Article

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Bortezomib Is Associated With Stress Responses and Growth Suppression in ELT3 Uterine Leiomyoma Cells

Hosouk Joung[1], Hyunju Liu[1,2,*]

[1]Department of Obstetrics and Gynecology, Chosun University College of Medicine, 61452 Gwangju, Republic of Korea
[2]Department of Obstetrics and Gynecology, Chosun University Hospital, 61453 Gwangju, Republic of Korea
*Correspondence: lhj@chosun.ac.kr (Hyunju Liu)
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Background: Uterine leiomyomas are the most frequently occurring benign tumors of the female reproductive system and are characterized by excessive proliferation, resistance to apoptosis, and abnormal extracellular matrix (ECM) accumulation. However, the current treatments are limited by recurrence, systemic side effects, and fertility loss, creating a need for novel therapeutic approaches. Bortezomib, a reversible proteasome inhibitor targeting the β5 subunit of the 26S proteasome, is approved for hematologic malignancies but has not been systematically evaluated in uterine leiomyomas. Therefore, the aim of this study was to investigate the effects of bortezomib on Eker rat leiomyoma tumor-derived 3 (ELT3) uterine leiomyoma cells and explore the underlying molecular mechanisms.

Methods: We investigated the effects of bortezomib on ELT3 leiomyoma cells. Cell viability and colony formation were assessed to determine the anti-proliferative effects. Apoptosis, cell cycle progression, and intracellular reactive oxygen species (ROS) levels were evaluated. Mechanistic studies included the evaluation of ubiquitin accumulation, ERK signaling, caspase-3 activation, poly(ADP-ribose) polymerase (PARP) cleavage, levels of ECM-related proteins, including Collagen I and Acta2, and autophagy markers, including microtubule-associated protein 1 light chain 3 (LC3) conversion and p62 degradation. Antioxidant co-treatment experiments were performed to assess ROS dependency.

Results: Bortezomib markedly reduced cell viability and colony formation, increased apoptotic cell death, and altered cell cycle distribution, with an increase in the gap 2/mitotic (G2/M)-phase population. Co-treatment with antioxidants attenuated apoptosis, suggesting that the cytotoxic effect was mediated by ROS, which were also elevated intracellularly. Mechanistically, bortezomib promoted ubiquitin accumulation and was associated with changes in ERK-associated signaling involved in leiomyoma cell proliferation, activation of caspase-3 and PARP cleavage, downregulation of ECM proteins, and modulation of autophagy, as evidenced by LC3 conversion and differential patterns of p62 expression over time.

Conclusion: Bortezomib exerts multifaceted antitumor effects on uterine leiomyoma cells by targeting proliferation, apoptosis, oxidative stress, ECM deposition, and autophagy, thereby providing mechanistic insights and supporting its possible use as a treatment for uterine leiomyomas.

Keywords: uterine; leiomyoma; fibroid; bortezomib; antitumor

Introduction

Up to 70% of women of reproductive age develop uterine leiomyomas, which are the most common benign tumors of the female reproductive tract [1]. Although histologically benign, these tumors are frequently associated with abnormal bleeding and pelvic pain along with impaired fertility and poor pregnancy outcomes, imposing a substantial burden on the health and quality of life [2,3]. Current therapeutic options include surgical procedures, such as myomectomy and hysterectomy, and pharmacological interventions aimed at hormone modulation [4,5]. However, these approaches are often limited by high recurrence rates, systemic side effects, and irreversible loss of fertility, underscoring the need for novel targeted fertility-preserving therapies [4,6].

The pathophysiology of uterine leiomyomas is complex and involves multiple mechanisms [7,8,9]. Dysregulated cell proliferation, resistance to apoptosis, and excessive accumulation of extracellular matrix (ECM) are hallmark features of fibroid growth [9,10]. Aberrant activation of intracellular signaling pathways, particularly ERK signaling, contributes to tumor cell growth [9]. Oxidative stress and impaired protein homeostasis (proteostasis) are increasingly recognized as contributing factors to disease onset and progression [10,11,12]. These pathological characteristics suggest that therapeutic strategies that simultaneously target cell proliferation, apoptosis, ECM accumulation, and stress response pathways may provide significant clinical benefits.

Bortezomib, a first-in-class and Food and Drug Administration (FDA)-approved proteasome inhibitor, re-

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versibly targets the β5 subunit and inhibits chymotrypsin-like activity [13]. This inhibition prevents proteasomal degradation, leading to the accumulation of ubiquitinated proteins, proteasomal imbalance, and apoptotic pathways [14]. Clinically, bortezomib has been successfully used to treat blood cancers, including multiple myeloma and mantle cell lymphoma [14]. In addition to hematological cancers, proteasome inhibitors have also attracted increasing interest as potential therapeutic agents for solid tumors and other proliferative disorders [15]. However, its therapeutic relevance in uterine leiomyomas remains unclear.

In this study, we examined the therapeutic potential of bortezomib in uterine leiomyomas, using Eker rat leiomyoma tumor-derived 3 (ELT3) cells as a representative model. Extending proteasome research on fibroids to include FDA-approved inhibitors may help establish new management strategies.

Materials and Methods

Cell Culture and Reagents

ELT3 cells, which are adherent cells with epithelial-like morphology, were obtained from the American Type Culture Collection (ATCC; CRL-3371; Manassas, VA, USA). Mycoplasma contamination was tested using the MycoStrip-Mycoplasma Detection Kit (REP-MYS-10; InvivoGen, San Diego, CA, USA) according to the manufacturer's instructions, and no mycoplasma contamination was detected. ELT3 cells were grown in DMEM (LM001-05; Welgene, Gyeongsan, Republic of Korea) containing with 10% FBS (SH30919-03; Hyclone, Logan, UT, USA) and 1% penicillin-streptomycin (15140122; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C under 5% CO_2. Bortezomib (5.04314; Sigma-Aldrich, St. Louis, MO, USA) was used as the experimental agent, and DMSO (DMSO100; LPS Solution, Daejeon, Republic of Korea) served as the vehicle control. N-acetyl-L-cysteine (NAC, A7250; Sigma-Aldrich, St. Louis, MO, USA) and catalase (CAT, C1345; Sigma-Aldrich, St. Louis, MO, USA) were used to evaluate the involvement of oxidative stress in ELT3 cells [16,17]. NAC was administered at a final concentration of 5 mM for 24 or 48 h, and CAT was administered at a final concentration of 50 U/ml for 24 or 48 h.

