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PRECLINICAL STUDY

Diminished feedback regulation of proteasome expression and resistance to proteasome inhibitors in breast cancer cells

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Abstract Clinical trials with proteasome inhibitor Bortezomib (also named Velcade or PS-341) has shown promising results for some cancers. However, other types of cancers including breast cancer do not respond well to Bortezomib. To understand the cause of the drug resistance, we compared the regulation of proteasome expression and the sensitivity to proteasome inhibitors between human breast cancer cells and nontumorigenic mammary epithelial cells. We found that, while the endogenous expression level is much higher, the potential of feedback expression in response to proteasome inhibitors is much lower in the breast cancer cells. Furthermore, the breast cancer cells are much more resistant to proteasome inhibitors compared to the nontumorigenic mammary epithelial cells. Biochemical analysis showed that the pathway of Bortezomib-induced apoptosis is apparently defective in the breast cancer cells. Together, these results provide an explanation for the inefficacy of Bortezomib in the clinical trials for breast cancer patients. The likelihood of combination therapy with Bortezomib and other anti-cancer agents for breast cancer is also discussed.

Keywords Proteasome · Proteasome inhibitor · Feedback regulation · Drug resistance · Breast cancer

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Introduction

Protein degradation by the proteasome is one of the major regulatory mechanisms in the cell [1]. Aberrant proteasome activity is implicated in the pathogenesis of cancers [2]. In vitro studies have demonstrated that inhibition of the proteasome activity induces apoptosis in cancer cells, suggesting the potential utilization of proteasome inhibitors in cancer therapy [3–5]. Indeed, clinical trials with proteasome inhibitor Bortezomib (also named Velcade or PS-341) have been approved by the U.S. Food and Drug Administration and shown promising results in certain types of cancers including hematological malignancies [6–8]. However, Bortezomib appeared to have little efficacy in treating other cancers including metastatic breast cancer [9–13]. It is unclear why different cancers respond so differently to the same proteasome inhibitor. The underlying mechanism of the drug resistance to Bortezomib has not been explored.

The proteasome expression is regulated by a feedback mechanism, which was originally identified in *Saccharomyces cerevisiae* and subsequently found conserved in higher eukaryotes including humans [14–20]. For instance, inhibition of the proteasome activity by proteasome inhibitors induces upregulation of the proteasome genes in mammalian cells [17, 18]. This feedback mechanism is physiologically relevant as pretreatment with low concentration of proteasome inhibitor was shown to protect neuronal cells from oxidative injury [18]. In addition, the Rpn4-mediated proteasome feedback regulation is involved in drug resistance to Bortezomib in *S. cerevisiae* [16]. However, it has never been closely examined whether or not this feedback regulation is different between normal and cancer cells. More importantly, it is unknown whether the resistance to Bortezomib

in some cancers is related to the feedback regulation of proteasome expression.

In this study we compared the feedback regulation of proteasome expression and the sensitivity to proteasome inhibitors between breast cancer cells and nontumorigenic breast epithelial cells. We found that, while the endogenous abundance of proteasome is much higher, the feedback expression is diminished in the breast cancer cells. Moreover, the breast cancer cells are much more resistant to proteasome inhibitors than the nontumorigenic breast epithelial cells. This information may explain the inefficacy of Bortezomib in the clinical trials for breast cancer. The potential mechanisms of the drug resistance and the possibility of combination therapy using Bortezomib and other anti-cancer agents are discussed.

Materials and methods

Cell culture and reagents

Nontumorigenic mammary epithelial cell line MCF-10A and its transformed derivative cell line MCF-10CA1α were obtained from the cell bank at Karmanos Cancer Institute (also see ref. 22). Breast cancer cell lines MCF-7, MDA-MB-231 and MDA-MD-453 were maintained in our institute. Human lung sarcoma HT-1080, SV40-transformed human fibroblast VA-13, and nontransformed human fibroblast IMR90 cell lines were provided by Dr. Avraham Raz (Karmanos Cancer Institute). All mammary cell lines were maintained in DMEM/F12 (Invitrogen, CA) with 5% horse serum (Invitrogen, CA), 10 μg/ml of insulin (Sigma-Aldrich, MO), 20 ng/ml of epidermal growth factor (EGF) (Invitrogen, CA), 0.5 μg/ml of hydrocortisone (Sigma-Aldrich, MO) and 100 ng/ml of cholera toxin (Calbiochem, CA). The other cell lines were grown in DMEM/F12 with 10% fetal bovine serum (Invitrogen, CA). Proteasome inhibitor MG-132 (carbobenzoxyl-L-leucyl-L-leucyl-L-leucinal) (Boston BioChem, MA) was prepared as 10 mM stock in DMSO and diluted with growth medium for treatments. Bortezomib was a gift from Dr. Omer Kucuk (Karmanos Cancer Institute) and prepared as stock in PBS. Chloroquine and ammonium chloride were prepared in PBS. Cisplatin was purchased from Sigma-Aldrich.

