An autophagy inhibitor enhances the inhibition of cell proliferation induced by a proteasome inhibitor in MCF-7 cells

FENG YAO*, GUANNAN WANG*, WEN WEI, YI TU, HEXIANG TONG and SHENGRONG SUN

Department of Breast and Thyroid Surgery, Renmin Hospital of Wuhan University, Wuhan, Hubei, P.R. China

Received June 28, 2011; Accepted September 14, 2011

DOI: 10.3892/mmr.2011.590

Abstract. The ubiquitin-proteasome system and the autophagy-lysosome pathway are the two main routes for eukaryotic intracellular protein clearance. Inhibition of proteasome activity leads to cell death. Due to the dual roles of autophagy in tumor cell survival and death, the effect of suppression of autophagy on breast cancer cells remains to be elucidated. We investigated whether inhibition of the proteasome is capable of inducing autophagy, and we assessed the effect of combined inhibition of the proteasome and autophagy on human breast cancer MCF-7 cells. The proteasome inhibitor bortezomib was used to induce autophagy in MCF-7 cells, and the effect of autophagy on the proliferation of MCF-7 cells was investigated using the autophagy inhibitor 3-MA. Cell viability was measured by MTT assay. The expression of autophagy-related proteins was determined by Western blot analysis and the GFP-LC3 redistribution was detected using a fluorescence microscope after MCF-7 cells were infected with a GFP-LC3-expressing adenovirus. MCF-7 cell proliferation was inhibited and autophagy was activated in the same dose-dependent manner. Bortezomib induced a dose-dependent increase in LC3-II. However, when MCF-7 cells were co-treated with bortezomib and 3-MA, 3-MA blocked the increase in LC3-II protein expression and led to a significant inhibition of cell proliferation. Inhibition of the proteasome may induce autophagy in human breast cancer MCF-7 cells and 3-MA could inhibit autophagy induced by the proteasome inhibitor. A combination of 3-MA and bortezomib increases cell death. These findings indicate that suppression of the two intracellular degradation systems may shed new light on breast cancer control.

Correspondence to: Dr Shengrong Sun, Department of Breast and Thyroid Surgery, Renmin Hospital of Wuhan University, Wuhan, Hubei 430060, P.R. China
E-mail: sun137@sina.com

*Contributed equally

Key words: proteasome inhibitors, autophagy, cell death

Introduction

Protein degradation is an essential cellular function that, when dysregulated or impaired, may lead to a wide variety of disease states, such as cancer. The ubiquitin-proteasome system (UPS) and the autophagy-lysosome are the two major intracellular protein degradation systems (1,2). The UPS serves as the primary route of degradation for thousands of short-lived proteins (3). UPS-mediated catabolism is essential to maintain amino acid pools in acute starvation and contributes significantly to the degradation of defective proteins (4-6). Autophagy is primarily responsible for degrading long-lived proteins and maintaining amino acid pools in the setting of chronic starvation. Autophagy also plays a housekeeping role in removing misfolded or aggregated proteins, clearing damaged organelles and eliminating intracellular pathogens. Thus, autophagy is generally thought of as a survival mechanism (7).

Inhibition of proteasome activity has become a new chemotherapy strategy and a number of chemicals and natural compounds have proven to be effective at inducing tumor cell death by inhibiting proteasome activity (8,9). Currently, one proteasome inhibitor, bortezomib (Velcade®), has been approved for treating relapsed multiple myeloma. Autophagy induction is greatly altered in cancer tissue. The tumor microenvironment is commonly poor in nutrients and oxygen, favoring autophagy induction (10). However, human cancer frequently displays inactivating mutations in pro-autophagy genes and the activation of anti-autophagy genes (10,11). These changes contribute differentially to the autophagic capacity in tumors, depicting the duality of autophagy in cancer.

In the present study, we investigated whether the proteasome inhibitor bortezomib induces autophagy in human MCF-7 breast cancer cells. We showed that treatment of MCF-7 cells with bortezomib induced autophagy. Furthermore, inhibition of autophagy by the autophagy inhibitor 3-MA enhanced inhibition of cell proliferation by bortezomib alone. These findings indicated the benefits of suppressing the two cellular degradation systems as a novel tumor-specific therapeutic strategy in breast cancer.