Assessment of Cell Viability and Cytotoxicity

ELT3 cells (5.0×10^3/well) were plated into 96-well plates, cultured for 24 h, and subsequently exposed to bortezomib. For the cell viability assay, 5 mg/mL MTT reagent (M2128; Sigma-Aldrich, St. Louis, MO, USA) was added, followed by incubation at 37 °C for 2 h. Formazan was dissolved in 150 µL DMSO, and absorbance at 570 nm was measured with an INNO microplate reader (LTek, Seongnam, Republic of Korea). The relative cell viability was calculated as follows: (absorbance of treated group/absorbance of control group) × 100. The LDH Plus Cytotoxicity Assay Kit (GBL-P500; Dyne Bio, Seongnam, Republic of Korea) was used to evaluate cytotoxicity according to the manufacturer's instructions, and the absorbance was measured at 490 nm. The relative cytotoxicity was expressed as the ratio of the absorbance of the treated group to that of the control group.

Colony Formation Assay

ELT3 cells were seeded in 6-well plates and treated with various doses of bortezomib. After 4 d, the colonies were stained with 2% crystal violet (ab232855; Abcam, Cambridge, UK) for 20 min, washed, air-dried, and solubilized. Absorbance was measured at 595 nm to determine the effect of bortezomib on colony formation. The relative colony formation rate was calculated as follows: (absorbance of the treated group/absorbance of the control group) × 100.

Apoptosis Assay Using Flow Cytometry

Apoptosis in ELT3 cells was evaluated using the Muse Annexin V & Dead Cell Kit (MCH100105; Cytek Biosciences, Fremont, CA, USA) according to the manufacturer's instructions. The cells were seeded in 6-well plates and exposed to bortezomib. The harvested cells were incubated with Annexin V reagent for 20 min at room temperature in the dark and then analyzed using a Guava Muse Cell Analyzer (0500-3115; Luminex, Austin, TX, USA).

Cell Cycle Analysis

Cells were collected, pelleted by centrifugation, and fixed with 70% ethanol at −20 °C for 3 h, and incubated with 200 µL of Muse Cell Cycle Reagent (MCH100106; Cytek Biosciences, Fremont, CA, USA). After 30 min of incubation in the dark, the stained cells were analyzed for cell cycle using a Guava Muse Cell Analyzer.

Flow Cytometric Analysis of ROS

Intracellular ROS levels were analyzed using the Muse Oxidative Stress Kit (MCH100111; Cytek Biosciences, Fremont, CA, USA). Cells were plated in at 5×10^4/well in 6-well plates and treated with 0–100 nM bortezomib for 24 or 48 h. The collected cells were incubated with Muse Oxidative Stress Reagent in 1× assay buffer for 30 min at 37 °C in the dark and then analyzed using a Guava Muse Cell Analyzer.

Autophagy Analysis Using Flow Cytometry

Bortezomib-induced autophagy was evaluated using a Muse Autophagy LC3-Antibody Based Kit (MCH200109; Cytek Biosciences, Fremont, CA, USA) according to the manufacturer's protocol. In this assay, selective permeabilization removes soluble cytosolic microtubule-associated protein 1 light chain 3 (LC3), whereas LC3 associated with autophagosomes is preserved, allowing the preferen-

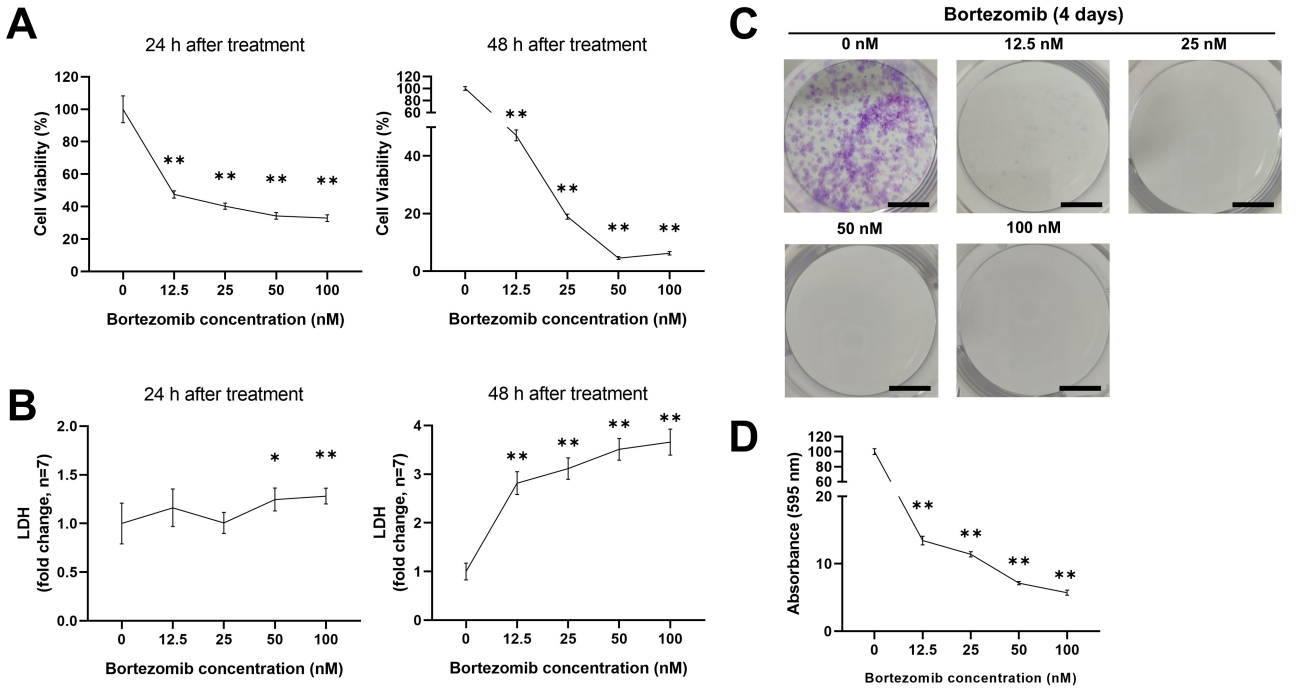

Fig. 1. Bortezomib reduces viability and colony growth while increasing LDH release in ELT3 cells. (A,B) MTT assay and LDH activity after 24 and 48 h of bortezomib exposure. (C) Colony growth of ELT3 cells after bortezomib exposure (12.5, 25, 50, and 100 nM) for 4 d. Upper panel: representative crystal violet-stained colonies (scale bar = 10 mm). (D) Lower panel: quantification of absorbance at 595 nm. Results are expressed as mean ± SD from seven technical replicates for A and B and three technical replicates for D; significance was determined using Dunnett's test. *$p < 0.05$, **$p < 0.01$ versus control. LDH, lactate dehydrogenase; SD, standard deviation.

tial measurement of lipidated LC3-II. Briefly, treated and control cells were stained with an anti-LC3-Alexa Fluor 555 antibody for 30 min, resuspended in 1× assay buffer, and analyzed using a Muse Cell Analyzer. Autophagy was quantified based on the fluorescence ratio of untreated cells.