Real-time reverse transcription-polymerase chain reactions

Total RNA was isolated using Trizol Reagent (Invitrogen, CA) and treated with DNase I to eliminate contaminated genomic DNA. Reverse transcription and real-time PCR was performed using the SYBR Green RT-PCR Reagents (Applied Biosystems, CA) according to the manufacturer's instruction. PCR amplification was carried out in a GeneAmp 5700 Sequence Detection System (Applied Biosystems, CA) under universal thermal cycling conditions (2 min at 50°C, 10 min at 95°C followed by 15 s at 95°C/1 min at 60°C for 40 cycles). Relative quantification was defined using the comparative Ct (threshold cycle) method. Primers for PSMA5 were 5′-AGCAATTGGCTC TGCTTCAG-3′ and 5′-GCATTCAGCTTCTCCTCCAT-3′. Primers for PSMD3 were 5′-GGCCUATCTCGCGCTC GTGTG-3′ and 5′-ACGCGUGATGTGGAAGGCAGCAT-3′. GAPDH was used as an endogenous control amplified with primers 5′-CAGCCTCAAGATCATCAGCA-3′ and 5′-TGTGGTCATGAGTCCTTCCA-3′.

Cell viability analysis

Cells were inoculated in 96-well plates at 4,000 ~ 6,000 cells per well 24 h prior to the treatment. Cell viability was measured using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H- tetrazolium bromide (MTT) colorimetric assay. MTT was from Sigma-Aldrich. The values given were the means ± S.E. from three to six independent experiments.

Proteasome activity assay

Cells were washed 3 times with ice-cold PBS and scraped for centrifugation. Cell pellets were resuspended in lysis buffer (50 mM Tris–Cl, pH 7.5, 10 mM $MgCl_2$, 100 mM KCl, 1% Triton X-100, 10% glycerol, 1 mM DTT, 1X protease inhibitor cocktail) and mixed with rotation at 4°C for 2 h. Extract was centrifuged at 13,200 rpm for 10 min at 4°C. Protein concentration was determined using Bradford Assay Kit (Bio-Rad, CA). Chymotrypsin-like activity of the 20S proteasome was measured by incubation of 40 μM Suc-Leu-Leu-Val-Tyr-AMC (Boston BioChem, MA) with 20 μg cell extract in 100 μl assay buffer (50 mM Tris–Cl, pH 7.5) at 37°C for 1 h. The released fluorescence signals were monitored with an excitation filter of 355 nm and an emission filter of 460 nm using VersaFluot Fluorometer (Bio-Rad, CA).

Immunoblotting analysis

Cell extracts prepared above were subjected to SDS-PAGE (12% gel) and electroblotted onto PVDF membranes. The membranes were probed with antibodies against 20S proteasome subunits PSMA5, PSMD3 (BostonBioChem, MA), ubiquitin (Santa Cruz Biotechnology, CA), cleaved Caspase-3 (Cell Signaling Technology, MA), Bax (Santa Cruz Biotechnology, CA) and β-actin (Sigma, MO),

respectively. The secondary antibodies Alexa Fluor 680 anti-mouse Ig G and IRDye 800 anti-rabbit Ig G (Molecular Probes, OR) were used for the Odyssey Infrared Imaging System (LI-COR Biosciences, NE). In some blots, the signals were detected by horseradish peroxidase-conjugated secondary antibody and Visualizer Western Blot Detection Kit (Upstate, NY).

Data analysis

Statistical analyses were conducted using Student's test. Differences were considered significant at $P < 0.05$ versus control.

Results and discussion

Higher endogenous level but lower feedback potential of proteasome expression in breast cancer cells

