Bortezomib overcomes TRAIL resistance in Burkitt's lymphoma by enhancing apoptosis via reactive oxygen species-mediated DR5 upregulation and MAPK pathway activation

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Abstract. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) selectively kills tumor cells and exerts minimal toxic effects on normal cells. However, resistance to TRAIL-induced apoptosis is a major obstacle in the clinical application of TRAIL. Bortezomib can enhance the tumor-killing effect of TRAIL in certain tumors, but understanding in Burkitt's lymphoma (BL), especially in TRAIL-resistant BL cells, is limited. Therefore, the present study aimed to assess the synergistic effect of bortezomib and TRAIL in BL. Using the Raji cell line, the least susceptible to TRAIL among BL cell lines, the present study assessed the effect and mechanism by which bortezomib reverses resistance to TRAIL. Cell proliferation inhibition was assessed using the Cell Counting Kit-8 assay. Apoptosis was assessed by flow cytometry. Intracellular reactive oxygen species (ROS) were detected with DCFH-DA, and mitochondrial membrane potential was measured using JC-1. Expression levels of apoptosis proteins and MAPK signaling pathway-related proteins were analyzed by western blot. Combination of bortezomib and TRAIL strongly and synergistically inhibited Raji and CA46 BL cell proliferation. Furthermore, the combination of bortezomib and TRAIL was associated with the following: Induction of apoptosis; increased levels of ROS; presence of mitochondrial membrane potential disorders; upregulation of the levels of apoptosis-related proteins cleaved caspase 8/9/3 and cleaved poly (ADP-ribose) polymerase; and downregulation of the levels of antiapoptotic factors Bcl-2 and Bcl-xl. In addition, bortezomib induced a significant increase in the expression levels of death receptor 5 (DR5), a TRAIL receptor. Pretreatment with the antioxidant N-acetylcysteine inhibited not only ROS upregulation but also DR5 upregulation induced by bortezomib in Raji cells. Furthermore, the present study revealed that the combination of bortezomib and TRAIL could regulate the levels of MAPK signaling pathway-related proteins, such as phosphorylated extracellular signal-regulated kinase 1/2, phosphorylated (p-)p38, p-c-Jun, p-activating transcription factor 2 and phosphorylated stress-activated protein kinase/c-Jun N-terminal kinase. Therefore, the results indicate that bortezomib may enhance Raji cell sensitivity to TRAIL via ROS-dependent upregulation of DR5, induce apoptosis through the MAPK signaling pathway, and subsequently inhibit cell proliferation. Additionally, bortezomib combined with TRAIL had a potential synergistic apoptosis-inducing effect in TRAIL-resistant BL cells.

Introduction

Burkitt's lymphoma (BL) is a rapidly developing, aggressive B-cell non-Hodgkin lymphoma that is more prevalent in pediatric patients than in adults (1). It is estimated that there were 553,389 new cases and 250,679 deaths worldwide in 2022 (2). The 5-year net survival rate was >80% in high-income countries, and <50% in certain regions of Central and South America (3). The current mainstay of treatment for BL is multidrug chemotherapy, which employs doxorubicin alkylating agents, vincristine and etoposide. However, prolonged high-dose and high-intensity chemotherapy is less tolerated by patients, frequently leading to clinical toxicity and treatment-related complications. Concurrently, these regimens impede bone marrow function, suppress the immune response and induce clinical infections during treatment (4-6). Therefore, it is necessary to develop a novel type of treatment that is less immunosuppressive and more tolerable.

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Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL; also referred to as Apo2L) is a member of the TNF superfamily and a promising antitumor medication due to its unique ability to destroy cancer cells whilst maintaining normal cells (7). TRAIL induces apoptosis by binding to the receptors TRAIL-R1 [(death receptor 4 (DR4)] and TRAIL-R2 [(death receptor 5 (DR5)], leading to the formation of the death-inducing signaling complex and subsequent activation of caspase 8. As the apical caspase in this extrinsic pathway, activated caspase-8 directly cleaves and activates the effector caspase 3/7, which serve as the ultimate executioners of the apoptotic program and can be activated by both extrinsic and intrinsic pathways (8).

Although TRAIL has the capacity to induce apoptosis in certain tumor cells, certain malignant lymphomas are resistant to TRAIL-induced apoptosis (9). Clinical combination therapies to increase the sensitivity of cancer cells to TRAIL include chemotherapy drugs, radiation therapy or immunotherapy, as well as several signal transduction modulators and small molecule inhibitors, providing the optimal combination for the use of TRAIL in cancer treatment (10-14). The use of proteasome inhibitors has also been reported to be a promising strategy to enhance the sensitivity of cancer cells to TRAIL (15).

Bortezomib (PS-341; Velcade) is the first selective proteasome inhibitor approved by the US Food and Drug Administration for the treatment of multiple myeloma and relapsed mantle cell lymphoma (16). Bortezomib has been reported to synergistically enhance TRAIL-induced apoptosis in multiple drug-resistant cancer cells, such as SNU-216 gastric cancer cells (17), B16F10 melanoma cells and CT26 colon carcinoma cells (18). The induction of apoptosis by bortezomib in primary chronic lymphocytic leukemia cells and the BJAB BL cell line has been reported to be associated with the upregulation of TRAIL and its death receptors DR4 and DR5 (19). However, to the best of our knowledge, the synergistic antitumor effects of bortezomib and TRAIL in drug-resistant BL cells, and the underlying mechanism, have not yet been elucidated. Our previous study reported that several BL cell lines, including Raji and CA46 cells, were insensitive to TRAIL, with Raji cells exhibiting the lowest sensitivity to TRAIL (20). Therefore, in the present study, Raji cells were selected to explore the synergistic inhibitory effect of bortezomib and TRAIL, and the underlying mechanism, in order to provide a novel therapeutic strategy for TRAIL-insensitive BL cells.