Protein Isolation and Western Blotting

Protein isolation and western blotting were performed as described previously [18]. After treatment, cells were lysed in a protease inhibitor-containing RadioImmunoPrecipitation Assay buffer (RIPA) buffer (R2002; Biosesang, Seongnam, Republic of Korea). Equal amounts of protein were resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to PVDF membranes (IPVH00010; Merck Millipore, Burlington, MA, USA). The membranes were then blocked with 5% skim milk (262100; BD Difco, Sparks, MD, USA) for 1 h, and incubated overnight at 4 °C with primary antibodies. The primary antibodies from Cell Signaling Technology (CST) were used: phospho-ERK1/2 (9106S), ERK1/2 (4695S), PARP (9542S), caspase-3 (9662S), cleaved caspase-3 (9664S), collagen I (72026T), α-SMA/Acta2 (19245S), p62/SQSTM1 (5114T), and LC3B (2775S). Additional antibodies included β-actin (sc-47778), GAPDH (sc-166574) from Santa Cruz Biotechnology (Dallas, TX, USA), and ubiquitin (BML-PW0930) from Enzo Biochem (Farmingdale, NY, USA). After incubation with horseradish peroxidase-conjugated secondary antibodies (7074S or 7076S; CST, Danvers, MA, USA) for 1 h, protein signals were visualized using a Western Pico ECL Kit (PICO-250; LPS Solution, Daejeon, Republic of Korea) and Azure c280 imaging system (Azure Biosystems, Dublin, CA, USA).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Software, Boston, MA, USA). Data are shown as the mean ± standard deviation (SD) from at least three independent experiments. Comparisons among groups were made using one-way ANOVA, followed by Tukey's or Dunnett's post-hoc tests. Statistical significance was defined as *$p < 0.05$, **$p < 0.01$.

Results

Bortezomib Suppresses Viability and Clonogenic Growth of ELT3 Cells

To investigate whether bortezomib induced cytotoxicity, ELT3 cells were exposed to various concentrations of bortezomib. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay results demonstrated a marked decline in cell viability, with survival drop-

ping to 32.9 ± 2.06% after 24 h of treatment at 100 nM and further to 6.2 ± 0.62% after 48 h ($p < 0.01$; Fig. 1A). These observations indicated that bortezomib exerts an inhibitory effect on ELT3 cells, suggesting its therapeutic potential. LDH release assays were performed to confirm cytotoxicity. At 100 nM, bortezomib increased the LDH activity by approximately 1.3 fold at 24 h and 3.6 fold at 48 h, indicating significant membrane damage ($p < 0.01$; Fig. 1B). Consistently, colony formation assays revealed a marked reduction in the proliferative capacity, as crystal violet staining showed smaller and fewer colonies with 12.5, 25, 50, and 100 nM bortezomib over four days (Fig. 1C, upper panel). Quantitative evaluation further confirmed a nearly 90% reduction in the absorbance at 595 nm at 50 and 100 nM, underscoring its strong inhibitory effect on clonogenic growth ($p < 0.01$; Fig. 1D, lower panel). Collectively, these findings demonstrate that higher doses of bortezomib significantly decrease cell viability, enhance LDH release, and markedly suppress the colony-forming ability of ELT3 cells.

Bortezomib Triggers Apoptosis in ELT3 Cells

Although bortezomib reduced ELT3 cell viability at 12.5 nM, the concentration range required to induce apoptotic and molecular responses has not been fully established. Therefore, 25, 50, and 100 nM bortezomib were selected for subsequent mechanistic analyses to evaluate the dose- and time-dependent cellular responses. To evaluate the apoptotic effect of bortezomib in ELT3 cells, Annexin V flow cytometry was performed to assess apoptosis after treatment with 25, 50, or 100 nM bortezomib for 24 or 48 h. After 24 h, the apoptosis increased to 9.3 ± 0.39%, 14.0 ± 0.21%, and 18.9 ± 1.45% at 25, 50, and 100 nM, respectively, compared with the control group (2.3 ± 0.14%; $p < 0.01$; Fig. 2A, upper panel). Prolonged treatment for 48 h further increased apoptosis to 23.9 ± 2.82%, 32.4 ± 2.51%, and 33.8 ± 2.36%, respectively, at the same concentrations compared with the control group (5.5 ± 0.7%; $p < 0.01$; Fig. 2A, lower panel). Taken together, these results showed that apoptosis in ELT3 cells increased with bortezomib dose and treatment time.

Bortezomib Alters Cell Cycle Distribution in ELT3 Cells

To examine whether the bortezomib-induced growth inhibition was linked to cell cycle regulation, ELT3 cells were treated with increasing concentrations of bortezomib for 24 or 48 h (Fig. 2B). After 24 h, the proportion of cells in the G0/G1 phase decreased from 30.5 ± 0.7% in the control to 24.9 ± 0.69%, 25.0 ± 1.45%, and 25.8 ± 0.85% with 25, 50, and 100 nM, respectively ($p < 0.01$; Fig. 2C, left panel). Conversely, the percentage of cells in the G2/M phase increased from 62.7 ± 0.55% in the control group to 67.5 ± 0.80% in the 25 nM group ($p < 0.01$). Prolonged exposure for 48 h further accentuated these effects; the fraction of cells in the G0/G1 phase decreased from 31.3 ± 0.10% to 19.9 ± 0.26%, 13.5 ± 0.37%, and 16.0 ± 1.32% ($p < 0.01$), whereas the fraction of cells in the G2/M phase increased from 60.8 ± 1.26% to 69.7 ± 1.57%, 75.9 ± 0.49%, and 70.8 ± 1.90%, respectively, at the same concentrations ($p < 0.01$; Fig. 2C, right panel). Together, these results indicated that bortezomib altered the cell cycle distribution in ELT3 cells, as evidenced by an increase in the G2/M-phase population.

