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RESEARCH ARTICLE

The Senescence Program is Reduced in Proteasome Inhibitor Bortezomib-Resistant PC3 Prostate Cancer Cell Line

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ABSTRACT

Objective: Senescence may act as an antitumor mechanism by preventing the proliferation of cancer cells. Here we investigated the hypothesis that PC3 prostate cancer cells resistant to bortezomib respond differently to proteasomal inhibition with respect to induction of the senescence program as compared to the parental cells.

Materials and Methods: The degree of senescence was measured by β-galactosidase activity and the level of senescence-associated p16 INK4a by Western blotting after treatment of cells with varying concentrations of bortezomib. In addition, the senescence-associated secretory phenotype was analyzed by Human Cytokine Antibody Array.

Results: It is reported that the basal level of senescence was lower in resistant cells compared to non-resistant cells. It was found that the basal level of the senescence marker p16 INK4a was lower in bortezomib-resistant cells than in parent non-resistant cells. Moreover, p16 INK4a was significantly reduced in both cells under conditions of 100 nM bortezomib treatment, a finding suggesting that the reduced senescence after proteasomal inhibition was likely due to the reduced levels of p16 INK4a. Finally, it is reported here for the first time that basal levels of the proteins NAP2, FGF-6, MIP-3α, and PARC are significantly increased in the resistant cells compared to the parental cells.

Conclusion: Overall, the results suggest that inhibition of senescence may play an important function in the development of resistance to bortezomib.

Keywords: Bortezomib, cancer, prostate, proteasome, p16 INK4a, senescence

INTRODUCTION

Senescence, also known as cellular aging, is a dynamic cellular program that restricts the proliferation of old or damaged cells.[1,2] Characteristic features of senescent cells include the absence of proliferation markers and expression of the senescence-induced β-galactosidase.[3] Recent studies show that senescence is a highly stable cell cycle arrest and can be induced *in vitro* or *in vivo* in actively proliferating cells in response to proliferative stress or DNA damage.[2,4] Senescence stimuli also include oncogenic stress, ionizing agents, reactive oxygen species, and chemotherapeutic agents.[4,5] Because senescence can function as an antitumor mechanism, the integrity of the senescence program can also significantly affect cancer development and treatment outcomes.[2] In the absence of simple molecular or biochemical assays, there are few studies investigating the mechanism of senescence. Moreover, some of the detected senescence markers are not specific to the senescence program; for example, upregulation of certain cyclin-dependent kinase inhibitors (CDKi) also occurs in quiescent cells.[4,6] Interestingly, several viral oncogenes (SV40 large T antigen, HPV E6, and E7 proteins) are known to bypass the cellular senescence program by inactivating the tumor suppressor genes p53 and Rb.[7,8] However, it is very interesting to note that in the presence and stimulation of p53 and p16 INK4a proteins, it has been observed that the Ras oncogene stimulates the senescence response, which has been defined as oncogene-induced senescence.[9] Examination of the Ras signal transduction pathway suggests that the Raf/MEK/MAPK cascade is involved in stimulating the senescence program.[10] These results suggest that induction of the same pathway under different cellular conditions can stimulate not only cellular transformation but also the senescence program. The studies in Ras-expressing senes-

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cent cells have shown that target proteins of the ERK/MAPK pathway are degraded during the induction of senescence. Detailed analyzes have also shown that the degradation of many of these proteins is mediated by the proteasomal pathway.[11,12] These results indicate that the ubiquitin-mediated proteasomal pathway, a selective protein degradation pathway in eukaryotic cells, is also actively involved in the senescence program. In addition, many *in vivo* and *in vitro* studies have shown that inhibitors of the ubiquitin-proteasomal pathway prevent malignant cell proliferation.[13–15]

The proteasome inhibitor bortezomib is currently used in clinics to treat hematologic tumors (e.g., multiple myeloma).[16,17] Our previous studies have also shown that the proteasome inhibitor bortezomib is a promising chemotherapeutic agent against cells of solid tumors (e.g., prostate and breast cancer), and has significantly lower IC_{50} values compared with commonly used chemotherapeutic agents (e.g., 5-fluorouracil, cisplatin, and etoposide).[18,19] However, as observed with many chemotherapeutic agents, resistance mechanisms to bortezomib develop during the course of treatment.[20] To elucidate the resistance mechanisms, we developed a bortezomib-resistant PC3 prostate cancer cell as a model cell line by gradually increasing the bortezomib concentration. The results showed that bortezomib-resistant PC3 cells were approximately 4.3-fold more resistant to bortezomib compared with the parent cells.[21] As mentioned previously, it is also known that the senescence response may play an important role in chemotherapy resistance.[22] It is known that the molecular pathways and causes of cancer and the senescence program also interact and overlap considerably.[23] Therefore, the main purpose of this research was to accurately describe the potential role of senescence mechanisms in the development of resistance to the proteasome inhibitor bortezomib. We first decided to investigate whether there was a differential response in the induction of senescence between parental and bortezomib-resistant cells. Although induction of senescence appears to be a barrier to malignant transformation, recent studies suggest that senescence induction also has tumor-progressive potential because of the senescence-associated secretory phenotype, which contains more than 40 secretory factors (e.g.,IL-6, IL-8 and MMP-1).[24–27] Indeed, this is the first time that the senescence program has been reported to be reduced in both nonresistant and resistant cells in response to bortezomib treatment. In addition, basal levels of the proteins NAP-2, FGF-6, MIP-3α, and PARC are significantly increased in untreated bortezomib-resistant PC3 cells compared with untreated parental cells. Upon exposure to 100 nM bortezomib, the expression of NAP-2, FGF-6, MIP-3α, and PARC proteins was significantly increased in the resistant cells compared with bortezomib-treated parental cells.