Materials and methods

Materials. TRAIL (cat. no. HY-P77256, purity, ≥95%) and bortezomib (cat. no. HY-10227, purity, ≥99%) were purchased from MedChemExpress. RPMI 1640 medium and FBS were purchased from Gibco (Thermo Fisher Scientific, Inc.). A Cell Counting Kit-8 (CCK-8) was purchased from Biosharp Life Sciences. ProteinSafe™ phosphatase inhibitor cocktail and protease inhibitor cocktail (EDTA-free) were purchased from TransGen Biotech Co., Ltd. An Annexin V-FITC/PI apoptosis detection kit, a mitochondrial membrane potential (MMP) assay kit with JC-1, a reactive oxygen species (ROS) kit, N-acetylcysteine (NAC) and RIPA lysis buffer were purchased from Beyotime Biotechnology. BCA and supersensitive ECL kits were purchased from Oriscience Biotechnology Co., Ltd. PVDF membranes were purchased from Merck KGaA. The phycoerythrin (PE)-CD262 (DR5) monoclonal antibodies (cat. no. 12-9908-42) and PE-CD261 (DR4) monoclonal antibodies (cat. no. 12-6644-42) were purchased from eBioscience (Thermo Fisher Scientific, Inc.). Antibodies against PARP (cat. no. 9532), cleaved PARP (cat. no. 5625), caspase 8 (cat. no. 4790), cleaved caspase 8 (cat. no. 9496), caspase 9 (cat. no. 9504), cleaved caspase 9 (cat. no. 7237), caspase 3 (cat. no. 9662), cleaved caspase 3 (cat. no. 9664), Bcl-xl (cat. no. 2764), Bcl-2 (cat. no. 3498), phosphorylated (p-) p38 (cat. no. 4511), p- stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK; cat. no. 4668), p-c-Jun (cat. no. 3270), p-activating transcription factor 2 (ATF2; cat. no. 27934), p-extracellular signal-regulated kinase (ERK)1/2 (cat. no. 4695), C/EBP homologous protein (CHOP; cat. no. 2895), GAPDH (cat. no. 5174) and HRP-conjugated goat anti-rabbit antibodies (cat. no. 7074) were purchased from Cell Signaling Technology Inc. with GAPDH as the internal reference protein. GAPDH antibodies were diluted at a ratio of 1:10,000, whilst all other antibodies were diluted at a ratio of 1:1,000. All other chemicals used were of analytical grade.

Cell culture. Raji (cat. no. CCL-86; American Type Culture Collection) and CA46 (cat. no. CRL-1648; American Type Culture Collection) BL cell lines were cryopreserved in a liquid nitrogen tank in the laboratory. Cells were cultured in RPMI 1640 medium supplemented with 10% FBS under humidified conditions with 5% CO_2 at 37˚C, according to the culture conditions recommended by American Type Culture Collection. Cells in the logarithmic growth phase were used in subsequent experiments.

Cell proliferation assay. Cell viability was determined using a CCK-8 assay. The TRAIL stock solution was prepared by dissolving the powder via sonication according to the manufacturer's protocol, which suggests a stock solution of ≥100 μg/ml in ddH_2O. Additionally, there should be no crystals when determining the maximum working concentration. Viable Raji and CA46 cells were seeded in a 96-well plate, incubated with $1x10^5$, $5x10^4$, $2.5x10^4$, $1.25x10^4$, $6.25x10^3$, $3.125x10^3$ or 0 ng/ml TRAIL, and incubated with 200, 100, 50, 25, 12.5, 6.25 or 0 nM bortezomib and/or 100 ng/ml TRAIL, followed by the addition of CCK-8 to each well and incubation for another 2 h at 37˚C with 5% CO_2. The absorbance at 450 nm was measured using a Powerwave XS multiplate reader (BioTek; Agilent Technologies, Inc.). Data acquisition and half-maximal inhibitory concentration analysis were performed using GraphPad Prism 9.0 software (Dotmatics). The following formula was used to determine the cell inhibition rate: Cell inhibition rate (%)=[1-optical density (OD) mean value of the experimental group/OD mean value of the control group] x100%.

Apoptosis analysis. Viable Raji cells treated with 100, 50, 25 or 0 nM bortezomib and/or 100 ng/ml TRAIL for 24 h at 37˚C. A total of $2x10^5$ cells/well were harvested and washed with ice-cold PBS. The cells were stained with 200 μl staining buffer containing 10 μl annexin V-FITC and 5 μl PI, followed by incubation at 25˚C for 20 min, after which the proportion of

apoptotic cells was detected and analyzed using flow cytometry (Quanteon; ACEA Biosciences, Inc.). NovoExpress software (version 1.6.1, Agilent Technologies) was used for data analysis. Morphological changes in Raji cells were observed under an inverted microscope (Olympus Corporation) after treatment with bortezomib and TRAIL.

MMP assay. Changes in the MMP were detected by staining cells with the fluorescent probe JC-1. Raji cells treated with 100, 50, 25 or 0 nM bortezomib and/or 100 ng/ml TRAIL for 24 h at 37˚C with $2x10^5$ cells/well were harvested, washed with ice-cold PBS and stained with 5 mg/ml JC-1 at 37˚C for 30 min in the dark. Data acquisition and analysis of the MMP were performed using flow cytometry. The analyte reporter was the fluorescent probe JC-1, which exhibits a shift from red fluorescence (J-aggregates, ~590 nm emission) to green fluorescence (monomers, ~527 nm emission) upon mitochondrial membrane depolarization. Data acquisition was performed using a NovoCyte Quanteon flow cytometer (ACEA Biosciences, Inc.), and data analysis was conducted with NovoExpress software (version 1.6.1, Agilent Technologies).

Intracellular ROS assay. NAC, a ROS scavenger and antioxidant, was used to assess intracellular ROS generation (21). Raji cells were divided into two groups: i) Pretreatment with NAC for 1 h at 37˚C; and ii) untreated (control) group. Subsequently, both groups were exposed to varying concentrations of bortezomib (0, 25, 50 or 100 nM) and/or TRAIL (100 ng/ml) for 24 h at 37˚C, after which the cells were harvested, washed with PBS, mixed with 10 μM 2'-7'-dichlorodihydrofluorescein diacetate and incubated in the dark at 37˚C for 30 min. Data acquisition and analysis of ROS were performed using flow cytometry. The analyte reporter was the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate. Data acquisition was performed using a NovoCyte Quanteon flow cytometer (ACEA Biosciences, Inc.), and data analysis was conducted with NovoExpress software (version 1.6.1, Agilent Technologies).