Bortezomib Induces Apoptosis via ROS-Dependent Mechanisms in ELT3 Cells

Reactive oxygen species (ROS) are highly reactive molecules that induce oxidative stress and cellular damage [19]. To determine whether bortezomib affects ROS production, the cells were exposed to 0, 25, 50, or 100 nM bortezomib for 24 or 48 h. After 24 h, the proportion of ROS-positive cells increased to 36.1 ± 0.75%, 51.1 ± 0.99%, and 60.6 ± 3.09% at 25, 50, and 100 nM, respectively, compared with that in the untreated controls (17.85 ± 1.38%; $p < 0.01$; Fig. 3A, upper panel). Consistent results were observed at 48 h, with ROS levels increasing by 37.2 ± 3.42%, 52.6 ± 2.76%, and 53.3 ± 1.80% at the same concentrations, respectively (19.37 ± 1.62%; $p < 0.01$; Fig. 3A, lower panel). To investigate whether ROS accumulation mediates apoptosis, ELT3 cells were co-treated with the antioxidants NAC or CAT in the presence of 100 nM bortezomib. As shown in Fig. 3B, NAC significantly reduced apoptosis at both 24 and 48 h, whereas CAT primarily suppressed apoptosis after 48 h (Fig. 3C). Collectively, these results indicate that bortezomib causes apoptotic cell death in ELT3 cells, largely through an ROS-dependent mechanism.

Bortezomib Regulates Proliferation-, Apoptosis-, and ECM-Related Protein Expression in ELT3 Cells

Bortezomib is a well-characterized proteasome inhibitor [14,20]. Western blotting was performed to evaluate the effect on ubiquitin-conjugated proteins in ELT3 cells. Treatment with 25, 50, and 100 nM bortezomib for 24 or 48 h led to pronounced accumulation of ubiquitinated proteins compared with the untreated controls, indicating inhibition of proteasomal degradation (Fig. 4A). Next, we examined the ERK signaling pathway, a key driver of cell proliferation in uterine leiomyomas. Bortezomib treatment markedly reduced the p-ERK/t-ERK ratio after 48 h ($p < 0.01$; Fig. 4B). To further investigate the apoptotic mechanisms, we analyzed apoptosis-related proteins. Bortezomib enhanced caspase-3 activity and PARP cleavage, particularly at 50 and 100 nM for 24 and 48 h ($p < 0.05$; Fig. 4C). Since excessive ECM deposition, characterized by elevated Collagen I and Acta2 levels, is a hallmark of uterine leiomyomas, we also assessed ECM protein expression. Bortezomib significantly decreased Collagen I and Acta2 levels at both time points ($p < 0.05$; Fig. 4D). Collectively, these results indicated that bortezomib treatment was asso-

