Inhibition of autophagy enhances the anticancer activity of bortezomib in B-cell acute lymphoblastic leukemia cells

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Abstract: B-cell acute lymphoblastic leukemia (B-ALL) remains a challenging disease to treat in adults because of the high rates of relapse and refractory. Bortezomib, as a proteasome inhibitor, exerts obvious cytotoxicity against ALL cells and increases the sensitivity of ALL cells to conventional chemotherapeutic agents. We observed that bortezomib inhibited proliferation, induced apoptosis, arrested the cell cycle and induced autophagy in the Nalm-6 cell line and CD34+ primary cells. Additionally, we demonstrated that bortezomib promoted the disruption of the Bcl-2/Beclin-1 complex and increased the formation of the Beclin-1/PI3KC3 complex, leading to the initiation of autophagy. Autophagy inhibitors were employed in this study, and we found that autophagy inhibitors enhanced the anti-ALL activity of bortezomib. Taken together, these results revealed that autophagy protected B-ALL cells against the cytotoxicity of bortezomib and, in combination with autophagy inhibitors, can enhance the anticancer effects of bortezomib.

Keywords: Bortezomib, autophagy, autophagy inhibitors, anticancer effects, B-cell acute lymphoblastic leukemia

Introduction

B-cell acute lymphoblastic leukemia (B-ALL) is a heterogeneous group of diseases characterized by malignant proliferation of precursor B lymphocytes in the bone marrow. Over the years, different protocols have been exploited to ameliorate the outcome, but the rates of relapse and refractory are high, overall survival is low, and B-ALL remains a challenging disease to treat in adults [1].

Bortezomib, a dipeptidyl boronic acid analog, is a proteasome inhibitor authorized for use in the treatment of myeloma and mantle cell lymphoma [2, 3] that reversibly inhibits the 26S proteasome [4]. As reviewed elsewhere, the anticancer activity of proteasome inhibition involves several different mechanisms, including blocking cell cycle progression, inducing apoptosis, inhibiting cell growth, and anti-angiogenesis [5]. Previous studies have reported that bortezomib exerts marked cytotoxicity against ALL cells and increases the sensitivity of ALL cells to conventional chemotherapeutic agents, which is associated with the molecular mechanisms involved in Notch1, NF-kB, and PI3K/AKT signaling [6, 7]. Although the mechanism of bortezomib's anticancer activity is still not completely understood, it is a new treatment choice for patients with refractory or relapsed ALL, especially when treated in combination with conventional chemotheraphy or targeted agents.

Autophagy is characterized by the formation of autophagosomes, which are double-membrane vesicles that swallow cytoplasmic material. Sequentially, autophagosomes fuse with lysosomes and lysosomal enzymes degrade their contents [8, 9]. Recent studies suggest that autophagy may represent a novel therapeutic target for treating cancer. Bortezomib-induced autophagy has been reported in several types of cancer cells [10-12]; however, whether the exact molecular mechanism by which bortezomib acts against ALL, especially in B-ALL cells, is associated with autophagy has not been clearly defined.
In this study, we observed that bortezomib induced autophagy in the B-ALL cell line NALM-6 and primary cells from two patients, and we explored the effect of bortezomib on apoptosis and the cell cycle in the aforementioned cells. Furthermore, we also examined whether inhibition of autophagy would potentiate cell apoptosis when bortezomib was used.

**Materials and methods**

**Cells and cell culture**

The human precursor B cell lymphoblastic leukemia (B-ALL) cell line Nalm-6 from Leibniz institute DSMZ was cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS) at 37°C in a humidified incubator with 5% CO₂ and 95% air. Bone marrow mononuclear cells (BMMCs) from 2 B-ALL patients were isolated by Ficoll density gradient centrifugation. CD34 positive cells from BMMCs were isolated and purified with a CD34 selection kit (Miltenyi Biotec GmbH, Germany). B-ALL was diagnosed according to the MICM classification. The study was authorized by the institution’s review boards and ethics committees, and all patients gave written informed consent according to the Declaration of Helsinki. CD34⁺ cells were cultured in X-VIVO 15 (BioWhittaker, MD) containing a cytokine cocktail.