Analysis of DR5 expression on the cell surface. To assess whether bortezomib enhances TRAIL sensitivity via the ROS-dependent regulation of DR5 expression, Raji cells were divided into two groups: i) Pretreatment with NAC (10 mM) for 1 h at 37˚C; and ii) untreated group. Both groups were then exposed to increasing concentrations of bortezomib (0, 25, 50 or 100 nM) for 24 h at 37˚C, and then stained with PE-CD262 (DR5) monoclonal antibodies at 4˚C for 30 min in the dark. Data acquisition and analysis of DR5 expression in Raji cells were performed using flow cytometry. The analyte reporter was a PE-CD262 (DR5) monoclonal antibody (cat. no. 12-9908-42) was directly conjugated to PE. Data acquisition was performed using a NovoCyte Quanteon flow cytometer (ACEA Biosciences, Inc.), and analysis was conducted with NovoExpress software (version 1.6.1, Agilent Technologies).

Western blot analysis. Raji cells treated with 100, 50, 25 or 0 nM bortezomib and/or 100 ng/ml TRAIL for 24 h at 37˚C with $5x10^6$ cells were lysed with RIPA buffer containing protease inhibitors or phosphatase inhibitors, then lysed on ice for 30 min, mixed at 5-min intervals and centrifuged at 13,000 x g/min for 15 min at 4˚C. The protein supernatants were collected, and the protein concentration was determined using BCA kit. Proteins (50 μg) were separated using 12% SDS-PAGE. The proteins were subsequently transferred to PVDF membranes. The membranes were blocked with 5% BSA for 1 h at 37˚C, and incubated with primary antibodies overnight at 4˚C, and then with secondary antibodies conjugated to HRP for another 2 h at 37˚C. Subsequently, the PVDF membranes were developed with ECL emitting solution and images were captured using a gel imaging system (Bio-Rad Laboratories, Inc.). The integrated optical density (IOD) values of the protein blots were analyzed using ImageJ 1.54 analysis software (National Institutes of Health). GAPDH served as a loading control to normalize for equal protein loading across samples, and the IOD of the target protein/IOD of GAPDH ratio reflected the relative expression level of the target protein.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 10.0 software (Dotmatics). Each group of experiments was independently repeated three times, and the experimental results are presented as the mean ± SD. The differences between two groups were compared using t-tests with Welch's correction, whilst the differences between multiple groups were compared using one-way ANOVA and Dunnett's post hoc test. Although all multiple comparisons were formally assessed, only selected pairwise results are displayed in the figures for clarity. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Bortezomib enhances TRAIL sensitivity and synergistically inhibits proliferation in BL cells. According to established criteria, cell lines exhibiting <50% tumor growth inhibition following treatment with 100 ng/ml TRAIL are classified as TRAIL-insensitive (22). Cell viability assays revealed that the BL cell lines (Raji and CA46) were insensitive to TRAIL. When applied at a high concentration ($1x10^5$ ng/ml), TRAIL achieved a proliferation inhibition rate of ~50% in CA46 cells, whilst demonstrating notably less efficacy in Raji cells (<50% inhibition; Fig. 1A). However, when cells were treated with different concentrations of bortezomib combined with 100 ng/ml TRAIL for 24 h, the inhibition of Raji (Fig. 1B) and CA46 (Fig. 1C) cell proliferation noticeably improved.

According to the Chou-Talalay combination index (CI) method (23), synergistic, additive and antagonistic effects between drugs are indicated when CI<1, CI=1 and CI>1, respectively, whereas CI<0.9 indicates strong synergistic effects. Bortezomib and TRAIL demonstrated strong synergistic effects on Raji and CA46 cells (Table I). These results suggest that bortezomib enhanced TRAIL sensitivity and synergistically inhibited proliferation in BL cells. Consequently, the Raji cell line, the least TRAIL-sensitive strain, was selected as the model for subsequent experiments.

Combination of bortezomib and TRAIL could induce morphological changes in Raji cells. Morphological analysis of Raji cells treated for 24 h with 50 nM bortezomib and/or 100 ng/ml TRAIL was performed using inverted microscopy at a magnification of x10. Compared with no treatment, treatment with

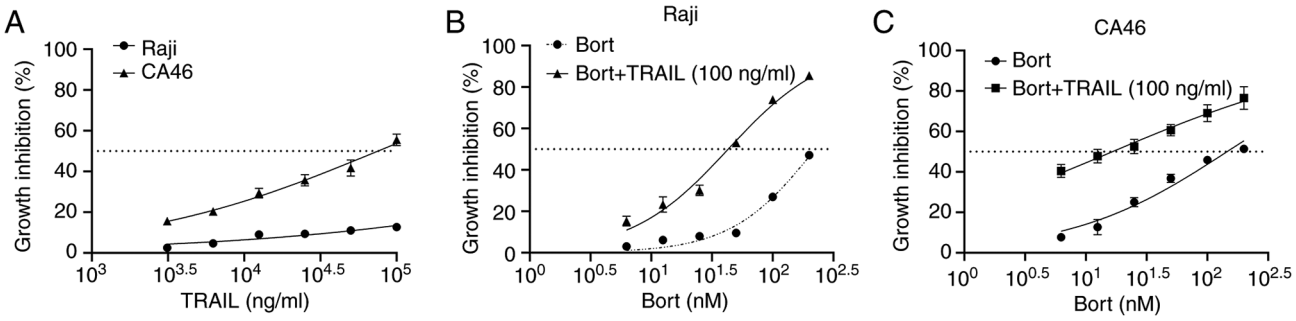

Figure 1. Bortezomib combined with TRAIL synergistically inhibits the proliferation of Burkitt's lymphoma cells. (A) Raji and CA46 cells were incubated with different concentrations of TRAIL for 24 h, and cell proliferation was assessed using a CCK-8 assay. (B) Raji and (C) CA46 cells were incubated with different concentrations of bortezomib alone or in combination with 100 ng/ml TRAIL for 24 h, and cell proliferation was evaluated using a CCK-8 assay. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; CCK-8, Cell Counting Kit-8; Bort, bortezomib.