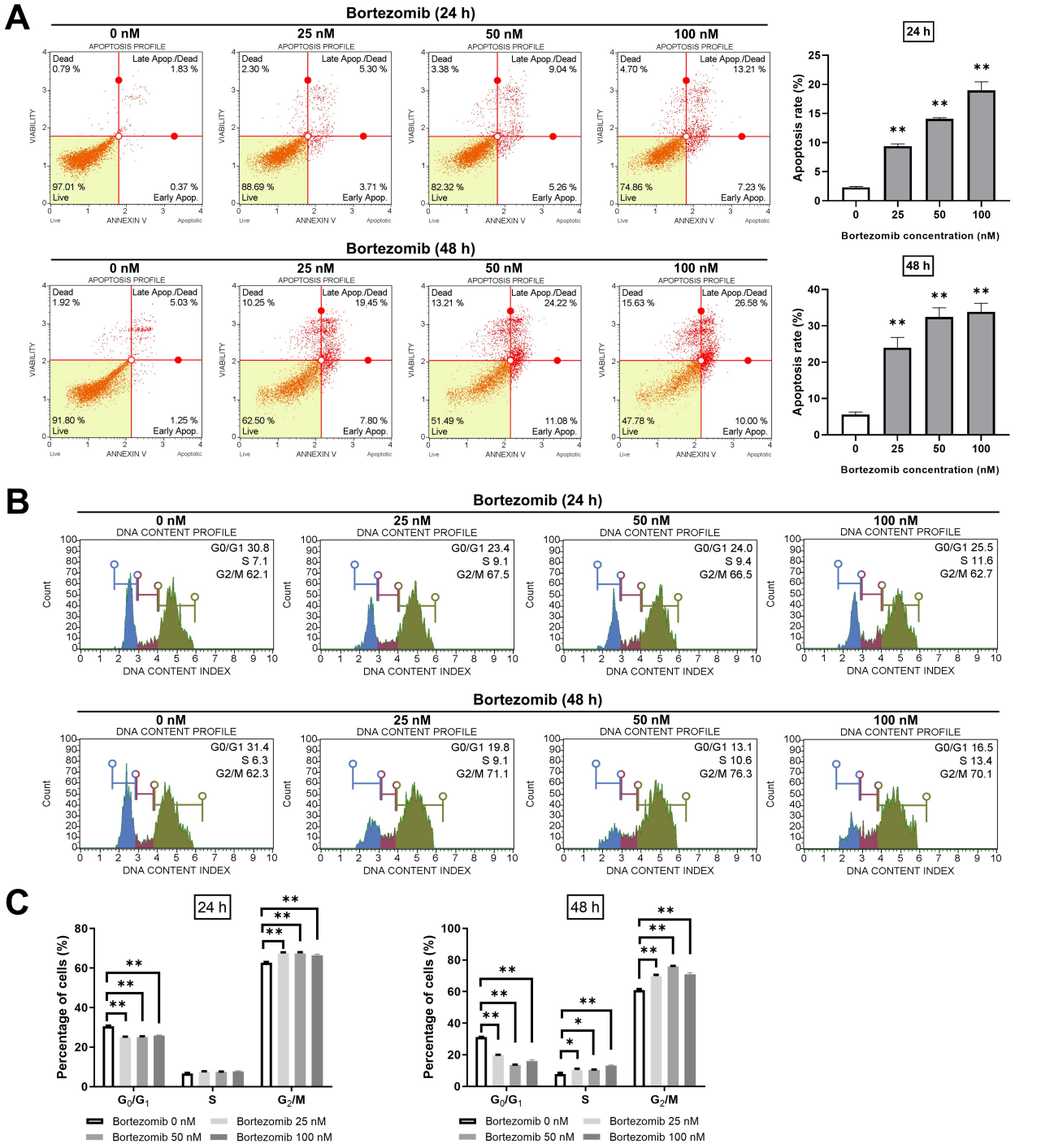

Fig. 2. ELT3 cell apoptosis and cell cycle distribution following bortezomib treatment. (A) Flow cytometric analysis of apoptosis using Annexin V staining after treatment with 25, 50, and 100 nM bortezomib for 24 and 48 h. (B) Representative histograms showing cell cycle profiles. (C) Quantification of cell populations at 24 h and 48 h. Data are shown as mean ± SD from three technical replicates. Dunnett's test was used to compare the treated groups with the control. *$p < 0.05$, **$p < 0.01$ versus control. SD, standard deviation.

ciated with ubiquitin accumulation, altered ERK-associated signaling, apoptotic pathway activation, and reduced ECM protein expression in ELT3 cells. These molecular changes may be associated with reduced growth and survival of ELT3 cells.

Bortezomib Induces Changes in Autophagy in ELT3 Cells

Autophagy is a fundamental cellular process that helps maintain homeostasis under both normal and stress conditions [21]. To investigate whether bortezomib modulates autophagy in ELT3 cells, LC3 expression was measured us-

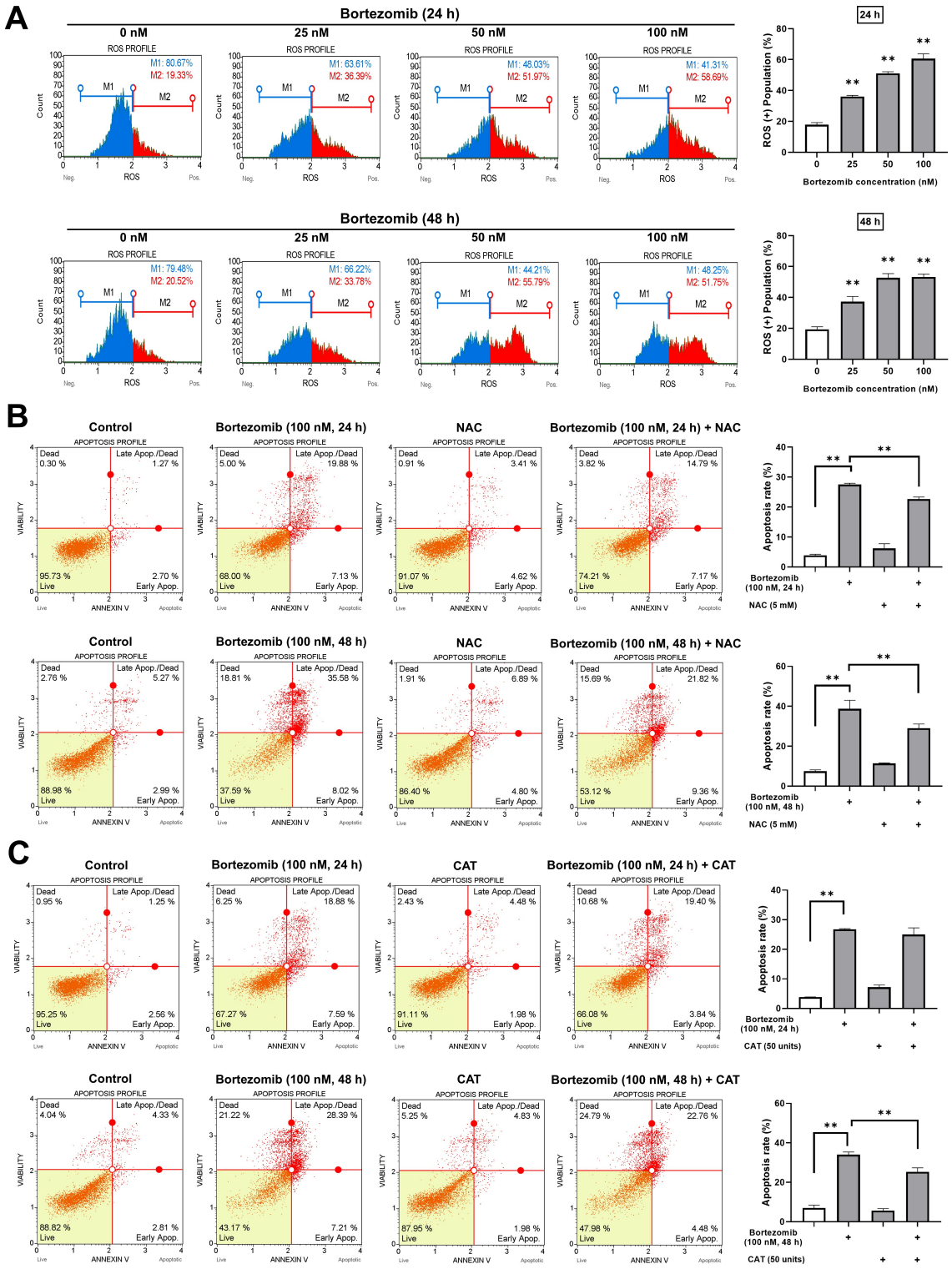

Fig. 3. Bortezomib increases ROS levels and induces ROS-dependent apoptosis in ELT3 cells. (A) Flow cytometric measurement of ROS levels in ELT3 cells after treatment with 0, 25, 50, or 100 nM bortezomib for 24 or 48 h. Representative ROS plots and the related statistical results are shown. (B,C) Apoptosis analysis of ELT3 cells co-treated with bortezomib (100 nM) and antioxidants, NAC or CAT, for 24 and 48 h. Data are shown as mean ± SD from three technical replicates. Dunnett's test was used in (A) to compare each treatment group with the control group, whereas Tukey's test was used in (B and C) for comparisons among all groups. $^{**}p < 0.01$. ROS, reactive oxygen species; NAC, N-acetylcysteine; CAT, catalase; SD, standard deviation.