**Drugs and antibodies**

The antibodies to LC3 and P62 were obtained from Novus (Littleton, CO). The antibodies to Beclin-1, Bcl-2, Caspase-3, cleaved Caspase-3 and Bax were obtained from Cell Signaling Technology (Danvers, MA). The antibody to cytochrome C was obtained from Santa Cruz Technology (Dallas, Texas). β-actin, MTT, monodansylcadaverine, E64D, leupeptin and 3-MA were purchased from Sigma-Aldrich (St. Louis, MO). SP600125, an inhibitor of JNK and Bortezomib were purchased from Selleck (Houston, TX). Horseradish peroxidase-conjugate secondary antibodies and FITC-labeled goat anti-rabbit IgG were purchased from Sigma-Aldrich (St. Louis, MO). SP600125, an inhibitor of JNK and Bortezomib were purchased from Cell Signaling Technology (Danvers, MA). The protein concentrations in the supernatant were determined with the BCA assay. Before immunoprecipitation, samples were precleared by adding 20 µl of Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, Dallas, Texas) and 1 µg of an appropriate control IgG; they were then

**Cell viability and apoptosis assays**

Cell viability was analyzed with MTT assays. Annexin V/propidium iodide (PI) staining assays were conducted according to the manufacturer's instructions. Annexin-V positive cells were measured using a FACScalibur™ flow cytometer (Becton Dickinson, San Jose, CA) and data were assessed using CellQuest™ software (BD Biosciences).

**Cell cycle analysis**

The cell cycle was assessed by propidium iodide (PI) staining and measured with a FACScalibur™ flow cytometer. The cell distribution of each phase of the cell cycle was evaluated with ModFit LT software (BD Biosciences).

**Western blot analysis**

The protein concentrations of cell lysates were measured using the BCA Protein assay (Pierce, Rockford, IL). Proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane and sequentially incubated overnight with primary antibodies at 4°C. After incubation with secondary antibodies, the signals were visualized by chemiluminescence using the SuperSignal reagent (Pierce, Rockford, IL).

**Immunofluorescence**

Nalm-6 and primary CD34⁺ cells were fixed and permeated and subsequently incubated overnight with an anti-LC3 antibody at 4°C, which was followed by staining with FITC-conjugated and DAPI. After three 5-minute washes with PBS containing 0.2% BSA, cells were spread on glass slides by centrifugation at 1000 rpm for 5 min using a cytopin system (StatSpin, Westwood, MA). Fluorescence signals were analyzed using an Olympus BX50 microscope. The average percentage of LC3 puncta positive cells was assessed from at least 50 cells for each experiment.

**Co-immunoprecipitation**

Cells were lysed in RIPA lysis buffer (Cell Signaling Technology, Danvers, MA). The protein concentrations in the supernatant were determined with the BCA assay. Before immunoprecipitation, samples were precleared by adding 20 µl of Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, Dallas, Texas) and 1 µg of an appropriate control IgG; they were then
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Results

Bortezomib induces autophagy in B-ALL cells

LC3 is widely used to monitor autophagy by analyzing the conversion of LC3-I to LC3-II, which is situated to the autophagosomal membrane. As shown in Figure 1A, Nalm-6 cells and CD34+ primary cells isolated from bone marrow mononuclear cells of B-ALL patients were exposed to increasing concentrations of bortezomib for 24 h or treated with 10 nM of bortezomib for 6, 12 or 24 h, and the LC3 and P62 levels were analyzed by Western blot. The Nalm-6 cells were untreated or treated with 0.1% DMSO for 12 h or with 10 nM bortezomib for 12 or 24 h and stained with monodansylcadaverine (MDC, 50 µM) or DAPI (blue) and LC3 (green); the cells were then observed with an Olympus BX50 microscope. The formation of autophagic vacuoles was defined by the accumulation of LC3, and the arrows point to autophagic vacuoles. The LC3 puncta-positive cells were calculated as described in materials and methods. The columns represent the average percent of LC3 puncta-positive cells from 3 independent experiments and were shown as the mean ± SD (**, p < 0.01 versus untreated). Representative images are shown in the left panel.

Statistical analysis

Results are expressed as the mean ± SD of three independent experiments. Two-group comparisons were performed using Student’s t-test. P values < 0.05 were defined as statistically significant. All data analyses were performed with GraphPad Prism 5.
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membranes [13, 14]. P62 is an autophagic receptor that interacts with LC3-II at the forming autophagosome, and it is degraded by autophagy, which makes P62 a useful marker of autophagy [15, 16]. To determine whether bortezomib is a direct activator of autophagic flux, we monitored the autophagy markers, including LC3-II and P62, in the presence of bortezomib. Treatment with bortezomib induced a dose-dependent and time-dependent increase in the expression of LC3-II, while down-regulating the P62 expression in the Nalm-6 cell line (Figure 1A). Furthermore, we tested the levels of LC3 in CD34+ primary cells treated with various concentrations of bortezomib and found that bortezomib dose-dependently induced autophagy in CD34+ primary cells from two B-ALL patients (Figure 1A). We next used monodansylcadaverine (MDC), a dye that stains autophagolysosomes [12]. MDC staining increased at 12 h and 24 h in Nalm-6 cells (Figure 1B). Additionally, LC3 puncta, an indicator of autophagosome formation, were examined by immunofluorescent staining, as shown.
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**Figure 1C** where LC3 puncta were significantly increased after treatment at 12 h and 24 h in Nalm-6 cells, whereas minimal autophagosome formation was present in the control group.

