through its induction of tumor cell proliferation, survival, and angiogenesis. In androgen-independent prostate cancer cells, IL-8 expression enhances tumorigenicity and metastasis. In this study, we show that proteasome inhibition by BZ greatly increases IL-8 expression in androgen-independent prostate cancer PC3 and DU145 cells. Of note, however, in both cell types, the level of IL-8 induction at the mRNA level was considerably higher than on the protein release level (Figs. 3, 4), indicating that in addition to inducing the IL-8 mRNA expression, proteasome inhibition has an inhibitory effect on protein(s) controlling mRNA processing, translation, or IL-8 release from cells. On the transcriptional level, proteasome inhibition seems to have two opposite effects on the NF-κB–dependent genes. One mechanism of action consists of the inhibition of NF-κB activity through the induction of cytoplasmic IκBα degradation and induction of the nuclear translocation and accumulation of IκBα, resulting in suppression of most of the NF-κB–dependent genes. The other, opposite, mechanism consists of the
increased expression of IL-8, and perhaps other genes regulated by p65 and IKKα (Fig. 8). Future studies should determine the exact mechanism of IKKα involvement in the enhanced p65 recruitment and IL-8 expression, as well as the mechanisms responsible for the IL-8 posttranscriptional regulation by proteasome inhibition in prostate cancer cells. Understanding the mechanisms of how proteasome inhibition and IKKα regulate IL-8 expression and secretion could lead to the development of new combination therapies targeting both IKKα and proteasome in androgen-independent prostate cancer and other solid tumors characterized by excessive IL-8 release.

Disclosures

The authors have no financial conflicts of interest.

References

BORTZEZOMIB INCREASES IL-8 IN PROSTATE CANCER CELLS