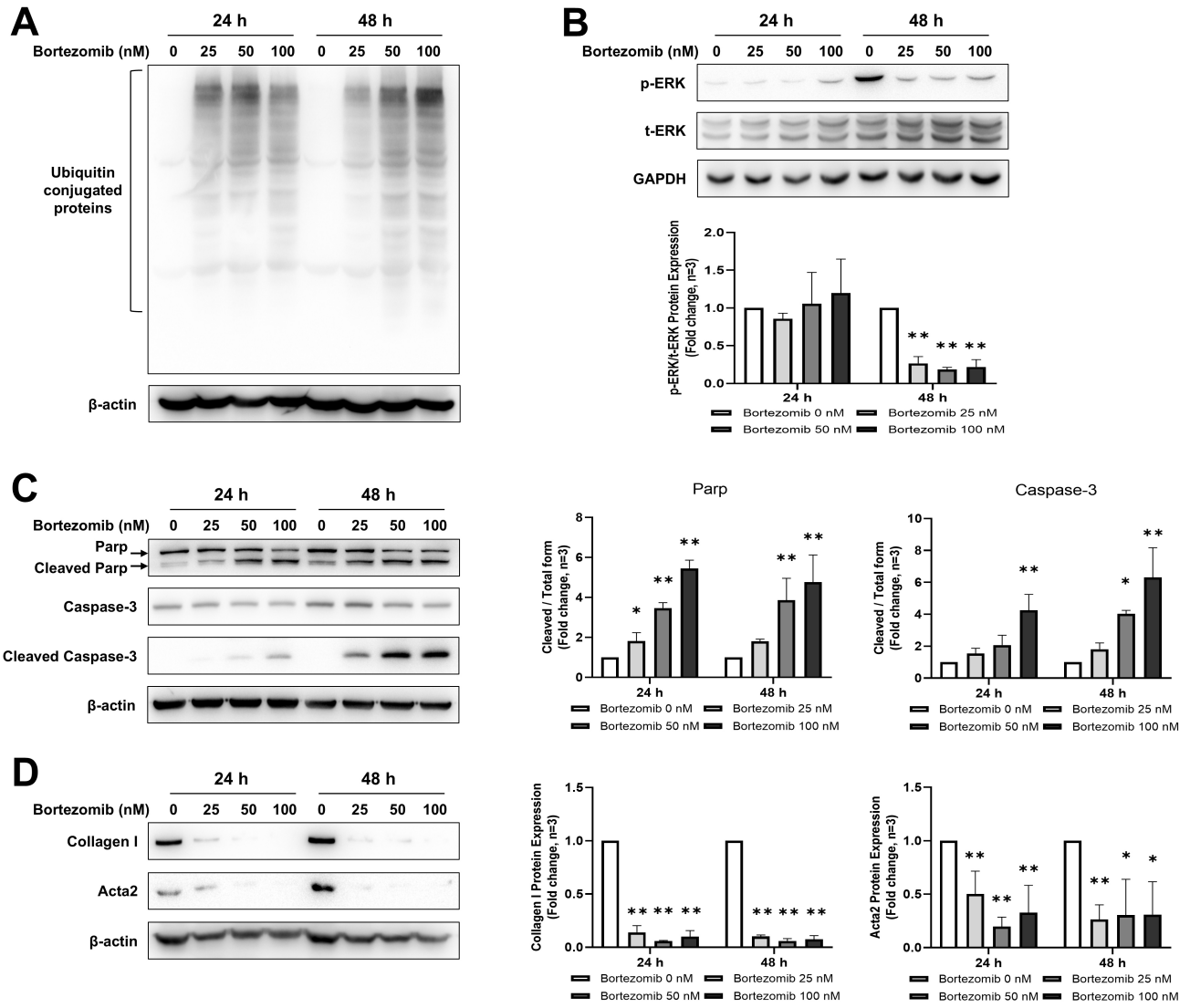

Fig. 4. Impact of bortezomib on ubiquitination, ERK-associated signaling, apoptotic proteins, and ECM proteins in ELT3 cells. (A) Cells were exposed to 25, 50, and 100 nM bortezomib for 24 or 48 h, and ubiquitin-conjugated proteins were analyzed by western blotting. β-actin or GAPDH was used as a loading control, as indicated. (B) Western blot analysis of p-ERK and t-ERK, with quantification of the p-ERK/t-ERK ratio. (C) Western blot analysis of caspase-3 and PARP to assess caspase-3 activation and PARP cleavage. (D) Western blot analysis of ECM-related proteins Collagen I and Acta2 following bortezomib treatment. Data are shown as mean ± SD from three independent biological experiments. Dunnett's test was used to compare each treatment group with the control group. *$p < 0.05$, **$p < 0.01$ versus control. p, phosphorylated form; t, total form; ERK, extracellular signal-regulated kinase; SD, standard deviation.

ing flow cytometry after treatment with 25, 50, or 100 nM bortezomib for 24 or 48 h. Bortezomib increased LC3 levels in a dose- and time-dependent manner, with the fluorescence intensity increasing 1.5 ± 0.10-, 1.7 ± 0.11-, and 1.8 ± 0.10-fold after 24 h, and further increased to 2.2 ± 0.15-, 2.8 ± 0.23-, and 3.4 ± 0.25-fold, respectively, at 48 h ($p < 0.01$; Fig. 5A). To confirm these results, western blotting was performed to assess LC3 and p62 protein levels. Notably, p62 expression increased transiently after 24 h, whereas a marked decrease was observed after 48 h of exposure to 25, 50, or 100 nM bortezomib, suggesting a dynamic response. In addition, LC3 conversion was evident, as demonstrated by the accumulation of LC3 II levels following treatment with 50 and 100 nM bortezomib for 24 and 48 h ($p < 0.01$; Fig. 5B). Taken together, these findings indicate that bortezomib is associated with changes in autophagy markers in ELT3 cells.