**Bortezomib-induced autophagy is dependent on the PI3KC3 signaling pathway and JNK activity**

The Beclin 1/PI3KC3 complex is one of the most important regulators essential for autophagosome formation, and inhibition of PI3KC3 activity will block autophagy [17, 18]. To determine whether bortezomib-induced autophagy in B-ALL cells is associated with the PI3KC3 signaling pathway, Nalm-6 or CD34+ primary cells were exposed to 3-methyladenine (3-MA), a specific inhibitor of PI3KC3, in the present or absent of bortezomib. We observed that the LC3-II level was increased for treatment with bortezomib alone and 3-MA down-regulated the bortezomib-induced LC3-II accumulation (**Figure 2A** and **2B**). To further study this finding, autophagosome formation was assessed in Nalm-6 and CD34+ primary cells on a fluorescent microscope after treatment with bortezomib alone or in combination with 3-MA. Consistent with the immunoblotting results, fluorescent microscopic analyses showed that bortezomib obviously increased LC3 puncta, an indicator of autophagic vacuoles, but this was inhibited by 3-MA (**Figure 2C**). These results indicated that bortezomib induces autophagy via the PI3KC3 signaling pathway.

Previous reports have suggested that phosphorylation of Bcl-2, which liberates Beclin-1, may also be a crucial mechanism for initiating autophagy [19]. The kinase c-JUN N-terminal kinase (JNK) can phosphorylate Bcl-2, leading to dissociation of Beclin 1 from Bcl-2, liberating beclin-1 to activate the autophagy pathway [20, 21]. Several studies have shown that bortezomib inhibits growth and induces apoptosis by activating the JNK enzyme [22, 23]. We next evaluated whether bortezomib induces autophagy by activating JNK; cells were co-treated with bortezomib and SP600125, a JNK inhibitor (JNKi), and the LC3-II level was examined by immunoblotting with an anti-Beclin-1 antibody and then evaluation of Beclin-1, Bcl-2, and PI3KC3 expression.
PI3KC3/Beclin-1 complex [25, 26]; therefore, the expression levels of Beclin-1 and PI3KC3 were measured by immunoblotting after bortezomib treatment, and we found that Beclin-1 and PI3KC3 were not induced by bortezomib in any of the examined concentrations and time points in Nalm-6 cells (Figure 3A). The study above showed that PI3KC3 is involved in bortezomib-induced autophagy. To further elucidate the detailed mechanism, we explored the effect on the formation of the Bcl-2/Beclin-1 complex and Beclin-1/PI3KC3 complex by treatment with bortezomib; Nalm-6 cells were treated with bortezomib and then the cell lysates were prepared for the co-immunoprecipitation with an anti-Beclin-1 antibody. As we expected, there was disruption of the Beclin-1/Bcl-2 complex and formation of the Beclin-1/PI3KC3 complex in the bortezomib-treated cells (Figure 3B). These results demonstrate that bortezomib induces autophagy via decreasing the interaction of the Beclin-1/Bcl-2 complex, while increasing the interaction of the Beclin-1/PI3KC3 complex.

**Bortezomib inhibits growth and induces apoptosis in B-ALL cells**

To assess the anti-ALL effect of bortezomib, Nalm-6 or CD34+ primary cells was exposed to bortezomib at different concentrations for 24 h. Cell growth and apoptosis were measured with the MTT assay and FACS analysis after Annexin V-FITC/propidium iodide (PI) staining, respectively. After treatment, inhibition of proliferation and apoptosis were markedly increased in a dose-dependent manner (Figure 4A and 4B). Furthermore, we examined the expression of proteins relative to apoptosis by immunoblotting. The expression of cleaved Caspase-3, Bax, and cytoplasm cytochrome C were up-regulated and Bcl-2 was down-regulated in Nalm-6 cells after treatment (Figure 4C). Consistently, the cleaved Caspase 3 level was up-regulated in CD34+ primary cells from 2 B-ALL patients (Figure 4D). These results suggest that bortezomib inhibits growth and induces apoptosis as well as has a therapeutic impact on B-ALL.