MATERIALS AND METHODS

Reagents

Cell culture reagents were purchased from Sigma-Aldrich (Steinheim, Germany). The protein assay dye was from BioRad, and the human cytokine antibody array was purchased from Abcam (Cambridge, UK). Senescence-associated β-galactosidase staining reagents were purchased from Cell Signaling Technology. The authenticity of the PC3 prostate cancer cell line (ATCC Cat# CRL-1435)[28] was verified by loss of the normal Y chromosome using RT-PCR with Applied Biosystem QuantifilerTM Trio DNA Quantitation Kit (Cat# 4482910), optimized, and validated as part of a complete DNA testing system targeting Y chromosome. Y-chromosome amplification results were analyzed using HID RT-PCR Analysis Software-Version 1.2.

Cell Lines

Bortezomib-resistant cells were developed as described in a previous study using a stepwise dose-escalation method, a common method for developing cell lines resistant to chemotherapeutic agents to achieve high fold resistance.[21,29]

Measurement of β-Galactosidase Activity

Senescence-associated β-galactosidase staining kit (Cat# 9860, Cell Signaling Technology) was used to measure β-galactosidase activity at pH 6.0. PC3-P and PC3-R cells were seeded in 6-well plates at 50,000 cells per well. Cells were grown to a logarithmic phase and treated with isotonic water, 10 nM, and 100 nM bortezomib for 48 h. Bortezomib concentrations were selected based on our previous IC_{50} determinations with IC_{50} values of 71 nM in 4T1 cells, 2.5 nM in B16F10 cells, and 53.4 nM in PC3 cells.[18,30] All agents and solutions were prepared and applied according to the manufacturer's protocol. After treatment with the drug, the medium was removed from all wells, and the cells were washed once with PBS. After washing, 1X fixative solution was added to each well, and the cells were fixed for 15 min at room temperature. After the washing steps, 1 ml of β-gal staining solution was added, and the plates were sealed and incubated overnight at 37°C in a CO_2-free incubator. The developing blue color was photographed under an inverted microscope and at 200X magnification. Senescence-associated β-gal activity (%) was calculated using the following formula: SA-β-gal (%) = (# of stained cells/total # of cells in the field of view) x 100

Western Blotting

Western blotting experiment was performed as described previously.[31,32] Briefly, cells were seeded in 6-well plates at 200,000 cells per well. Then, cells were grown to a logarith-

Table 1. Human cytokine antibody array map. The array is used for the concurrent detection of 60 human cytokine levels in the resistant and parental cells. POS – Positive control; NEG – Negative control; BLANK – No antibody.

	A	E	C	D	E	F	G	H	I	J	K	L	M	N
1.	Pos	Pos	Neg	Neg	Blank	Angio genin	BDNF	BLC	BMP-4	BMP-6	CKpS-1	CNTF	EGF	Eotaxin
2.	Pos	Pos	Neg	Neg	Blank	Angio genin	BDNF	BLC	BMP-4	BMP-6	CKpS-1	CNTF	EGF	Eotaxin
3.	Eotaxin-2	Eotaxin-3	FGF-6	FGF-7	Flt-3 Ligand	Fractalkine	GCP-2	GDNF	GM-CSF	1-309	IFN-γ	IGFBP-1	IGFBP- 2	IGFBP-4
4.	Eotaxin-2	Eotaxin-3	FGF-6	FGF-7	Flt-3 Ligand	Fractalkine	GCP-2	GDNF	GM-CSF	1-309	IFN-γ	IGFBP-1	IGFBP- 2	IGFBP-4
5.	IGF-1	IL-10	IL-13	IL-15	IL-16	IL-1α	IL-1β	IL-1ra	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7
6.	IGF-1	IL-10	IL-13	IL-15	IL-16	IL-1α	IL-1β	IL-1ra	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7
7.	Leptın	LIGHT	MCP-1	MCP-2	MCP-3	MCP-4	M-CSF	MDC	MIG	MIP-1 δ	MEP-3 α	NAP-2	NT-3	PARC
8	Leptın	LIGHT	MCP-1	MCP-2	MCP-3	MCP-4	M-CSF	MDC	MIG	MIP-1 δ	MEP-3 α	NAP-2	NT-3	PARC
9.	PDGF-BB	RAMTES	SCF	SDF-1	TARC	TGF- β1	TGF-β3	TNF-α	TNF-β	Blank	Blank	Blank	Blank	POS
10.	PDGF-BB	EANTES	SCF	SDF-1	TARC	TGF- β1	TGF-β3	TNF-α	TNF-β	Blank	Blank	Blank	Blank	POS

mic phase and treated with 10 nM and 100 nM bortezomib or isotonic solution for 24 and 48 h. Cells were lysed with RIPA buffer (Cat# R0278, Sigma-Aldrich) containing a 1X protease inhibitor cocktail (Cat# sc-29131). Quantification of protein amount was performed using the Bradford assay.[33] Equal amounts of protein (35 μg) were separated using 10% SDS-PAGE and then transferred to PVDF membranes (Cat# 1704156) using a Bio-Rad Trans-Blot Turbo Transfer System. Membranes were exposed to rabbit monoclonal anti-p16 INK4a (1:1000 dilution, Cat# 80772) or rabbit monoclonal anti-MMP-1 (1:1000 dilution, Cat# 54376). To determine equal protein loading, membranes were re-probed with anti-β-actin rabbit antibody (1:3000, Cat# ab8227, Abcam). After incubation with the primary antibody, an anti-rabbit HP-conjugated secondary antibody (1:3000, Cat# 7074) was added for 1 h in TBS-T. Membranes were incubated with LumiGLO reagent (Cat# 7072), and protein bands were visualized using a Bio-Rad ChemiDoc Imaging System. Protein bands were quantified after background correction using the Image J program. Graphs and statistical analyzes were obtained with the GraphPad Prism 5 program.