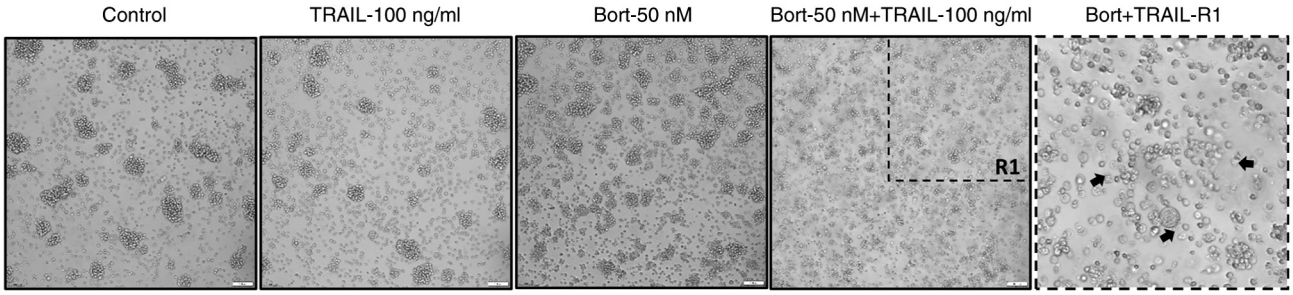

Figure 2. Morphological characterization of Raji cells after combination treatment with bortezomib and TRAIL. The combination of TRAIL and bortezomib was compared with TRAIL or bortezomib alone (indicated by arrows; magnification, x10). R1 indicates a high magnification image (x40) showing apoptotic features, including cell shrinkage, membrane rupture and apoptotic body formation. Scale bars, 20 and 5 μm (R1). TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; Bort, bortezomib.

100 ng/ml TRAIL alone did not induce notable morphological changes. By contrast, 50 nM bortezomib alone triggered characteristic apoptotic morphology, including cell shrinkage. The combination of bortezomib and TRAIL produced synergistic effects, as demonstrated by markedly reduced cell density and widespread cell death. At a higher magnification of x40, the R1 field displayed classical apoptotic hallmarks, including marked cell shrinkage, nuclear condensation, membrane blebbing and apoptotic body formation, confirming enhanced cytotoxicity (Fig. 2).

Bortezomib combined with TRAIL induces the apoptosis of Raji cells. To further determine the pathway by which bortezomib combined with TRAIL induced Raji cell death, annexin V-FITC/PI double staining was used to detect the apoptosis of Raji cells. Compared with that of cells treated with bortezomib alone, the apoptosis rate of Raji cells increased significantly in a dose-dependent manner when this was combined with 100 ng/ml TRAIL. Specifically, the apoptosis rate rose from 12.63±0.17% for 100 nM bortezomib alone to 80.82±0.20% for the combination treatment with TRAIL and bortezomib (Fig. 3A). Moreover, the expression levels of apoptosis-related proteins were detected using western blot, and the results revealed that compared with the bortezomib group the expression levels of PARP were significantly decreased when TRAIL was combined with bortezomib, and the levels of cleaved PARP and cleaved caspase 8/9/3 were significantly increased ($P<0.05$; Fig. 3B).

Bortezomib combined with TRAIL induces the apoptosis of Raji cells via the mitochondrial apoptosis pathway. The mitochondrial pathway is an important apoptotic pathway. To further evaluate whether the mitochondrial apoptotic pathway is involved in the increased sensitivity of Raji cells to TRAIL caused by bortezomib, changes of the MMP were determined using JC-1 staining. Compared with that of the TRAIL alone group, the MMP changed significantly from 7.71±0.15 to 85.64±0.18% when TRAIL was combined with 100 nM bortezomib (Fig. 4A). Furthermore, the western blot analysis results revealed that compared with the bortezomib group the expression levels of the antiapoptotic proteins Bcl-2 and Bcl-xl were significantly decreased when TRAIL was combined with bortezomib, ($P<0.05$; Fig. 4B). These results suggest that bortezomib combined with TRAIL induces apoptosis via the mitochondrial apoptotic pathway.

Bortezomib combined with TRAIL induces ROS production. ROS are considered to be potential modulators of apoptosis (24). The combination of TRAIL and bortezomib markedly increased ROS levels in Raji cells from 14.45±1.11% (100 nM bortezomib) to 36.56±0.77% (TRAIL combined with 100 nM bortezomib; Fig. 5A). Moreover, NAC is a widely used thiol-containing antioxidant that clears ROS from cells by interacting with OH and H_2O_2, thereby affecting ROS-mediated signaling pathways (25). The flow cytometry results revealed that NAC pretreatment significantly blocked the ROS production caused by bortezomib + TRAIL