**Bortezomib arrests the cell cycle at the G2/M phase**

We explored the effect of bortezomib on the cell cycle and found that bortezomib suppressed cell cycle progression at the G2/M phase. Our data showed that the percentage of cells in the G2/M phase was increased from 17.72% to 55% with increasing concentrations of bortezomib (Figure 5A and 5B), indicating that cell cycle arrest contributed to growth inhibition by bortezomib in the Nalm-6 cell line.

**Inhibition of autophagy enhances the anticancer activity of bortezomib**

To examine the effect of autophagy on the anticancer activity of bortezomib, we analyzed the cell viability, apoptosis and cell cycle after treatment with bortezomib alone or in combination with autophagy inhibitors, including 3-MA and JNKi. Both Nalm-6 and CD34+ cells had more significantly inhibited growth after treatment with bortezomib in combination with 3-MA or JNKi compared with bortezomib alone (Figure 6A). Furthermore, 3-MA and JNKi increased the bortezomib-induced apoptosis by flow cytometric analysis and up-regulated the expression of apoptosis proteins, including cleaved-Caspase 3 and cytoplasm cytochrome C (Figure 6B). Additionally, we investigated the cell cycle process of Nalm-6 cells that were exposed to either bortezomib alone or in combination with 3-MA or JNKi, and we found that only JNKi enhanced the effect of G2/M arrest by bortezomib (Figure 6C). These findings suggest that autophagy prevents Nalm-6 cells from apoptosis and promotes cells survival after bortezomib treatment.

Recent studies have shown that chloroquine, a lysosomal inhibitor, can potentiate the cytotoxicity of chemotherapy drugs by inhibiting autophagy in established cancers, including prostate cancer [27], colorectal cancer [28], chronic myeloid leukemia [29], etc. To investigate the effect of chloroquine in combination with bortezomib treatment in B-ALL, we examined autophagic flux, cell viability, and apoptosis proteins and observed that the LC3-II level was notably increased with the combination of bortezomib and chloroquine. Co-treatment with bortezomib and chloroquine markedly inhibited cell growth (Figure 6D); moreover, the expression of cleaved-Caspase 3 and cytoplasm cytochrome C, induced by bortezomib, were enhanced by treatment with chloroquine (Figure 6D). Taken together, our data indicated that the effect of bortezomib on B-ALL is partly attenuated by autophagy, and autophagy inhibitors can enhance the anti-B-ALL activity of bortezomib.
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A

Nalm-6

ALL#1

ALL#2

Bortezomib (nM)

Cell viability (%)

0 5 10 20

Cell viability (%)

0 5 10 20

Cell viability (%)

0 5 10 20

B

Bortezomib (nM)

PI

Annexin-V FITC

Annexin V positive cell (%)

0 5 10 20

C

Bortezomib (nM)

Caspase-3

0 5 10 20

Cleaved-Caspase-3

0 5 10 20

Bax

0 5 10 20

Bcl-2

0 5 10 20

Cytoplasm

0 5 10 20

Cytochrome C

0 5 10 20

β-actin

D

ALL#1

ALL#2

Cleaved-Caspase-3

0 5 10 20

β-actin

0 5 10 20

β-actin

0 5 10 20
Figure 4. Bortezomib shows anti-leukemia activity in ALL cells. A. Bortezomib inhibits the growth of Nalm-6 cells. Nalm-6 cells and CD34+ primary cells were exposed to increasing concentrations of bortezomib for 24 h. Cell viability was evaluated with the MTT assay. Data are shown as the mean ± SD (**, p < 0.01, ***, p < 0.001 versus control). B. Bortezomib induces apoptosis in Nalm-6 cells. Nalm-6 cells were treated with increasing concentrations of bortezomib for 24 h, which was followed by analysis of apoptosis by staining with PI and Annexin-V FITC. Annexin-V positive cells were measured by flow cytometry. Columns represent the average percent of Annexin-V positive cells from 3 independent experiments, which are shown as the mean ± SD (***, p < 0.001 versus control). Representative images are shown in the left panel. C and D. Nalm-6 cells and CD34+ primary cells were treated with increasing concentrations of bortezomib for 24 h, which was followed by Western blot analysis for the expressions of Caspase 3, cleaved Caspase 3, Bax, Bcl-2 and cytoplasm cytochrome C.