Proteasome expression is regulated by a feedback mechanism, which is conserved from yeast to humans [14–20]. To examine this feedback mechanism in human breast cancer cells, we treated the malignant breast epithelial MCF-7 cells with proteasome inhibitor MG-132 at different concentration for different time periods. As control, we also treated the nontumorigenic human mammary epithelial MCF-10A cells under the same conditions. Using real-time RT-PCR analysis, we measured the expression of two of the proteasome genes, PSMA5 and PMSD3, encoding one of the 19S and 20S subunits, respectively. As shown in Fig. 1a and b, treatment with MG-132 increased the transcription of the PSMA5 and PSMD3 genes in both cell lines in a time- and concentration-dependent manner. For instance, the expression of PSMA5 and PSMD3 reached the highest level in both cell lines after 12 h treatment (Fig. 1a). However, the scale of the feedback upregulation was significantly different between these two cell lines (Fig. 1c). The MCF-10A cells had a 3.0 fold increase in PSMA5 and PSMD3 expression after treated with 8.0 μM MG-132 for 12 h, whereas the increase in MCF-7 cells was only about 1.5 fold. Similar results were obtained when another proteasome inhibitor, LLnL, was used (data not shown). In line with the real-time RT-PCR results, immunoblotting analysis detected a marked increase of PSMA5 protein abundance in MCF-10A after treated with 4.0 μM MG-132 for 16 h (Fig. 1b, upper panel). By contrast, the PSMA5 protein level was not elevated noticeably in MCF-7 by the same treatment (Fig. 1b, upper panel). A similar differential feedback response was observed when these 2 cell lines were treated with Bortezomib (Fig. 1b, lower panel, Fig. 1d). Note that real-time RT-PCR analysis did show a modest increase of PSMA5 and PSMD3 mRNA expression in MCF-7 in response to MG-132 treatment (Fig. 1a, c). The failure to detect an increase in PSMA5 protein level in MCF-7 is likely due to inhibition of protein synthesis by the proteasome inhibitors [21]. On the other hand, the inhibitory effect of the proteasome inhibitors on protein synthesis is transient and partial, which explains why the increase of PSMA5 protein abundance can be observed in MCF-10A cells, which have a much stronger feedback upregulation of the proteasome genes than MCF-7 cells. Similar to MCF-7, other breast cancer cells MDA-MB-231 and MDA-MB-453 did not respond to Bortezomib with an increase in PSMA5 and PSMD3 protein expression (Fig. 1d). Thus, the feedback potential of proteasome expression is lower in breast cancer cells than in nontumorigenic breast epithelial cells.

It is interesting to note that in the absence of proteasome inhibitors the endogenous protein levels of PSMA5 and PSMD3 were much higher in the breast cancer cells than in MCF-10A (Fig. 1b, d). Real-time RT-PCR analysis also indicated that the mRNA levels of PSMA5 and PSMD3 were significantly higher in MCF-7 than in MCF-10A (data not shown). It is likely that the lower potential of feedback response to proteasome inhibitors is directly related to the higher steady-state level of the proteasome in the breast cancer cells.

The differential response to proteasome inhibitors by MCF-10A and the breast cancer cells promoted us to examine if this difference also exists between MCF-10A and a well-characterized transformed derivative cell line, MCF-10CA1α [22]. Specifically, we compared the PMSA5 and PMSD3 protein levels between these two cell lines in the presence and absence of Bortezomib treatment. As shown in Fig. 1d, there was no statistically significant difference in either endogenous or feedback expression between these two cell lines. These observations are consistent with a recent report showing that these two cell lines have similar abundance and activity of proteasome [23]. Thus, the regulation of proteasome expression in the transformed MCF-10CA1α cells derived from xenografts in immunodeficient mice is different from the breast cancer cells utilized in this study, which were directly isolated from cancer patients.

Breast cancer cells are resistant to proteasome inhibitors

To assess the therapeutic effect of proteasome inhibitors in breast cancer cells, we first compared the sensitivity of MCF-7 and MCF-10A to MG-132 by measuring cell viability using MTT assay. As shown in Fig. 2a, MCF-7 cells were much more resistant to MG-132 compared to MCF-10A cells. Similarly, MCF-7 showed much stronger resistance to Bortezomib than MCF-10A (Fig. 2b). Inter-