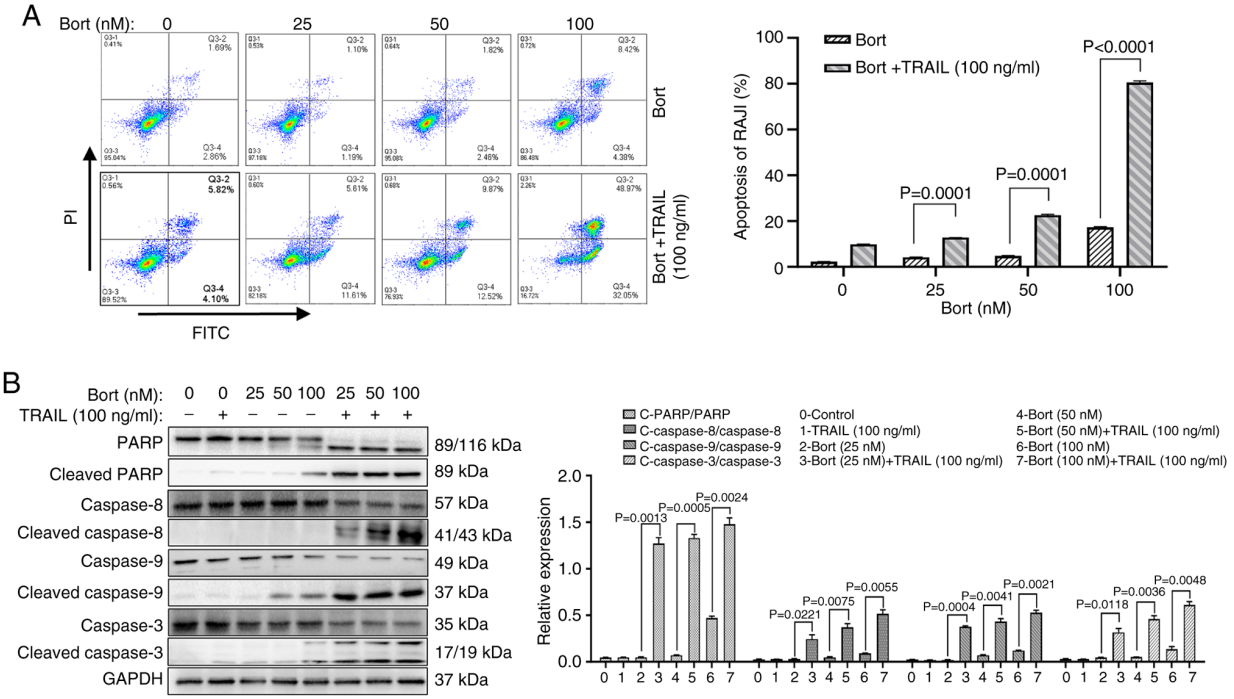

Figure 3. Bortezomib combined with TRAIL induces apoptosis in Raji cells. (A) Apoptotic effects of bortezomib and/or TRAIL in Raji cells were determined using flow cytometry via dual staining with annexin V-FITC and PI. (B) Expression levels of apoptosis-related proteins were assessed using western blot. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; Bort, bortezomib.

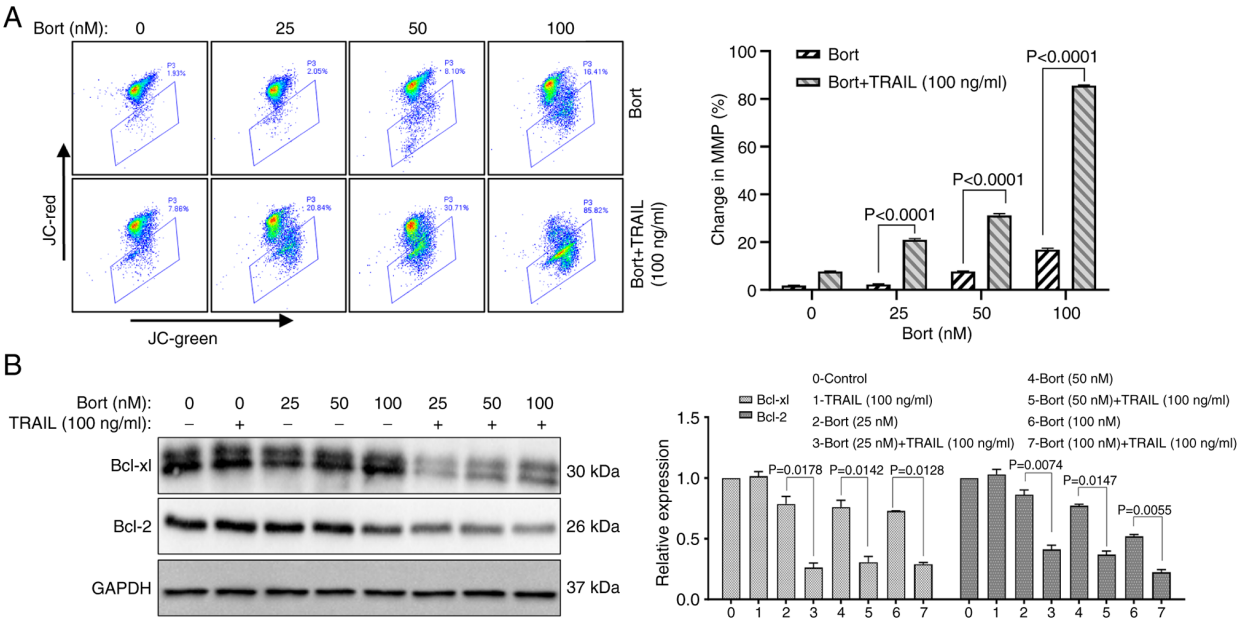

Figure 4. Bortezomib combined with TRAIL induces apoptosis via the mitochondrial pathway in Raji cells. (A) Changes of MMP in Raji cells were detected using flow cytometry. (B) Expression levels of mitochondrial pathway-related proteins were detected using western blot analysis. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; Bort, bortezomib; MMP, mitochondrial membrane potential.

compared with that in the group without NAC pretreatment (P<0.05; Fig. 5B).

Bortezomib-induced DR5 upregulation depends on ROS generation. DR5 is a receptor of TRAIL and serves a crucial role in TRAIL-induced apoptosis (26). Using staining with PE-CD262 (DR5) monoclonal antibodies, the present study revealed that compared with control, bortezomib significantly increased DR5 expression when in the presence of bortezomib at 100 nM in Raji cells, from 1.60±0.50% (control group) to 47.33±0.19% (in the presence of bortezomib at 100 nM). In addition, NAC pretreatment significantly blocked the DR5 upregulation induced by bortezomib compared with that in the group without NAC pretreatment (P<0.05; Fig. 6A). Furthermore, ROS generation is associated with endoplasmic reticulum stress (ERS) and CHOP, and the CHOP motif