Analysis of Human Cytokine Array

Changes in the levels of cytokines were analyzed using the Human Cytokine Antibody Array (Cat# ab169817, Abcam), which detects the expression of 60 different cytokines (Table 1).[34] First, PC3-P and PC3-R cells were seeded in 6-well plates at 100,000 cells per well. At a confluence level of approximately 70%, cells were treated with isotonic water (vehicle for bortezomib, control group) or 100 nM bortezomib for 24 h. After treatment, cells were washed with PBS and homogenized with a lysis buffer for protein extraction. Experimental steps were then performed according to the manufacturer's instructions. Briefly, after each membrane was placed in an 8-well plate, they were incubated with 1 ml of 1X blocking buffer containing 200 μg protein at room temperature for 2 h. After incubation, the membranes were washed 3X with wash buffer I and 2X with wash buffer II. After washing, membranes were incubated with a biotinylated antibody cocktail (1 ml) for 2 h. After extensive washing, membranes were treated with 1X HP-conjugated streptavidin for 2 h. Then, the membranes were treated with the detection buffer (500 μl) for 2 min (250 μl each of buffers C and B, mixed in a 1:1 ratio). Finally, the membranes were exposed to Kodak BioMax X-ray film in a darkroom. Quantification of individual protein spots was performed using the Image J program after background correction. Statistical analyzes were performed with GraphPad Prism 5.0 using One-way ANOVA and Bonferroni's post hoc test.

RESULTS

In our previous studies, we found that resistance to the proteasome inhibitor bortezomib in PC3 prostate cancer cells was not due to upregulation of the mature form of the proteasome subunit beta type-5 (PSMB5), the heat shock protein HSP70, or the multidrug resistance (MDR) transporters.[21,31] Because it is also known that an incomplete and heterogeneous senescence response may play a key role in chemotherapy resistance and produce more tumor-aggressive variants[22,35] we first wanted to investigate whether the senescence program is involved in the mechanism of bortezomib resistance. As shown in Figures 1 and 2A, SA-β-gal activity (as inferred from the number of blue-stained cells) was significantly higher in PC3-P cells compared with PC3-R cells ($p<0.001$) after 48 h of bortezomib exposure. Interestingly, SA-β-gal activity was significantly decreased in parental PC3-P cells after 10 nM and 100 nM bortezomib concentrations ($p<0.05$, $p<0.001$, respectively) (Figure 2A). Since the basal level of SA-β-gal activity was significantly lower in the resistant PC3-R cells, the dose-response effect was not observed in these cells (Figure 2A). These results suggest that the resistant cells may be less susceptible to induction of the senescence program. To confirm these results, we then treated the cells with the same concentrations of bortezomib for a relatively short period of time (i.e., 24 h). As shown in Figure 2B, SA-β-gal activity was again significantly lower in untreated resistant PC3-R cells compared with untreated parental PC3-P cells ($p<0.05$). Senescence-associated β-gal activity in 100

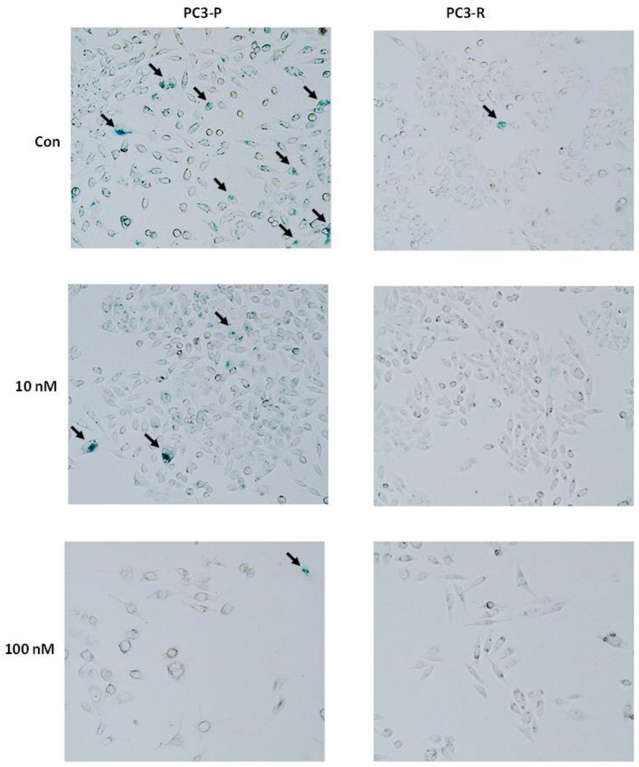

Figure 1. Determination of the level of senescence in parental PC3-P and resistant PC3-R cells. SA-β-gal activity assay was used to determine the senescent cells at pH 6. 0. The photographs were taken under an inverted microscope at 200X magnification. The cells were treated with isotonic water, 10 nM or 100 nM bortezomib for 48 h.