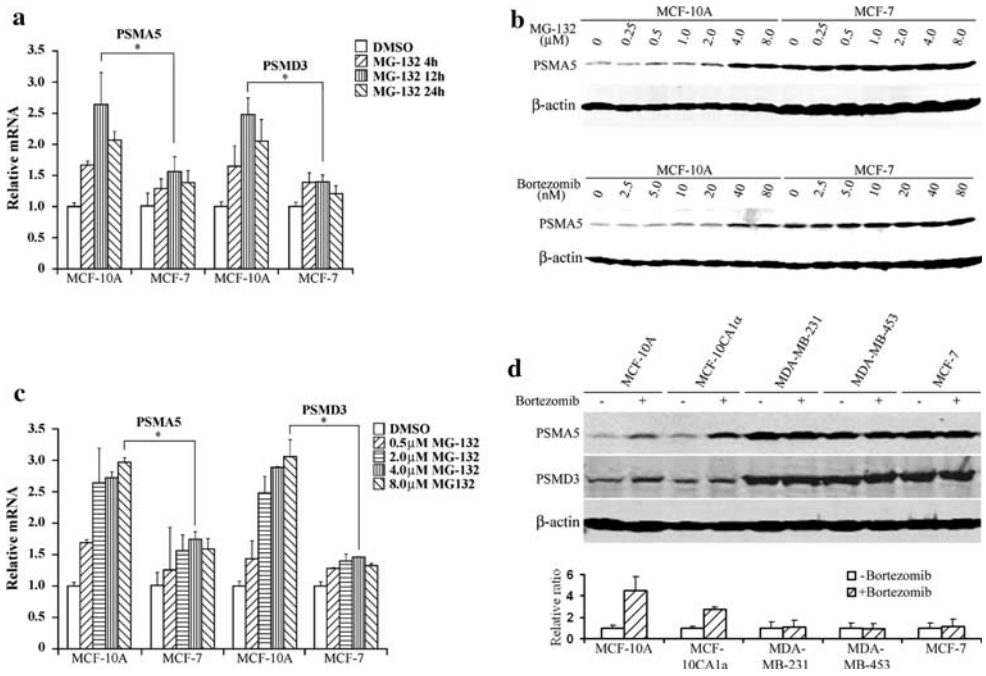

Fig. 1 Diminished feedback regulation of proteasome expression in breast cancer cells. (**a** and **c**) Real-time RT-PCR analysis of pSMA5 and pSMD3 expression in MCF-10A and MCF-7 cells treated with or without MG-132. MCF-10A and MCF-7 cells were treated with 2 μM MG-132 for 4, 12 and 24 h (**a**) or treated with different concentration of MG-132 for 12 h (**c**). Control cells were treated with DMSO for 12 hr. Transcripts of PSMA5 and PSMD3 were analyzed by real-time RT-PCR. GAPDH was used as endogenous control. Values were normalized against the DMSO control that was set as 1.0. Asterisks indicate $P < 0.05$. (**b**) Immunoblotting analysis of the PMSA5 protein in MCF-10A and MCF-7 cells treated with or without proteasome inhibitors. MCF-10A and MCF-7 cells were treated with different concentration of MG-132 or Bortezomib for 16 h. The protein expression levels of PSMA5 and β-actin were detected by immunoblotting analysis with antibodies against PSMA5 and β-actin, respectively. (**d**) Breast cancer cells have a higher level of endogenous proteasome but a lower feedback potential compared to nontumorigenic mammary epithelial cells. Cells were treated with 40 nM Bortezomib or DMSO control for 16 h. The PSMA5, PSMD3 and β-actin proteins were detected by immunoblotting analysis and quantified by the Odyssey Infrared Imaging System. The relative ratios of PSMA5 to β-actin in different cell lines with or without Bortezomib treatment were presented here (lower panel)

estingly, the other two breast cancer cell lines, MDA-MB-231 and MDA-MB-453, were also more resistant to Bortezomib than MCF-10A, even though the resistance was weaker than that of MCF-7 (Fig. 2b). By contrast, MCF-10CA1α displayed a similar sensitivity to Bortezomib as the parental MCF-10A (Fig. 2b). Consistent with the MTT assay, cell death of MCF-10A and MCF-10CA1α were observed when treated with as low as 40 nM of Bortezomib, whereas a higher dosage of Bortezomib (at least 80 nM) was required to induce cell death in MDA-MB-453 and MDA-MB-231 (Fig. 2c). No obvious cell death of MCF-7 was observed when treated with 160 nM Bortezomib even though the morphology of the treated cells did change (Fig. 2c). Interestingly, cleavage of Caspase-3 was detected in MCF-10A and MCF-10CA1α but not in the breast cancer cells after treated with 40 nM Bortezomib for 16 h (Fig. 2d). Together, these results demonstrate that breast cancer cells are generally resistant to Bortezomib-induced apoptosis, which may explain at least in part why Bortezomib had little effect in treating breast cancer in the clinical trials [9]. It is noteworthy that resistance to Bortezomib is cell-type specific as human lung sarcoma cells HT-1080 and SV40-transformed human fibroblast cells VA-13 were more sensitive to Bortezomib compared to the nontransformed human fibroblast cells IMR-90 (Fig. 3), which is in contrast to the breast cancer cells versus MCF-10A. This observation is in line with the clinical trial data showing different efficacy of Bortezomib in different cancers [6–13].