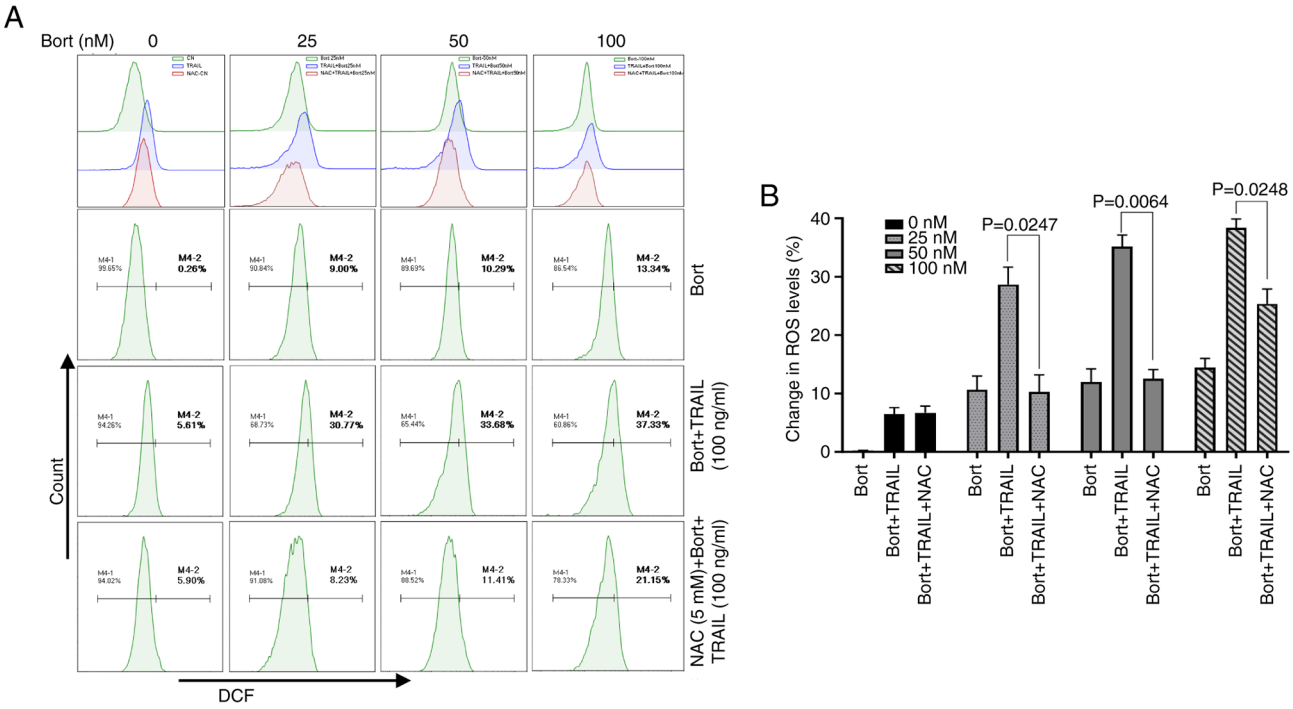

Figure 5. Bortezomib combined with TRAIL induces ROS production in Raji cells. (A) Intracellular ROS levels were assessed using the fluorescence dye DCFH-DA and flow cytometry analysis. (B) Changes in ROS were evaluated using histogram analysis. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; Bort, bortezomib; ROS, reactive oxygen species; NAC, N-acetylcysteine.

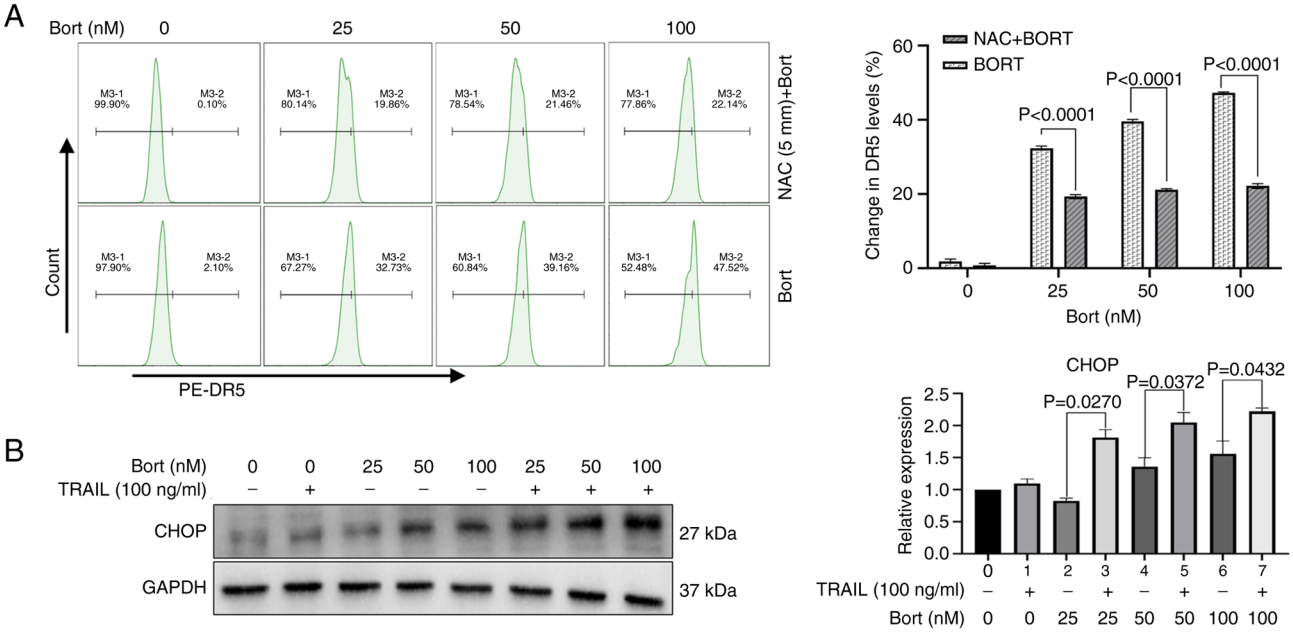

Figure 6. Effect of bortezomib combined with TRAIL on DR5 via ROS in Raji cells. (A) Expression of DR5 on the cell surface was detected using flow cytometry analysis with PE-CD262 (DR5) monoclonal antibodies. (B) Western blot was used to detect CHOP protein expression levels. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; Bort, bortezomib; DR5, death receptor 5; ROS, reactive oxygen species; CHOP, C/EBP homologous protein; NAC, N-acetylcysteine.

has been identified at the proximal region of the DR5 gene promoter (27). Western blot results identified that compared with that of the bortezomib group the expression levels of CHOP were significantly increased when TRAIL was combined with bortezomib (Fig. 6B). Based on these results, it was hypothesize that bortezomib sensitizes Raji cells to TRAIL by activating the ROS-CHOP-DR5 signaling axis, thereby promoting their synergistic effect in inducing apoptosis and inhibiting cell proliferation.

Bortezomib increases the sensitivity of Raji cells to TRAIL through the MAPK signaling pathway. The MAPK signaling

Table I. Combination index value of bortezomib and tumor necrosis factor-related apoptosis-inducing ligand combination therapy.