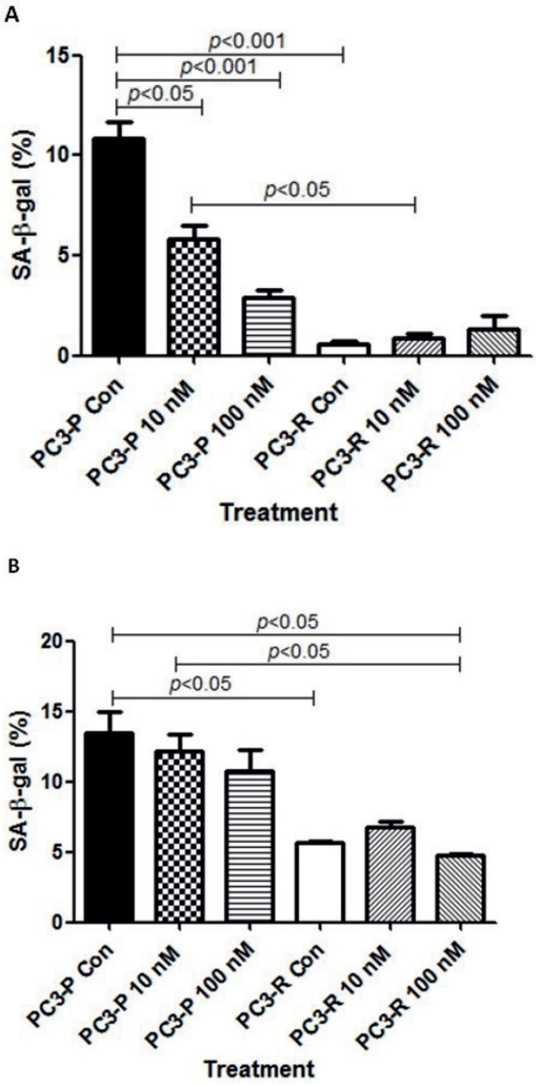

Figure 2. A) Quantitation of the level of senescence in parental PC3-P and resistant PC3-R cells after 48 h of bortezomib treatment. B) The analysis of the level of senescence in parental PC3-P and resistant PC3-R cells after 24 h of bortezomib treatment. The results are presented as means±SEM (n = 3). The groups were compared using one-way ANOVA with Bonferroni multiple comparison post-test. PC3-P, the parental PC3 cell; and PC3-R, bortezomib-resistant PC3 cell.

nM bortezomib-treated resistant PC3-R cells was also significantly lower compared with untreated PC3-P ($p<0.05$) or 10 nM bortezomib-treated PC3-P cells ($p<0.05$) (Figure 2B). We next examined the expression of the classic senescence marker p16 INK4a, as well as the expression of matrix metalloproteinase-1 (MMP-1), the abnormal expression of which has been associated with age-related diseases.[36] As shown in Figures 3A and B, although the expression of p16 INK4a was decreased in PC3-P cells after bortezomib treatment, there was no significant decrease in expression in PC3-R cells after 24 h of treatment compared with untreated PC3-R cells. A similar observation was made for the level of MMP-1 in both cells (Figures 4A and B). Interestingly, the basal level of p16 INK4a was approximately 40% lower in untreated PC3-R cells than in untreated PC3-P cells. After 48 h of treatment, both p16 INK4a and MMP-1 levels were significantly reduced in PC3-P and PC3-R cells after 100 nM bortezomib exposure (Figures 5A and B). Again, the basal level of p16 INK4a in untreated PC3-R cells was less than 50% compared with untreated PC3-P cells, a result consistent with the reduced SA-β-gal activity in untreated PC3-R cells. Finally, the Human Cytokine Antibody Array Kit was used to examine the changes in cytokine profiles in PC3-P and PC3-R cells after 48 h of treatment with 100 nM bortezomib. The 60 different cytokines detected are shown in Table 1. Bone morphogenetic proteins (BMPs) are involved in the development of bone formation as well as cell growth and apoptosis.[37] As shown in Figures 6 and 7, BMP-4 was decreased in PC3-P cells exposed to 100 nM bortezomib, whereas BMP-6 was decreased in both PC3-P and PC3-R cells after inhibition of the proteasome by 100 nM bortezomib. Interestingly, the basal level of BMP-6 was significantly higher in untreated PC3-R cells than in untreated parental cells ($p<0.05$). Similarly, CK β 8-1 [a CC chemokine that induces cell-cycle progression[38]] and ciliary neurotrophic factor (CNTF) were decreased in response to treatment with 100 nM bortezomib in both PC3-P and PC3-R cells (Figures 6 and 7). As shown in Figure 7, the basal level of CNTF was significantly higher in untreated PC3-R cells com-