Cause of resistance to proteasome inhibitors in breast cancer cells

Previous studies have shown that the feedback regulation of proteasome expression plays an important role in drug resistance to proteasome inhibitors in yeast [16]. We, however, found that MCF-10A and MCF-10CA1α cells with more profound feedback expression were actually more sensitive to proteasome inhibitors compared to the breast cancer cells (Figs. 1, 2). This observation suggests

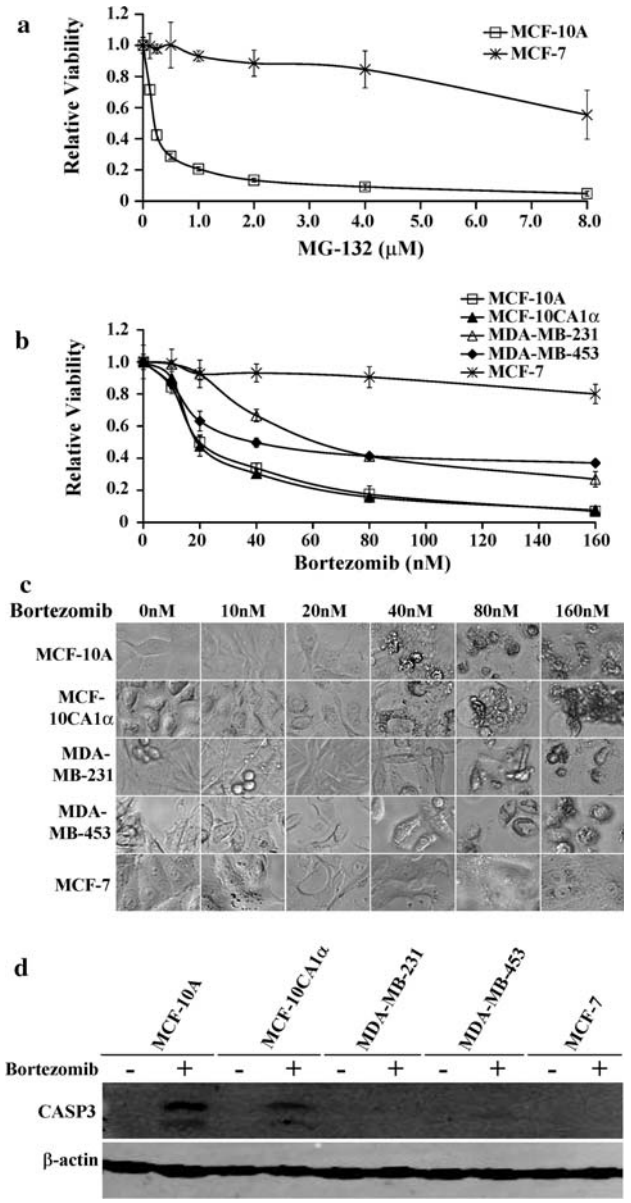

Fig. 2 Breast cancer cells are resistant to proteasome inhibitors. (**a**) MCF-10A and MCF-7 cells were treated with different concentration of MG-132 for 48 h. Cell viability was measured by MTT assay. (**b**) MTT assay for breast cancer cells and MCF-10A and MCF-CA1α cells treated with different concentration of Bortezomib for 48 h. (**c**) Morphology of the cells treated with Bortezomib as in (**b**). (**d**) Cleaved Caspase-3 products present in MCF-10A and MCF-CA1α but the breast cancer cells. Cell extracts used in Fig. 1d were applied to immunoblotting analysis with antibody specific to cleaved Caspase-3

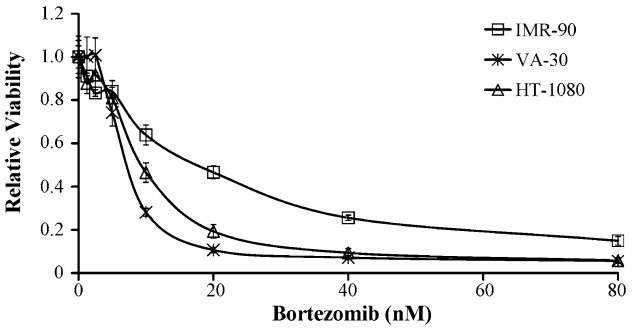

Fig. 3 Human lung sarcoma cells (HT-1080) and transformed fibroblast cells (VA-13) are more sensitive to Bortezomib than nontransformed human fibroblast cells (IMR-90). Cells were treated with different concentration of Bortezomib for 48 h. Cell viability was measured by MTT assay

that the resistance to proteasome inhibitors in breast cancer cells is unlikely contributed by the feedback regulation of proteasome expression. In support of this hypothesis, we found that pre-treatment of MCF-10A and MDA-MB-231 with low concentration of Bortezomib, which induced feedback expression of the proteasome, did not reduce the sensitivity of these cells to higher concentration of Bortezomib (data not shown).