A, Raji cells

Bortezomib, nM	TRAIL, ng/ml	Effect	CI[a]
200.00	100.0	0.85495	0.04971
100.00	100.0	0.73910	0.06212
50.00	100.0	0.53075	0.09787
25.00	100.0	0.30000	0.16464
12.50	100.0	0.23000	0.12932
6.25	100.0	0.15000	0.12527

B, CA46 cells

Bortezomib, nM	TRAIL, ng/ml	Effect	CI[a]
200.00	100.0	0.76543	0.24680
100.00	100.0	0.69000	0.22003
50.00	100.0	0.60597	0.19259
25.00	100.0	0.52500	0.15917
12.50	100.0	0.47773	0.10674
6.25	100.0	0.40460	0.08510

[a]CI>1.0, antagonism; CI <1.0, strong synergism; CI=1.0, additive effect. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; CI, combination index.

pathway can be activated by several stimuli, regulating physiological processes such as cell proliferation and death. It also serves an important role in the process of apoptosis (28). To assess the effect of bortezomib combined with TRAIL on the MAPK signaling pathway in Raji cells, western blot was used to detect the expression levels of MAPK signaling pathway-related proteins (Fig. 7A). Compared with that of the bortezomib group the levels of p-ERK1/2 in the MAPK/ERK signaling pathway were significantly decreased, and the levels of p-SAPK/JNK, p-c-Jun, p-ATF2 and p-p38 in the MAPK/p38 and MAPK/JNK signaling pathways were significantly increased when TRAIL was combined with bortezomib ($P<0.05$; Fig. 7B). These results suggest that bortezomib may increase sensitivity and regulate the apoptosis of Raji cells by activating the MAPK signaling pathway.

Discussion

TRAIL is a member of the TNF family that promotes apoptosis, and can selectively kill tumor cells through specific cell surface receptors, making it a promising antitumor drug. However, in malignant lymphoma, the development of TRAIL resistance is a major bottleneck limiting therapeutic effectiveness (9). Bortezomib, a proteasome inhibitor, exhibits great therapeutic potential for sensitizing tumor cells to TRAIL (16-18). Moreover, it has been reported that A549 cells with acquired bortezomib resistance exhibit phenotypic reversal of sensitivity to TRAIL (29).

Previous research has reported that the combination of TRAIL and bortezomib was able to induce cell death in TRAIL-resistant cancer cells via the intracellular TRAIL pathway (17,18). Our previous study demonstrated that BL cells have different sensitivities to TRAIL, and that the Raji cell line is the strain with the most pronounced resistance to TRAIL (20). Furthermore, the present study revealed a potential synergistic effect of bortezomib combined with TRAIL in promoting TRAIL-resistant BL cell apoptosis, and explored its molecular mechanisms. The findings demonstrated that bortezomib markedly increased the drug sensitivity of Raji and CA46 cells to TRAIL. Moreover, when bortezomib was combined with 100 ng/ml TRAIL, it significantly inhibited the proliferation of BL cells. Bortezomib also enhanced TRAIL-induced apoptosis in BL cells in a concentration-dependent manner.

The TRAIL pathway consists of two caspase-level pathways. After the initial activation of caspase 8 by TRAIL, the signals differentiate in two directions: i) Direct activation of caspase 3, without the involvement of mitochondria; and ii) apoptotic bodies (mitochondrial proteins, dATP and Apaf-1) are formed, resulting in the activation of caspase 9, followed by activation of caspase 3 (30). After bortezomib was combined with TRAIL in Raji cells, the levels of cleaved caspase 8/9/3 were increased, and the changes of the MMP, an important factor of mitochondrial dysfunction, significantly decreased. It has been reported that downregulation of Bcl-2 expression enhances the sensitivity of tumor cells to TRAIL, and decreased intracellular Bcl-xl levels are responsible for the acquisition of TRAIL resistance in tumor cells (31,32). The western blot results in the present study revealed that the expression levels of the antiapoptotic proteins Bcl-2 and Bcl-xl, key members of the Bcl-2 family that target the mitochondrial apoptotic pathway, were significantly decreased in Raji cells (33). These findings suggest that the mitochondrial apoptotic pathway serves a role in bortezomib sensitization of Raji cells to TRAIL-induced apoptosis.

Mitochondria are the primary sites of oxygen utilization in eukaryotic cells. Under stress (intracellular ROS accumulation), mitochondrial outer membrane permeability increases and the MMP decreases, leading to the release of proapoptotic proteins into the cytoplasm, the activation of caspases and ultimately apoptosis (34,35). Oxidative stress serves a crucial role as a mediator of cell death. Studies have reported that cancer chemopreventive drugs promote ROS generation, which upregulates DR4/DR5 and potentiates TRAIL pathway, ultimately increasing cancer cell sensitivity to TRAIL (36,37). Furthermore, the death receptor DR5 has an important function in the transduction of TRAIL activity and can activate signaling pathways associated with cell death in cancer cells (38). However, the reduced or lost expression of DR5 in cancer cells leads to TRAIL resistance (39). The present study demonstrated that bortezomib combined with TRAIL could significantly increase intracellular ROS levels and upregulate DR5 expression, whilst DR4 expression revealed no significant change (Fig S1). Furthermore, scavenging ROS with the antioxidant NAC abolished bortezomib-induced DR5 expression, suggesting that ROS serve a major role in the regulation of the TRAIL receptor DR5. In addition, the present study revealed that bortezomib combined with TRAIL enhanced the expression of the ERS-related marker protein CHOP. ROS, key