Materials and methods

Reagents. DMEM was from Thermol Scientific (Waltham, MA, USA), fetal bovine serum (FBS) was from Atlantic Biologicals (Lawrenceville, GA, USA), bortezomib was

from LC Laboratories (Woburn, MA, USA), 3-MA was from Sigma-Aldrich (St. Louis, MO, USA) and the cell proliferation reagent WST-1 was from Roche (Mannheim, Germany). All other chemicals were from Sigma-Aldrich or Invitrogen (Carlsbad, CA, USA).

The following antibodies were used. Horseradish peroxidase-conjugated goat anti-mouse/rabbit secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-β-catenin was from BD Transduction Laboratories (San Diego, CA, USA), anti-LC3 was from MBL Co. (Woburn, MA, USA) and anti-GAPDH was from Sigma.

Cell line and culture. The human breast cancer cell line, MCF-7, was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). MCF-7 cells were maintained in DMEM supplemented with 10% of FBS, 4 mM L-glutamine and 1X penicillin/streptomycin under standard conditions of temperature (37˚C) and carbon dioxide (5%). Cells in the mid-log phase were used in the experiments.

Cell proliferation assay. According to the procedure described in the data sheet of WST-1, MCF-7 cells were plated at a density of 10,000 cells per 100 μl per well in 96-well plates. Following treatment, the absorbance of the samples was measured at 450 nm with a Vmax kinetic microplate reader (Molecular Devices, Sunnyvale, CA, USA), using a background control as a blank. The reference wavelength was 650 nm.

Fluorescence microscopy for GFP-LC3 puncta. For GFP-LC3 redistribution, MCF-7 cells were infected with a GFP-LC3-expressing adenovirus. At 24 h post-infection, cells were treated with the drugs. GFP-LC3 was detected using a fluorescence microscope (Axiovert 200M, Carl Zeiss Light Microscopy, Göttingen, Germany). The percentage of GFP-LC3-positive cells with punctate staining was determined in three independent experiments. To quantify the number of GFP-LC3-positive autophagosomes per transfected cell, six random fields representing 200-300 cells were counted.

Western blotting. MCF-7 cells were washed twice with cold phosphate-buffered saline (PBS) and lysed with 1X loading buffer and a proteasome inhibitor cocktail. The total proteins were extracted for Western blotting. The concentration of proteins for each sample was determined using the bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Equal amounts of protein (30 μg/lane) were resolved by 14% SDS-PAGE and electrically transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% milk in PBS-T for 1 h at room temperature and then incubated overnight at 4˚C with the following primary antibodies: anti-β-catenin (1:2000) and anti-LC3 (1:1000). The membranes were then incubated with horseradish peroxidase-conjugated goat anti-mouse/rabbit secondary antibodies for 60 min at room temperature. ECL-Plus detection reagents (GE Healthcare, Piscataway, NJ, USA) and a VersaDoc imaging system (model 3000, Bio-Rad) were used to visualize and digitalize the Western blotting images. The densitometry of the Western blotting was determined with Quantity One software (Bio-Rad). The same-lane GAPDH level was employed as a protein loading control.

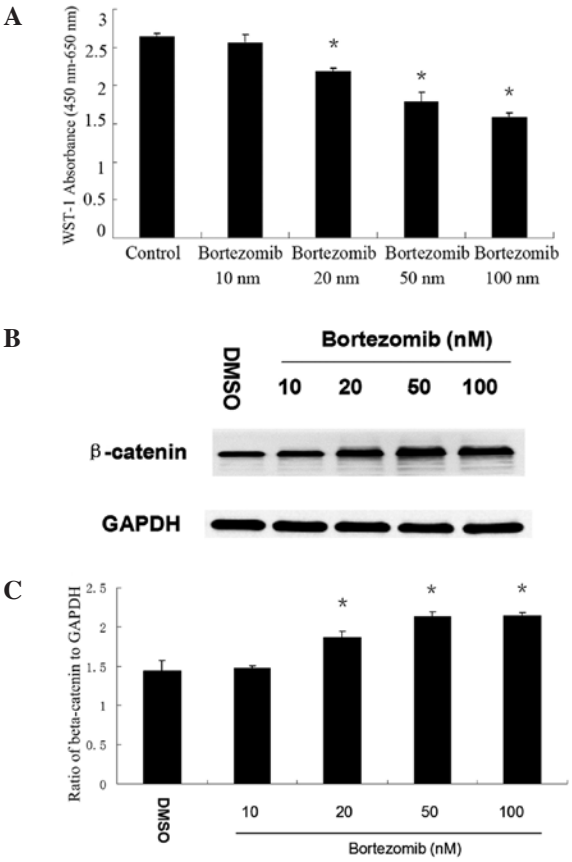

Figure 1. Inhibition of MCF-7 cell proliferation and proteasome activity by the proteasome inhibitor bortezomib. (A) Treatment with bortezomib for 48 h dose-dependently lowered cell proliferation as determined by MTT assay. (B) Bortezomib blocked the degradation of the endogenous proteasome substrate β-catenin in MCF-7 cells. (C) Relative amounts of β-catenin were described as the ratio of β-catenin to GAPDH. *P<0.01 compared with the control group.