Is it possible that the drug resistance stems from the higher endogenous proteasome abundance in the breast cancer cells? Conceivably, a higher dosage of proteasome inhibitor is required to inhibit the proteasome activity in the breast cancer cells. Our data, however, did not favor this argument. First, MCF-7 cells were much more resistant to Bortezomib than MDA-MB-231 cells even though the proteasome abundance and activity are comparable between these two cell lines (Figs. 1d, 4a). Second, treatment with 20 nM Bortezomib was apparently sufficient to inhibit most of the proteasome activity in all the cell lines used as shown by accumulation of polyubiquitinated proteins and direct measurement of the chymotrypsin-like activity of the proteasome (Fig. 4a, b). (The residual chymotrypsin-like activity in MCF-7 cells was modestly higher than that in other cell lines.) But the sensitivity to this dosage of Bortezomib was significantly different among these cell lines (Fig. 2b). In hindsight, these results also indicate that cell penetration and in vivo metabolism of Bortezomib do not contribute to the drug resistance. Thus, resistance to proteasome inhibitors is unlikely directly caused by high endogenous proteasome abundance in the breast cancer cells. In support of this argument, leukemia cells expressing high level of proteasome are actually very sensitive to Bortezomib [24, 25].

It has been shown that the proapoptotic protein Bax plays a critical role in apoptosis induced by proteasome inhibitors in colon cancer [26]. We decided to compare the steady-state protein level of Bax among the cell lines used in this study in the presence and absence of Bortezomib treatment. As expected, little Bax protein was observed in the absence of Bortezomib due to its rapid degradation by the proteasome (Fig. 4b). Interestingly, while it was readily detected in the MCF-10A and MCF-10CA1α cells in the

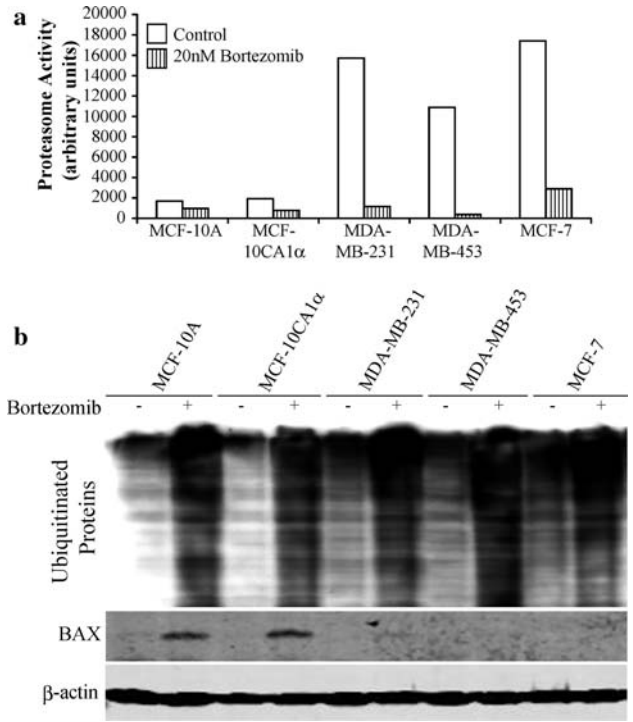

Fig. 4 Inhibition of the proteasome activity by Bortezomib in breast cancer cells. (**a**) Bortezomib efficiently inhibits the proteasome activity in all cell lines tested. Extracts prepared from cells treated with 20 nM Bortezomib or DMSO were analyzed for proteasome activity. (**b**) Immunoblotting analysis for ployubiquitinated proteins and the Bax protein in cells treated with or without Bortezomib

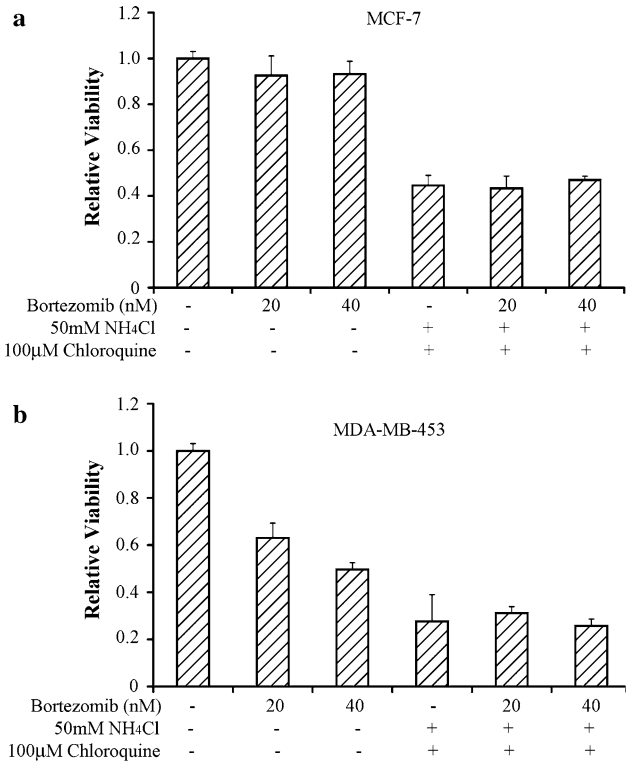

Fig. 5 No synergistic effect between Bortezomib and lysosome inhibitors in treating breast cancer cells. MCF-7 (**a**) and MDA-MB-453 (**b**) were treated with different combination of Bortezomib and ammonium chloride or chloroquine for 48 h. Cell viability was measured by MTT assay