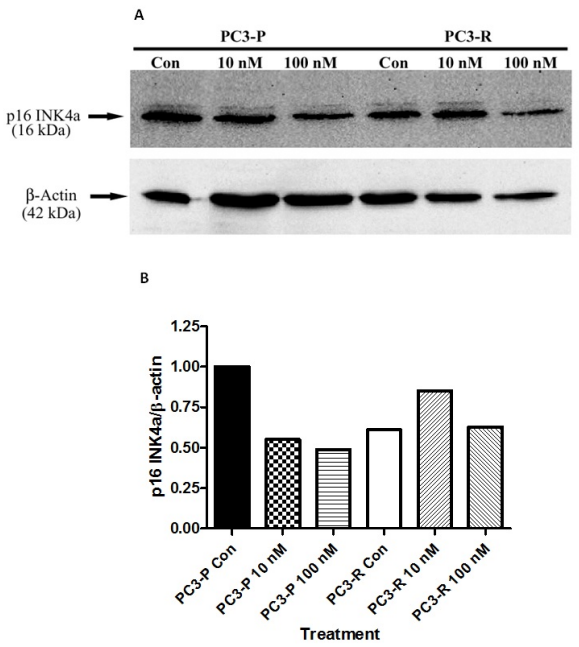

Figure 3. A) Examination of p16 INK4a protein in PC3-P and PC3-R prostate cells after 24 h of bortezomib treatment. Both cell lines were treated with 10 nM and 100 nM bortezomib for 24 h; the control cells were treated with isotonic water. 35 μg of protein was separated by 12% SDS-PAGE. Rabbit polyclonal anti-β-actin antibodies were used for loading controls. B) Quantification of Western blot results is seen in Figure 3A. p16 INK4a expression levels were normalized with β-actin levels.

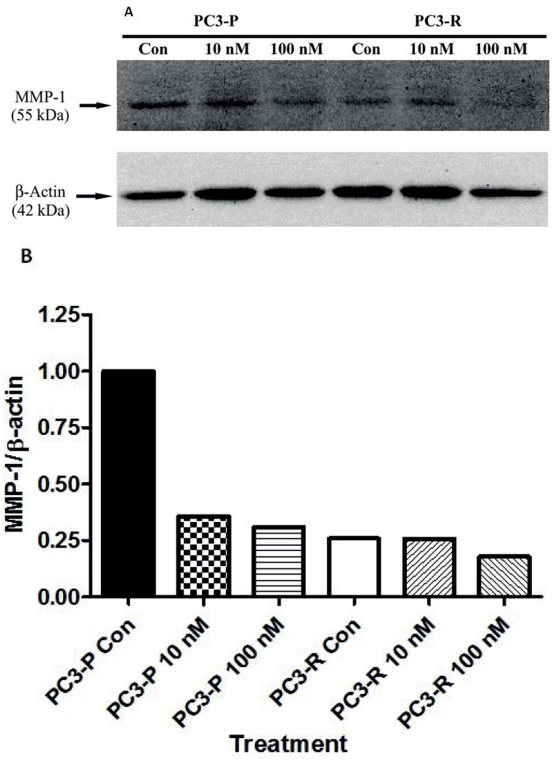

Figure 4. A) Examination of MMP-1 protein in PC3-P and PC3-R prostate cell lines after 24 h of bortezomib treatment. Both cell lines were treated with 10 nM and 100 nM bortezomib for 24 h; the control cells were treated with isotonic water. 35 μg of protein was separated by SDS-PAGE. Rabbit polyclonal anti-β-actin antibodies were used for loading controls. B) Quantification of Western blot results seen in Figure 4A. MMP-1 expression levels were normalized with β-actin levels. The result is representative of two experiments, each run in duplicate.

pared with untreated PC3-P cells, a result similar to that of BMP-6 described above. The levels of EGF and eotaxin proteins were also decreased in PC3-P cells after treatment with 100 nM bortezomib, with no significant effect on PC3-R cells (Figures 6 and 7). IGFBP-2 levels were reduced in both PC3-P and PC3-R cells after treatment with 100 nM bortezomib ($p<0.05$), but IGFBP-1 was reduced only in PC3-P cells after inhibition of the proteasome by bortezomib. IFN-γ (a dimerized soluble cytokine) was also significantly decreased in PC3-P cells after 100 nM bortezomib exposure ($p<0.05$). Although its level appeared to be reduced in the resistant cells, no significant changes were detected after statistical analysis (Figure 7). On the other hand, basal levels of FGF-6, MIP-3α, NAP-2 and PARC proteins were significantly higher in untreated PC3-R cells than in untreated PC3-P cells ($p<0.05$ in all cases, Figures 6 and 8). After exposure to 100 nM bortezomib, the expression of FGF-6, MIP-3α, NAP-2, and PARC proteins was similar and significantly higher in PC3-R cells than in PC3-P cells treated with 100 nM bortezomib ($p<0.05$ in all cases, Figure 8). The levels of eotaxin 3, IGF-1, MIP-1δ, NT-3, MCP-1, and MCP-4 were significantly increased in the resistant cells treated with 100 nM bortezomib compared to the untreated PC3-R cells as well as compared to the PC3-P cells treated with 100 nM bortezomib (Figure 8). No statistically significant differences in the expression levels of the other proteins shown on the cytokine array were detected between the experimental groups (Table 1 and Figure 6).

DISCUSSION

In the current study, we investigated whether there was a differential response in the degree of senescence program between parental and bortezomib-resistant cells after treatment with different concentrations of bortezomib. Previous studies have shown that bortezomib has both senescence-promoting and -inhibiting effects.[39,40] For example, Krwtowski et al reported that bortezomib caused a time-dependent increase in the senescence of normal fibroblasts, an effect that was particularly observed under hypoxic conditions.[40] On the other hand, exposure of rituximab-resistant cell lines (RRCL) derived from Raji cells (a continuous human cell line of hematopoietic origin) to bortezomib resulted in varying degrees of senescence inhibition and induced cell cycle arrest in G2-M phase associated with mitotic catastrophe.[39] Here, we showed for the first time that the degree of senescence was lower in bortezomib-resistant PC3 cells compared with parent cells treated with different doses of bortezomib at 24 or 48 h. These results suggest that the