Statistical analysis. Experimental data are shown as the mean ± SD. Statistical comparisons were carried out with the Student's t-test using SPSS 11.0 software. P<0.05 was considered to denote statistical significance.

Results

Bortezomib inhibited cell proliferation and proteasome activity. After MCF-7 cells were treated with bortezomib at the indicated concentrations for 48 h, cell viability was assessed by MTT assay. MCF-7 cell proliferation was inhibited by bortezomib in a dose-dependent manner (Fig. 1A). Compared with the control, 48-h treatment of bortezomib at a dose of 50 nM inhibited MCF-7 cell proliferation by approximately 32%.

To study the effect of blockade of UPS on MCF-7 cells by bortezomib, Western blot analysis was performed. β-catenin is an endogenous proteasome substrate (12). Inhibition of proteasome activity by bortezomib was confirmed by accumulation of this known proteasome substrate. After the MCF-7 cells were treated with bortezomib at the indicated concentrations for 48 h, β-catenin dramatically increased (Fig. 1B and C).

Bortezomib induced autophagy. As the amount of LC3 protein, particularly LC3-II, correlates with the extent of

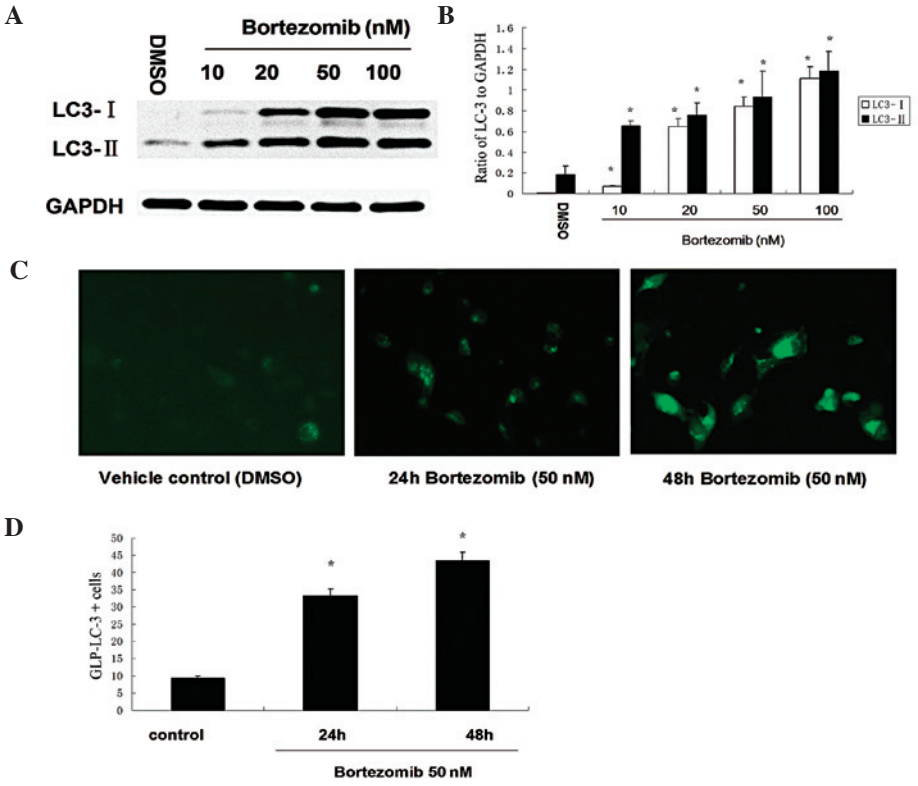

Figure 2. Induction of autophagy by bortezomib. (A) Bortezomib dose-dependently increased the protein expression of LC3-I and -II. (B) Relative amounts of LC3-I and LC3-II described as ratios of LC3-I and LC3-II to GAPDH. (C) MCF-7 cells were first infected with Ad-GFP-LC3 for 24 h and then treated with vehicle control, bortezomib (50 nM) for 24 h, or bortezomib (50 nM) for 48 h. Cells were then analyzed by fluorescence microscopy for LC3 translocation. The percentages of GFP-LC3-positive cells showing puncta were determined (columns, mean; bars, SD). (D) The percentage of GFP-LC3-positive cells in each group. *P<0.01 compared with the control group.