presence of Bortezomib, the Bax protein was barely seen in the breast cancer cells under the same conditions (Fig. 4b). This observation suggests that stabilization of the Bax protein by Bortezomib may lead to cell death of MCF-10A and MCF-10CA1α. Lack of Bax protein may play an important role in resistance to proteasome inhibitors in the breast cancer cells. Note that the proteasome activity in MDA-MB-231 and MDA-MB-453 was as low as that in MCF-10A and MCF-10CA1α in the presence of 20 nM Brotezomib (Fig. 4a), suggesting that the breast cancer cells may have a defect in expression rather than stabilization of the Bax protein.

In addition to the ubiquitin-proteasome system, lysosome is another major apparatus for protein degradation in the cell. To examine if lysosome may play a role in resistance to proteasome inhibitors through compensation of the loss of proteasome activity, we treated MCF-7 and MDA-MB-453 cells with Bortezomib in the presence or absence of lysosome inhibitors chloroquine and ammonium chloride, respectively (Fig. 5a, b). Although the lysosome inhibitors alone inhibited cell growth, no synergistic effect between Bortezomib and the lysosome inhibitors was detected. This observation suggests that lysosome is not involved in the drug resistance to proteasome inhibitors in the breast cancer cells.

Combination therapy with Bortezomib and other agents in breast cancer cells

Our experimental data demonstrated that Bortezomib alone had low efficacy against breast cancer cells. We then examined if combination of Bortezomib with other anticancer agents could have a better effect. It has been reported that hyperthermia may overcome drug resistance in many cancer cells [27, 28]. We, therefore, tested the combination effect of hyperthermia and Bortezomib on MDA-MB-231 and MDA-MB-453, controlled by MCF-10A. As shown in Fig. 6a and b, hyperthermia sensitized the breast cancer cells as well as MCF-10A cells to Bortezomib in a concentration- and time-dependent manner. At low concentration of Bortezomib (10 nM), the combination effect was stronger on breast cancer cells than on MCF-10A. However, MCF-10A cells were more sensitive to hyperthermia than the breast cancer cells at higher concentration of Bortezomib (20 nM). Treatment with hyperthermia at the time points of 6–7 h and 12–13 h after