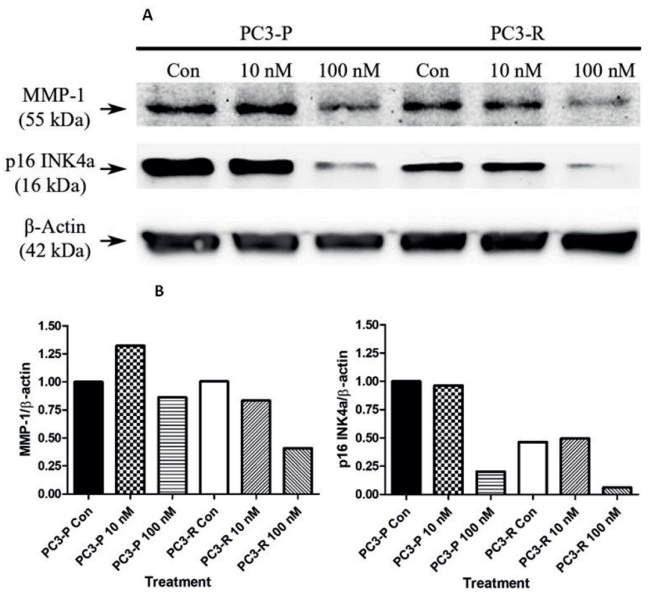

Figure 5. Analyses of MMP-1 and p16 INK4a proteins in PC3-P and PC3-R prostate cell lines after 48 h of bortezomib treatment. Both cell lines were treated with 10 nM and 100 nM bortezomib for 48 h; the control cells were treated with isotonic water. 35 µg of protein was separated by 12% SDS-PAGE. Rabbit polyclonal anti-β-actin antibodies were used for loading controls. B) Quantification of the level of MMP-1 and p16 INK4a seen in Figure 5A. MMP-1 (left panel) and p16 INK4a (right panel) expression levels were normalized with β-actin levels.

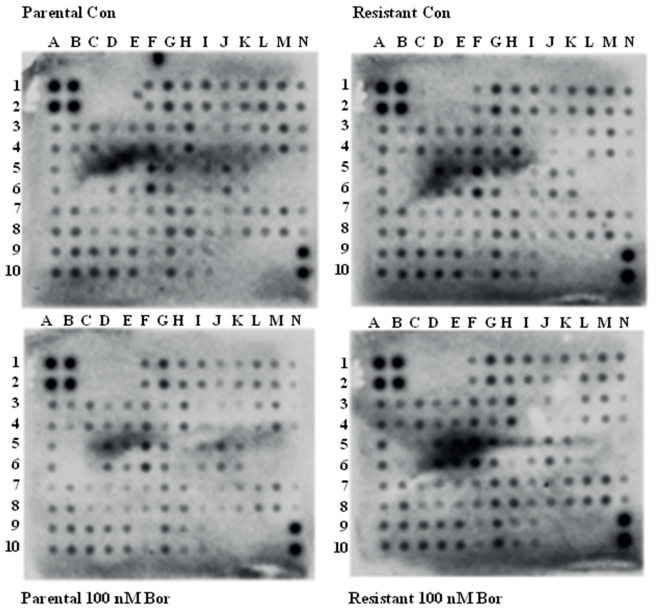

Figure 6. Cytokine array analysis in PC3-P and PC3-R prostate cell lines. Cytokine profiles were visualized by a Bio-Rad ChemiDoc Imaging System. The cells were treated with 100 nM bortezomib for 48 h; the control cells were treated with isotonic water.

inability to induce senescence may be a critical factor in resistance to chemotherapeutic agents, particularly bortezomib. p16 INK4a is a tumor suppressor and is also known to be required for the induction of senescence. In fact, it is probably the best-known biomarker for cellular senescence.[41] MMP-1 is also known to be associated with many age-associated disease pathologies and tumor progression and metastasis.[36] Therefore, we examined p16 INK4a and MMP-1 expressions by Western blot analysis to elucidate the mechanism of senescence in cells resistant to bortezomib. Consistent with the results shown in Figures 1 and 2, the basal level of p16 INK4a was lower in the resistant cells compared with the parental cells. Moreover, 100 nM bortezomib exposure decreased the expression of p16 INK4a in both cells, suggesting that the reduction in senescence after 100 nM bortezomib treatment may be due to the decreased expression of p16 INK4a. To the best of our knowledge, this is the first study to indicate that p16 INK4a expression is reduced in response to bortezomib exposure and thus is not degraded in a proteasomal-dependent manner. To further investigate the mechanisms leading to bortezomib resistance and the changes in the senescence program, the expression levels of 60 different cytokines were analyzed after treatment of parental and resistant cells with 100 nM bortezomib. BMP-4 protein was significantly reduced in parental cells treated with 100 nM bortezomib. It has been previously reported that the BMP-4 signaling pathway can induce senescence and downregulate the growth of A549 lung cancer cells.[42] IFN-γ was also reduced in parental cells treated with 100 nM bortezomib. A previous study reported that IFN-γ and TNF are able to induce stable growth arrest, which is similar to senescence in cancer cells.[43] In light of these previous studies, it can be concluded that the reduction in senescence in the parental cells after bortezomib treatment may be due to the decreased expression of BMP-4 and IFN-γ. On the other hand, the only cytokines detected in higher expression in the resistant cells compared with the parental cells were NAP-2 (also called CXCL7), FGF-6, MIP-3α, and PARC. NAP-2 is able to interact with both core and linker histones and transfer histones to DNA templates.[44] Previous studies have shown that NAP-2 plays a central role in mediating the interaction of mesenchymal stem cells (MSCs) with cancer stem cells (CSCs) and in maintaining increased numbers of CSCs in the tumor.[45,46] NAP-2 also forms a complex with the epidermal growth factor receptor (EGFR), activating the EGFR signaling pathway.[47] It is therefore critical for tumor cell proliferation and survival; indeed, it is associated with highly metastatic lesions and aggressive prostate cancer.[48,49] Thus, it is proposed here that NAP-2 may also play an important role in resistance to the proteasome inhibitor bortezomib, as well as in reduced senescence in resistant cells. FGF is known to promote self-renewing proliferation and inhibit cellular senescence[50], a finding that supports the observations presented here. Shang et al reported that MIP-3α is among the seven cytokines that consistently induce senescence in HUVECs.[51] Although this result seems to dispute the findings presented here regarding the expression level of MIP-3α, the conflicting observations could be due to the cell-specific effects. Moreover, the effects observed in different cells may certainly be caused by the combinations of