Discussion

The results summarized in Fig. 6 suggest that bortezomib has biological effects on ELT3 uterine leiomyoma cells. Bortezomib treatment was associated with reduced cell viability and clonogenic growth, increased apopto-

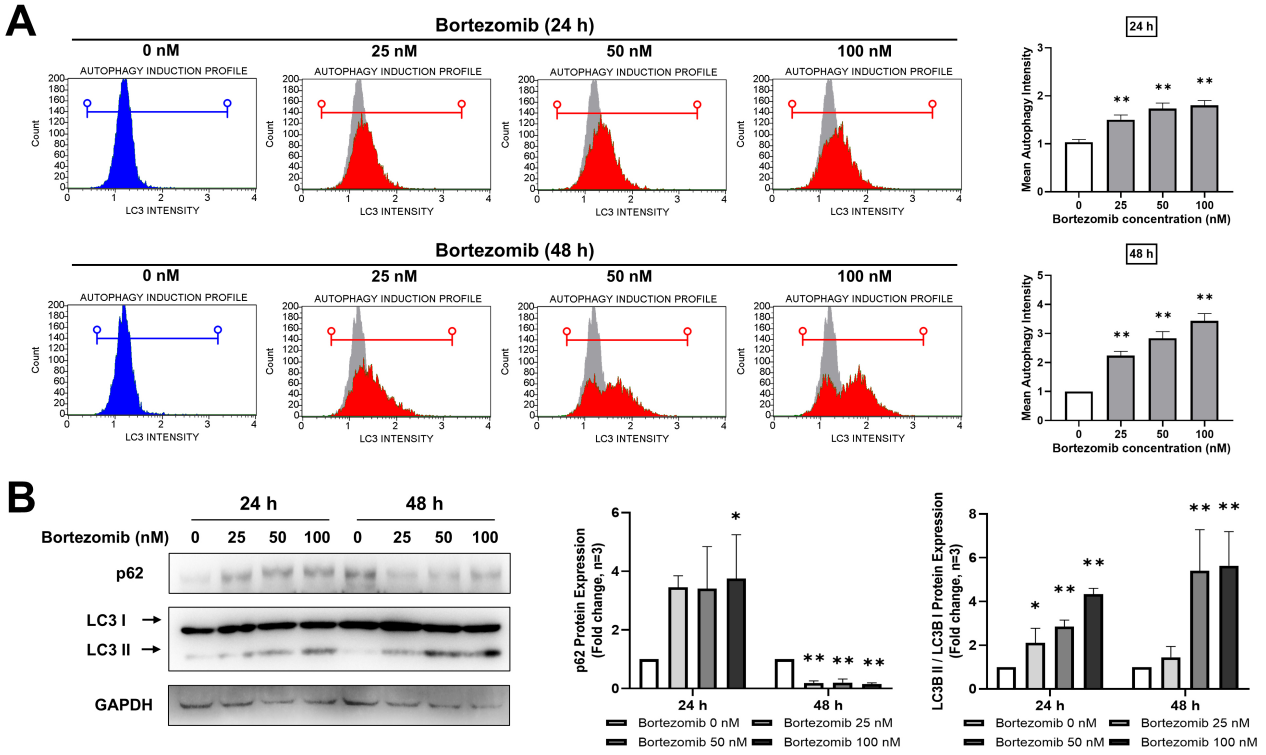

Fig. 5. Effects of bortezomib on autophagy markers in ELT3 cells. (A) Flow cytometric analysis of LC3 expression in ELT3 cells exposed to bortezomib (25, 50, and 100 nM) for 24 and 48 h. (B) Western blot analysis of p62 and LC3, with band intensities measured using ImageJ software. Data are shown as mean ± SD from three technical replicates (A) and three independent biological experiments (B). Dunnett's test was used to compare each treatment group with the control group. $*p < 0.05$, $**p < 0.01$ versus control. LC3, microtubule-associated protein 1 light chain 3; SD, standard deviation.

sis, increased G2/M-phase cell population, increased ROS generation, changes in ERK-associated signaling, reduced ECM protein expression, and modulation of autophagy-associated markers.

Bortezomib was selected for this investigation because it is the first proteasome inhibitor approved by the Food and Drug Administration (FDA), and has been successfully used to treat blood cancers [22,23,24]. Its clinical availability and well-characterized pharmacological profile provide a strong foundation for drug repurposing for non-malignant conditions such as uterine leiomyomas. This study was designed as an exploratory investigation to determine whether bortezomib can modulate key cellular processes related to leiomyoma pathology, before conducting more in-depth studies on proteasome-related pathway dysregulation in uterine leiomyomas. Mechanistically, bortezomib inhibits proteasomal degradation, induces ubiquitin accumulation, disrupts proteostasis, and activates downstream stress responses. In the present study, bortezomib treatment was associated with apoptotic cell death, increased G2/M cell population, ROS generation, changes in ERK-associated signaling, reduced ECM protein expression, and changes in autophagy-related markers.

Although bortezomib increased LC3-associated signaling and LC3-II accumulation in ELT3 cells, caution is warranted regarding the pattern of p62 expression. The transient increase in p62 at 24 h suggests that its regulation is dynamic and influenced by factors other than autophagic degradation, such as cellular stress or transcriptional regulation. In contrast, a decrease in p62 levels at 48 h may reflect a subsequent change in autophagy-associated processing. In addition, the concurrent reduction in ERK-related signaling and changes in autophagy markers observed after bortezomib treatment may reflect complex cellular stress responses induced by proteasome inhibition rather than a simple linear relationship between ERK signaling and autophagy.

Unlike malignant tumors, uterine leiomyomas are benign smooth muscle tumors that are characterized by excessive ECM deposition and fibrotic remodeling. Therefore, the therapeutic relevance of bortezomib in uterine leiomyomas may not be limited to its anti-proliferative or pro-apoptotic effects, but may also involve the modulation of ECM-associated pathological processes. In the present study, bortezomib reduced the expression of ECM-related proteins, suggesting its potential relevance in targeting the fibrotic characteristics of uterine leiomyomas. Taken to-