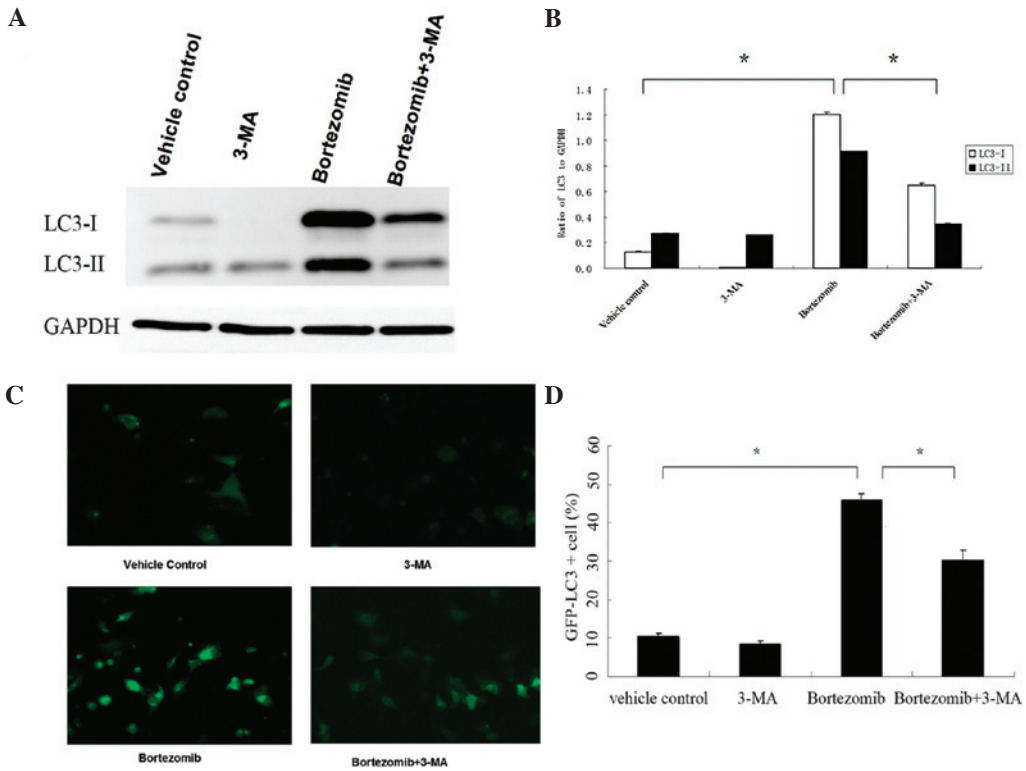

Figure 3. Blockade of bortezomib-induced autophagy by 3-MA. (A) 3-MA blocked the increase in LC3-II protein expression induced by bortezomib. (B) Relative amounts of LC3-I and LC3-II, described as ratios of LC3-I and LC3-II to GAPDH. (C) MCF-7 cells were first infected with Ad-GFP-LC3 for 24 h and then treated with vehicle control, bortezomib (50 nM), 3-MA (10 mM),or a combination of bortezomib (50 nM) and 3-MA (10 mM) for 48 h. Cells were then analyzed by fluorescence microscopy for LC3 translocation. The percentages of GFP-LC3-positive cells showing puncta were determined (columns, mean; bars, SD). (D) The percentage of GFP-LC3-positive cells in each group. *P<0.01.