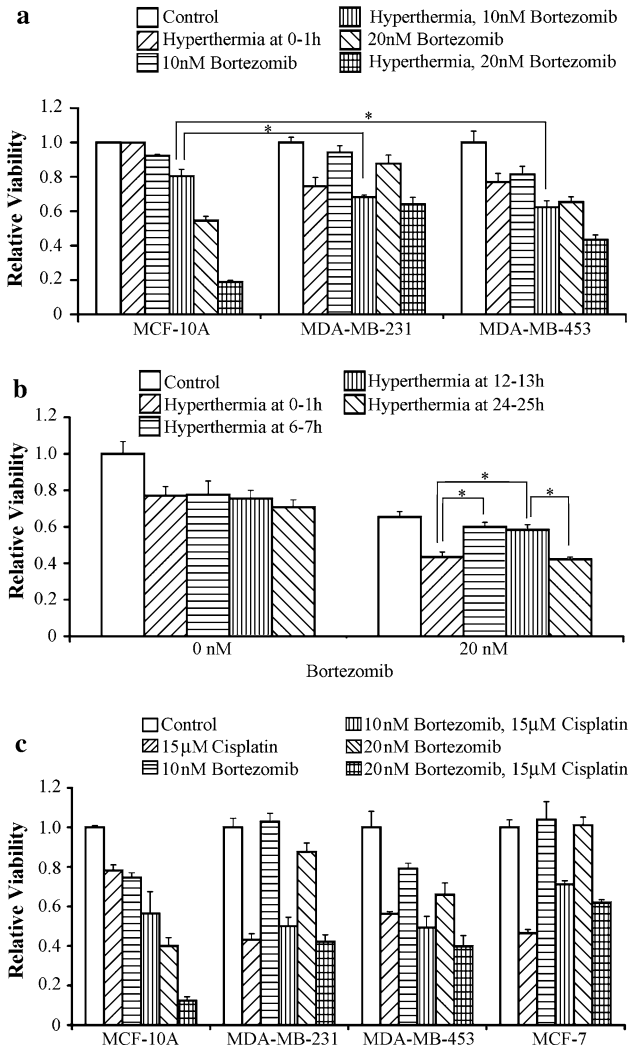

Fig. 6 Combinational treatment of breast cancer cells with Bortezomib and hyperthermia and cisplatin. (**a**) MCF-10A, MDA-MB-231 and MDA-MB-453 cells were treated with or without hyperthermia at 43°C for 1 h immediately after addition of 10 nM or 20 nM Bortezomib. The cells were further incubated at 37°C for 47 h. Control cells were incubated at 37°C for 48 h. Cell viability was measured by MTT assay. Asterisks denote $P < 0.05$. (**b**) MDA-MB-453 cells were treated with or without hyperthermia at 43°C for 1 h at different time points after addition of 20 nM Bortezomib. The cells were kept at 37°C for 47 h except during hyperthermia. Control cells were incubated at 37°C for 48 h. Cell viability was measured by MTT assay. (**c**) MCF-10A, MDA-MB-231, MDA-MB-453 and MCF-7 cells were treated with or without 15μM cisplatin in the presence of 0, 10 or 20 nM Bortezomib for 48 h. Cell viability was measured by MTT assay

addition of Bortezomib had a less combination effect compared to the hyperthermia conducted at the first hr or after 24 h (Fig. 6b). Thus, the timing of hyperthermia is critical for the therapeutic effect of Bortezomib. It will be of interest to further investigate the interplay between hyperthermia and Bortezomib as heat shock proteins may be involved in drug resistance to proteasome inhibitors as well as other anti-cancer drugs [29].

Cisplatin is a widely used DNA-damaging agent in chemotherapy because cells deficient in DNA repair are hypersensitive to cisplatin [30]. Recent studies have shown that Bortezomib sensitizes pancreatic cancer cells and squamous cell carcinoma cells to cisplatin [31, 32]. We wanted to examine if Bortezomib could also enhance the therapeutic effect of cisplatin on breast cancer cells. As shown in Fig. 6c, Bortezomib had a marginal effect on the sensitivity of MDA-MB-453 to cisplatin, but virtually no effect on MDA-MB-231. In fact, the cytotoxic effect of cisplatin was even attenuated by Bortezomib in MCF-7. By contrast, Bortezomib at 20 nM markedly sensitized MCF-10A cells to cisplatin. Thus, the combination effect of Bortezomib and cisplatin appears to be insignificant in treating breast cancer cells.

In this study, we demonstrated that the proteasome expression is aberrant in breast cancer cells. On the one hand, the endogenous abundance and the activity of the proteasome are much higher in breast cancer cells than in nontumorigenic breast epithelial cells. On the other hand, the feedback upregulation in response to proteasome inhibitors is weakened in the breast cancer cells. Although aberrant proteasome activity has been observed in many cancer cells, we report here for the first time that the feedback regulation of proteasome expression is diminished in breast cancer cells. It will be of interest to examine if this feedback defect also exists in other cancers. Moreover, it remains to be investigated if the lower potential of feedback expression contributes to the pathogenesis of breast cancer.

Another interesting finding in this study is that several breast cancer cell lines are more resistant to proteasome inhibitors including Bortezomib than nontumorigenic breast epithelial cells. This observation may explain the inefficacy of Bortezomib in clinical trials for breast cancer patients [9]. Our finding also emphasizes the importance of including nontumorigenic cells as control in drug sensitivity analysis before clinical trials. While the molecular mechanisms of the resistance to proteasome inhibitors remain to be elucidated, our data suggest that the pathway of Bortezomib-induced apoptosis may be defective in the breast cancer cells. This information is important for the design of combination therapy using Bortezomib and other anti-cancer agents. For instance, rejuvenation of the Bortezomib-induced apoptosis pathway may enhance the therapeutic effect of Bortezomib on breast cancer. Our studies using Bortezomib in combination with hyperthermia and cisplatin suggest that combination therapy with Bortezomib and other anti-cancer agents may be beneficial in treating breast cancer. However, the regimen to achieve the therapeutic effect can be complicated. Some treatment may evoke protection mechanism and even attenuate the biological effect

of Bortezomib. It has been shown that Vitamin C abrogates the anti-cancer activity of Bortezomib through a direct binding mechanism [33]. Further investigation of the mechanisms underlying the drug resistance to proteasome inhibitors will be required for development of more efficient anti-breast cancer agents targeting the proteasome.

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