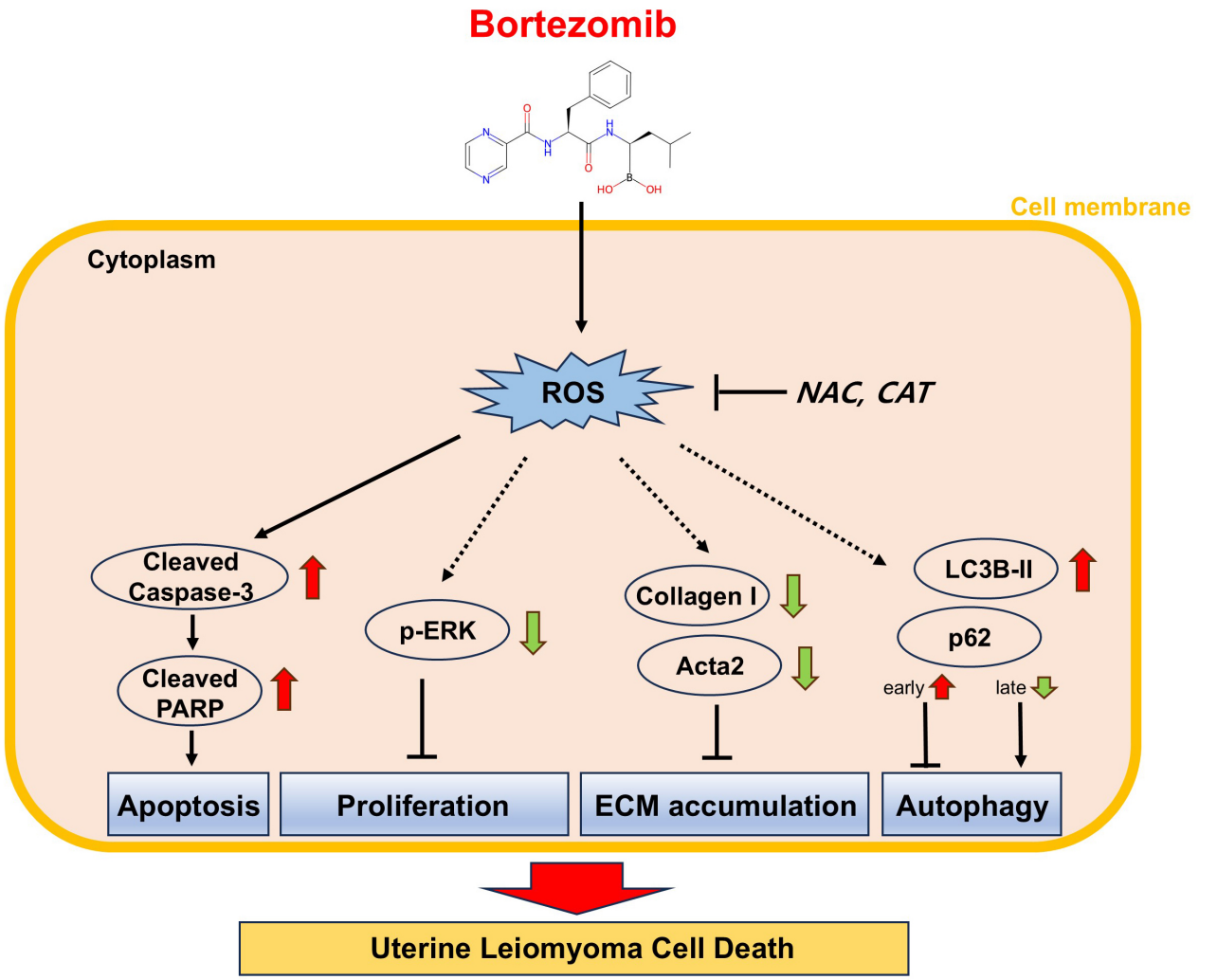

Fig. 6. Schematic summary of the proposed mechanism of bortezomib action in ELT3 uterine leiomyoma cells. Bortezomib inhibits proteasomal degradation and is associated with ubiquitin accumulation, altered ERK-associated signaling, reduced proliferation, apoptotic pathway activation, increased ROS production, reduced ECM protein expression, and changes in autophagy-associated markers in ELT3 cells. Up and down arrows indicate increased and decreased expression, respectively. Solid arrows indicate experimentally supported effects observed in this study, whereas dotted arrows indicate potential or indirect interactions that require further investigation. The schematic figure was prepared using Microsoft PowerPoint.

gether, these findings suggest that bortezomib may offer therapeutic potential by targeting fibroid-specific processes such as ECM accumulation.

Our findings are consistent with those of previous studies, which reported that proteasome inhibition is associated with apoptosis induction and changes in cell cycle distribution in malignant tumors, including multiple myeloma and solid cancers [23,25,26]. The observed activation of caspase-3 and PARP cleavage further confirmed the involvement of classical apoptotic pathways [27,28]. Importantly, leiomyomas are resistant to apoptosis, which contributes to their growth and persistence. Thus, the ability of bortezomib to overcome apoptosis resistance is a significant observation that underscores its therapeutic potential [29]. Moreover, the observed increase in the G2/M-phase population may reflect an altered cell cycle distribution, as reported in other bortezomib-treated cancer models [30,31].

Despite these promising findings, this study has some limitations. This study was performed *in vitro* using ELT3 cells, and *in vivo* validation in animal models or patient-derived uterine leiomyoma tissues is essential to confirm its translational potential. In addition, bortezomib is clinically associated with dose-limiting toxicities, which may limit its direct application in benign diseases. Furthermore, the current experimental design did not establish direct causal relationships among the observed molecular changes or directly demonstrate the abnormal activation or dysfunction of proteasome-related pathways in uterine leiomyomas.

Further studies are required to elucidate the mechanistic links between these pathways and the role of proteasome-related dysregulation in the pathogenesis of leiomyoma. Nevertheless, these findings suggest that bortezomib is a potential therapeutic candidate for uterine leiomyomas as it modulates multiple pathological processes, including proliferation, apoptosis, oxidative stress, ECM accumulation, and autophagy. Overall, this study provides new mechanistic insights into proteasome inhibition in uterine fibroids and supports further evaluation of bortezomib as a novel treatment strategy.

Conclusion

Bortezomib treatment is associated with reduced proliferation and clonogenic growth of uterine leiomyoma cells. These effects were accompanied by increased ROS generation, changes in ERK-associated signaling, accumulation of ubiquitinated proteins, reduced expression of ECM components, and changes in autophagy-associated markers. Overall, these findings suggest that bortezomib may modulate multiple cellular and molecular processes related to leiomyoma pathology and may be a potential therapeutic candidate for uterine leiomyomas.

Availability of Data and Materials

The data underlying this study are available from the corresponding author upon reasonable request.

Author Contributions

HL and HJ contributed to the study conception, design, data acquisition, analysis, and manuscript preparation. HL was responsible for funding acquisition. HL and HJ were involved in drafting the manuscript and revising it critically for important intellectual content. Both authors read and approved the final manuscript. Both authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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