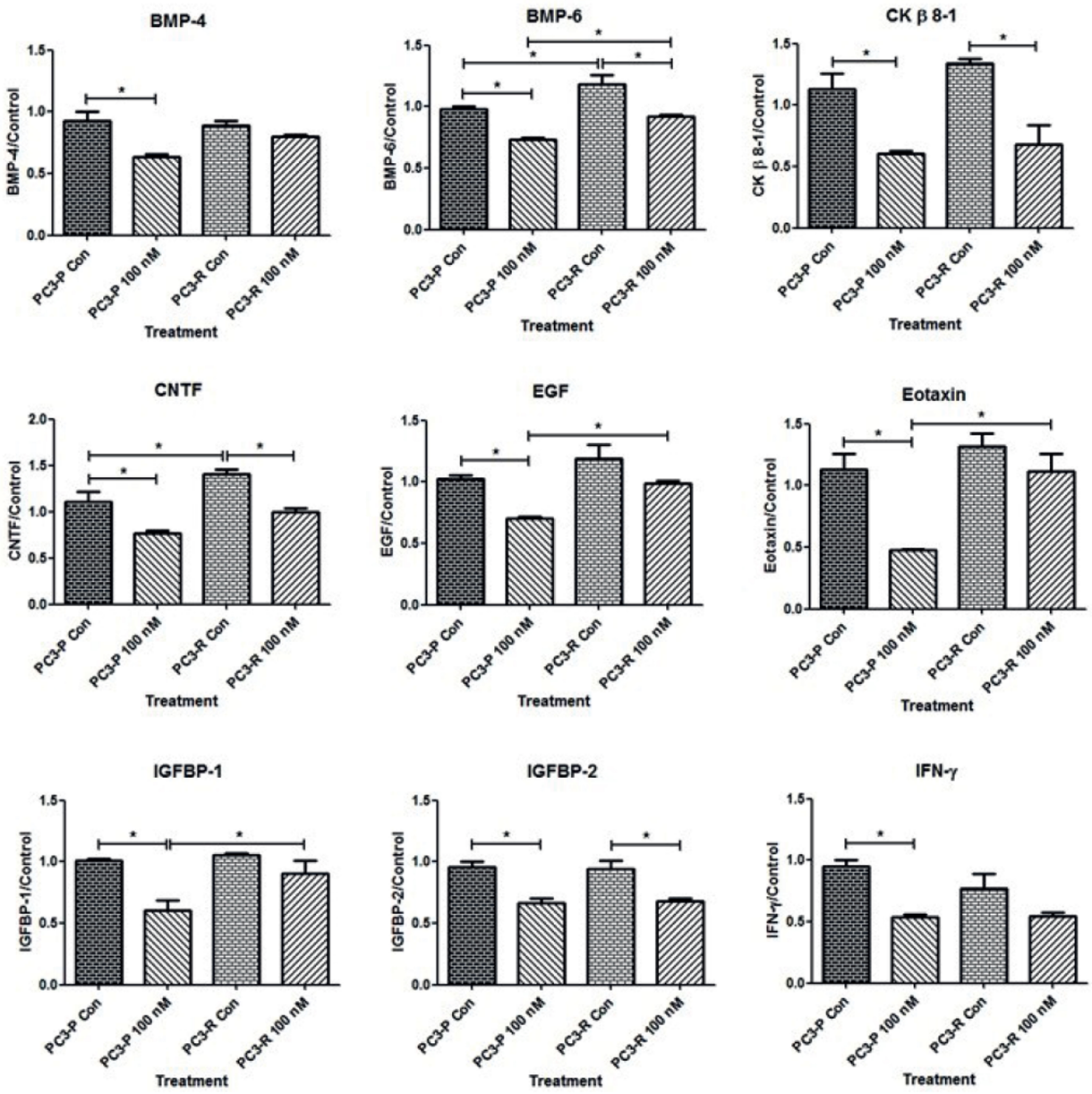

Figure 7. Graphical representations of cytokines with reduced levels between the experimental groups. The results are presented as means ±SD (n = 2). To determine the statistical significance, One-way ANOVA and Bonferroni's post hoc tests were applied using GraphPad Prism 5 program. *represents $p<0.05$, **represents $p<0.01$.

cytokines induced under certain conditions. Nevertheless, the results presented here provide important insights into the regulatory mechanisms of MIP-3α and other cytokines that are down- or up-regulated under proteasomal inhibition. There is limited information on the regulation and expression of PARC (also known as CCL18) under different circumstances. In one of these rare studies, Struyf et al reported that serum levels of PARC/CCL18 are elevated in childhood acute lymphoblastic leukemia and thus may represent a novel leukemia marker.[52] Therefore, this study provides crucial information not only on the regulatory mechanism of PARC under inhibition of the ubiquitin-proteasome pathway, but also on the possible role of the PARC protein in the development of resistance to proteasomal inhibitors as well as in the induction of senescence.