Figure 5. Bortezomib arrested the cell cycle progression at the G2/M phase in Nalm-6 cells. Nalm-6 cells were exposed to increasing concentrations of bortezomib for 24 h, which was followed by staining with PI; then, the DNA content was assayed for cell cycle analysis by flow cytometry. Columns represent the average percent of cells in the G2/M phase from 3 independent experiments, which were shown as the mean ± SD (**, p < 0.01, ***, p < 0.001 versus control). Representative images are shown in the upper panel.
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Discussion

The outcomes of refractory or relapsed ALL are still disappointing. Bortezomib is a new treatment choice for these refractory or relapsed patients. However, the molecular mechanism of the anti-ALL effect of bortezomib is not clear. In this study, we demonstrated that bortezomib...
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inhibited cell growth, induced apoptosis, arrested the cell cycle and induced autophagy in Nalm-6 cells and CD34+ primary cells. Additionally, the inhibition of autophagy could enhance the anti-ALL function of bortezomib; meanwhile, we explored bortezomib-induced autophagy in association with the interaction of three types of proteins, including PI3KC3, Beclin 1 and Bcl-2.

The ubiquitin proteasome system (UPS) plays a key role in maintaining cellular protein homeostasis through the selective degradation of damaged, misfolded and short-lived regulatory proteins that control essential cellular processes [30]. Dysfunction of this system has been related to transformation and oncogenesis; therefore, the UPS becomes an attractive target for the anticancer therapies. Bortezomib, as a reversible proteasome inhibitor, blocked the degradation of intracellular proteins, affecting multiple cellular processes, including cell cycle progression, cell apoptosis, endoplasmic reticulum stress, angiogenesis, and DNA repair, which contribute to the anti-tumor effect of bortezomib [31]. Currently, the therapeutic strategies, including bortezomib alone or in combination with other chemotherapy drugs for treating acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) [32-35], have led to further investigation of its utility in these malignancies. In this study, we observed that bortezomib inhibited cell growth in both the Nalm-6 cell line and CD34+ primary cells. To explore the reasons for inhibition of cell proliferation, we examined apoptosis, the cell cycle and the autophagy process in B-ALL cells and observed that bortezomib increased Bax and decreased Bcl-2, promoting the release of cytochrome C and activated mitochondrial apoptotic pathway, resulting in cell death. Additionally, the cell cycle process was arrested at the G2/M phase by bortezomib, which also contributed to cell growth inhibition. Moreover, we found that bortezomib induced autophagy in the aforementioned cells, and we further explore the molecular mechanism of bortezomib-induced autophagy and whether autophagy plays a pro-survival or pro-death role in B-ALL cells.

Recent findings have shown that in cells undergoing starvation-induced autophagy, JNK1 phosphorylates serine 70 on Bcl-2, leading to disruption of the Bcl-2/Beclin-1 complex [16, 17] and releasing Beclin-1 to form a complex with the class III phosphatidylinositol 3-kinase (PI3KC3), which engages in the early stage of autophagic vesicle formation [15]. In our study, bortezomib downregulated the Bcl-2 expression, liberating Beclin-1 from Bcl-2 and increasing Beclin-1/PI3KC3 complex formation. Because PI3KC3 positively regulates autophagy activation while Bcl-2 negatively regulates autophagy activation, our results demonstrated that bortezomib decreased the interaction between Beclin-1 and Bcl-2, antagonized the inhibition of autophagy by Bcl-2 and facilitated the formation of the Beclin-1/PI3KC3 complex, activating autophagy.

The role of autophagy in cell survival and cell death is like a “double-edged sword” [36]. On the one hand, autophagy maintains the nutrient and energy homeostasis in the case of exposure to various stresses conditions. On the other hand, autophagy might permit cancer cells to become chemotherapy resistant or too much autophagy might lead to undesirable cell death [37]. Therefore, we asked whether bortezomib-induced autophagy exerts a pro-survival or pro-death role in B-ALL cells; to do so, we used autophagy inhibitors to block the autophagy process, which was followed by assessing the effect on cell growth, apoptosis and cell cycle process. Our findings suggested that inhibition of autophagy enhanced the growth inhibition and cell cycle arrest by bortezomib, promoting apoptosis by up-regulating the expressions of cleaved Caspase 3 and cytoplasmic cytochrome C, indicating that bortezomib-induced autophagy promotes B-ALL cell survival and plays a protective role, and an inhibitor of autophagy could enhance the cytotoxicity of bortezomib treatment.

In conclusion, this study is the first report that bortezomib induces autophagy in B-ALL cells by increasing formation of the PI3KC3/Beclin 1 complex and, in combination with autophagy inhibitors, can enhance the anticancer activity of bortezomib. This study increases our understanding of bortezomib in the treatment of hematological malignancies.

Disclosure of conflict of interest

None.

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