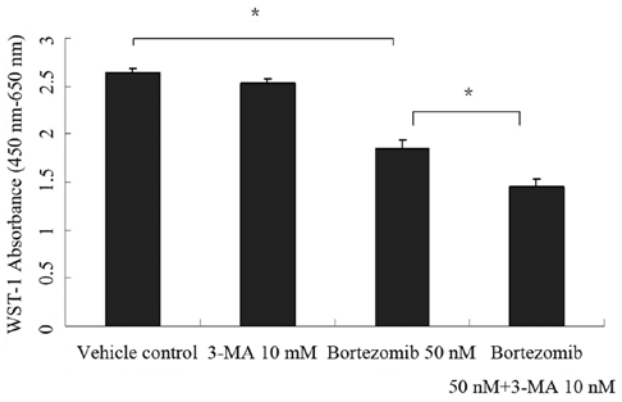

Figure 4. Enhancement of the effects of bortezomib on cell proliferation by 3-MA. Result of MTT assays. Compared with the bortezomib group, cell viability decreased significantly at 48 h after co-treatment with 3-MA. *P<0.01.

autophagy, the effect of bortezomib on LC3 protein expression and the alteration of GFP-LC3 punctation in MCF-7 cells were studied. Treatment of MCF-7 cells with bortezomib induced a dose-dependent increase in LC3-II (Fig. 2A and B) and a time-dependent accumulation of GFP-LC3 puncta (Fig. 2C and D).

3-MA blocked autophagy induced by bortezomib. To determine whether the autophagy induced by bortezomib is blocked by 3-MA, we co-treated the MCF-7 cells with bortezomib and 3-MA. We showed that 3-MA blocked the increase in LC3-II protein expression (Fig. 3A and B) and the accumulation of GFP-LC3 puncta induced by bortezomib (Fig. 3C and D), indicating that 3-MA blocked the autophagy induced by bortezomib.

To determine whether the antitumor effects of bortezomib are enhanced by the modulation of autophagy, MCF-7 cells were treated with the combination of bortezomib and 3-MA at the indicated concentrations for 48 h, and cell viability was assessed by MTT assay.

Bortezomib alone retarded cell proliferation compared with the control group (Fig. 4). However, the combination of bortezomib and 3-MA led to a significant inhibition of MCF-7 cell proliferation, indicating that suppression of the proteasome together with the compensatory autophagy caused maximal stress and demise to cancer cells.

Discussion

Recent studies have shown that proteasome inhibitors serve as a new and promising class of anticancer agents (13-17). In this study we showed that MCF-7 cell proliferation was inhibited by bortezomib in a dose-dependent manner. Furthermore, we found that autophagy was activated after MCF-7 cells were incubated with bortezomib as evidenced by the increased percentages of GFP-LC3-positive cells showing puncta, and up-regulation of LC3-II protein.

Recent studies have reported that impairment of the UPS leads to increased autophagic function (18-20). They revealed interaction between the UPS and autophagy (18,21). Autophagy is a self-degradative process that is crucial for balancing sources of energy at critical times in development and in response to nutrient stress. It is also an evolutionarily conserved self-digestion process responsible for the turnover of whole organelles and macromolecules (22,23). It is possible that autophagy induced by the inhibition of proteasome function may serve as a compensatory mechanism in response to protein accumulation to alleviate the toxicity of proteasome inhibitors (20,24), suggesting a coordinated and complementary relationship between these degradation systems.

To determine whether the autophagy induced by bortezomib is blocked by the autophagy inhibitor 3-MA, we co-treated the MCF-7 cells with bortezomib and 3-MA. We showed that 3-MA blocked the autophagy induced by bortezomib. Moreover, to determine whether the antitumor effects of bortezomib are enhanced by the modulation of autophagy, MCF-7 cells were treated with the combination of bortezomib and 3-MA, and cell viability was assessed by MTT assay. Notably, 3-MA alone also had an inhibitory effect; however, the combination of bortezomib and 3-MA led to a significant inhibition of MCF-7 cell proliferation, indicating that suppression of the proteasome together with the compensatory autophagy caused maximal stress and demise to cancer cells.

Single use of proteasome inhibitors may not be effective for resistant myeloma and for solid tumors (25-27). A number of regimes have been examined that suppress autophagy to enhance cancer cell death (28-30). It appears that simultaneous inhibition of autophagy and the proteasome could offer a unique advantage based on this study.

It has to be pointed out that suppressing autophagy is not a generic strategy to enhance therapeutic efficacy for all types of cancer. In certain cases, cytotoxic effects, rather than cytoprotective effects, were shown to be caused by autophagy (10).

In conclusion, we provide evidence that the proteasome inhibitor bortezomib produces *in vitro* growth inhibition in human MCF-7 cells, and inhibition of the proteasome activates autophagy. Moreover, we demonstrated that 3-MA blocked the autophagy induced by bortezomib and the combined use of bortezomib and 3-MA enhanced cell death.

Whether these findings can be translated into chemoprophylactic and therapeutic options for the treatment of breast cancers, however, awaits further clinical investigation.

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