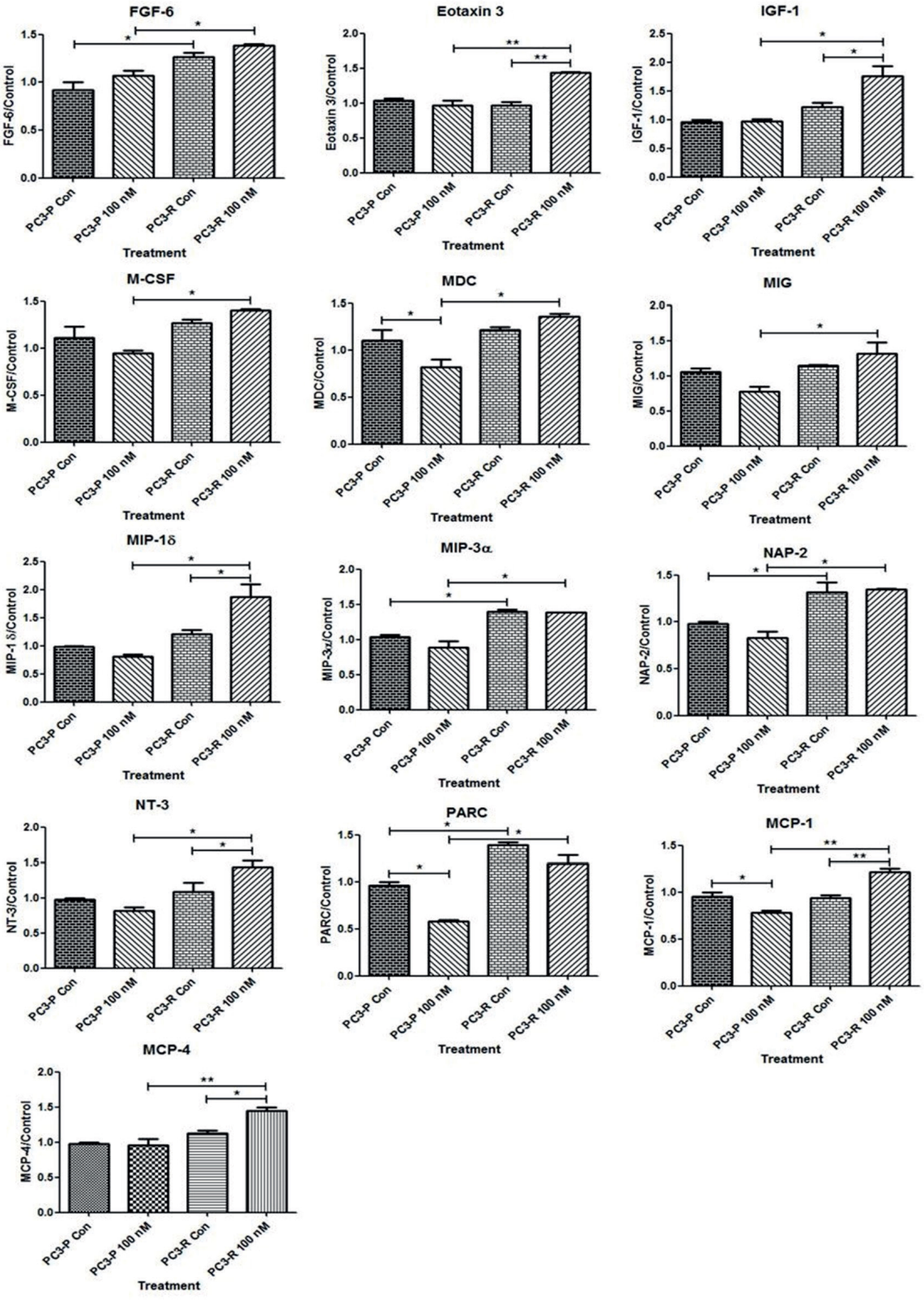

Figure 8. Graphical representations of cytokines with increased levels between the experimental groups. The results are means ±SD (n = 2). One-way ANOVA and Bonferroni's post hoc tests were performed using GraphPad Prism 5 program. *represents $p<0.05$, **represents $p<0.01$.

CONCLUSION

In summary, as discussed in detail above, it is well known that there are many conflicting studies on the role of senescence in tumor progression. In addition, there are few studies on the potential role of senescence in the development of resistance to chemotherapeutic agents, particularly the proteasome inhibitor bortezomib. Interestingly, the results presented here suggest that the basal level of senescence is decreased in bortezomib-resistant cells compared with parental cells. Furthermore, the experimental data suggest that senescence is downregulated in response to different concentrations of bortezomib in both resistant and nonresistant cells. In light of the data presented here, it is anticipated that a detailed investigation of the mechanisms of senescence, as well as the cytokines studied here, may provide further insight for the development of novel therapeutic targets and mechanisms for cancer cells resistant to common chemotherapeutic agents, particularly the proteasome inhibitor bortezomib.

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