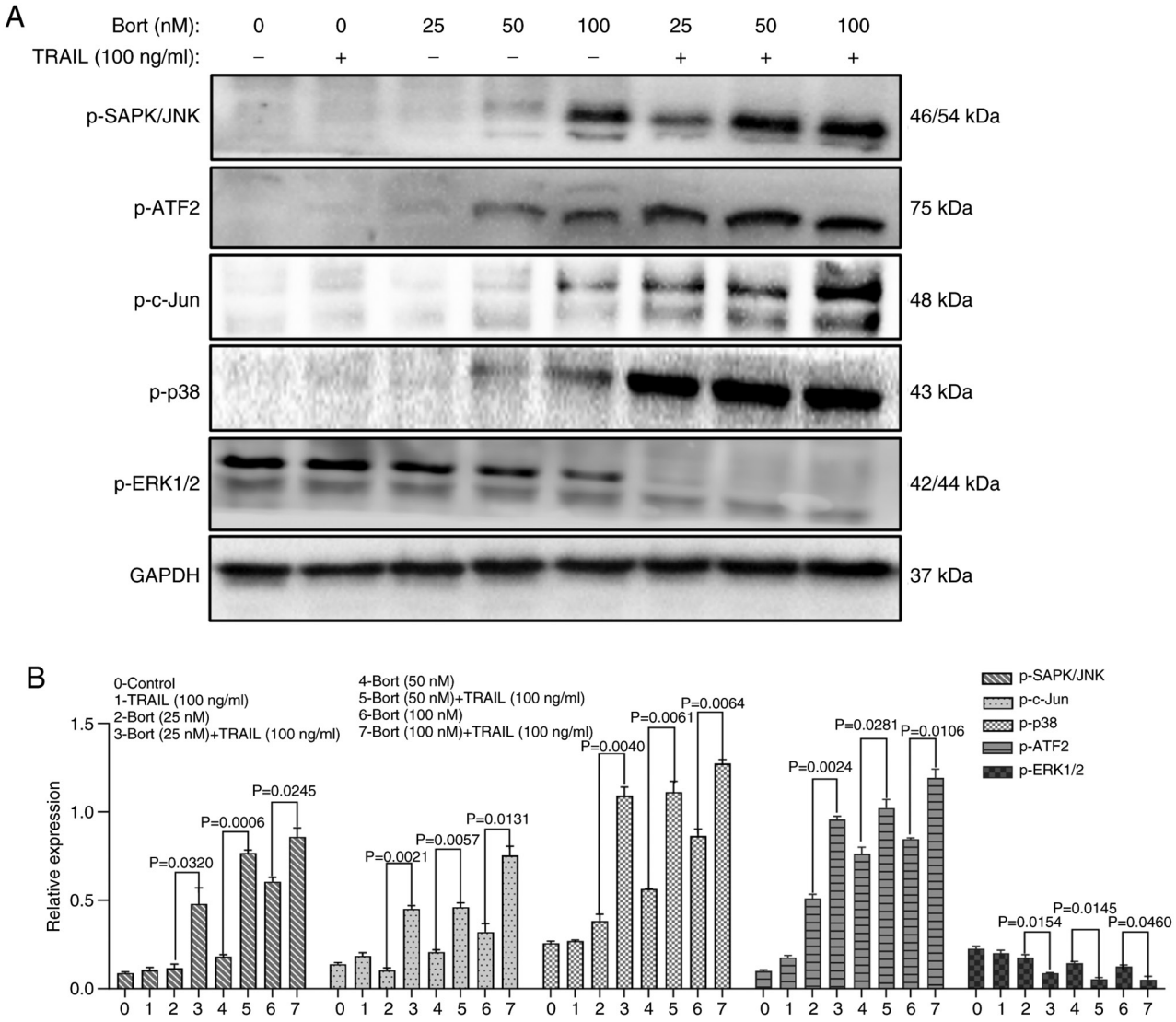

Figure 7. Effect of bortezomib combined with TRAIL on the MAPK signaling pathway in Raji cells. (A) MAPK signaling pathway relative protein expression in Raji cells treated with bortezomib and/or TRAIL was detected using western blot analysis. (B) Histogram analysis of protein expression. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; Bort, bortezomib; SAPK/JNK, stress-activated protein kinase/c-Jun N-terminal kinase; ATF2, activating transcription factor 2; ERK, extracellular signal-regulated kinase; p-, phosphorylated.

regulators of endoplasmic reticulum function and unfolded protein response activation, can induce apoptosis by inducing ERS and activating the downstream effector CHOP (40,41). It has been reported that CHOP can directly regulate the expression of DR5 through the CHOP binding site on the 5' side of the DR5 promoter (27). Therefore, we hypothesize that the ROS-mediated CHOP-dependent DR5 pathway may be involved in bortezomib-induced sensitization to TRAIL.

Additionally, it has been reported that ROS act as upstream signaling messengers that trigger the MAPK cascade (42), and that the MAPK signaling pathway is involved in the regulation of cell proliferation, differentiation, apoptosis, the inflammatory response and vascular development in the human body. ERK, JNK and p38 kinase belong to the most common mammalian MAPK subclass (43). Studies have reported that dysregulation of the MAPK system alters normal physiological processes and is frequently involved in tumorigenesis, development and drug resistance, and thus, the MAPK system is considered to be a viable target for cancer therapy (44,45). It is well established that the activation of MAPK family members (such as ERK, JNK and p38) requires dual phosphorylation, a process in which both threonine and tyrosine residues within the activation loop are phosphorylated by upstream kinases (MEK or MKK), resulting in full kinase activity (46). Accordingly, the phosphorylated forms of these kinases (such as p-ERK, p-JNK and p-p38) serve as reliable indicators of pathway activation. In the present study, following combination treatment with bortezomib and TRAIL, western blot was used to assess the expression levels of phosphorylated (activated) forms of key proteins within the MAPK signaling pathway. However, the total protein expression levels were not simultaneously detected due to insufficient consideration in the experimental design. The results revealed that the levels of p-ERK1/2 in the MAPK/ERK signaling pathway were significantly decreased., and the levels of p-p38 in the MAPK/p38 signaling pathway, and the levels of p-SAPK/JNK, p-c-Jun and p-ATF2 in the MAPK/JNK signaling pathway, were significantly increased. These findings suggest that bortezomib

enhanced the sensitivity of Raji cells to TRAIL by activating the MAPK signaling pathway and synergistically inducing apoptosis.

In summary, the mechanism by which bortezomib increased the sensitivity of Raji cells to TRAIL and synergistically induced apoptosis is hypothesized to be as follows: In Raji cells, bortezomib combined with TRAIL can increase the level of oxidative stress, increase the level of ROS, decrease the MMP, inhibit the expression of the antiapoptotic factor Bcl-2 family, activate the caspase cascades of caspase 8/9, caspase 3 and PARP, and lead to the apoptosis of Raji cells through the mitochondrial pathway. Furthermore, the increase in intracellular ROS may trigger ERS, and then induce DR5 upregulation through the transcription factor CHOP, thus increasing the sensitivity of Raji cells to TRAIL. Finally, the increase in the sensitivity of Raji cells to bortezomib may regulate the apoptosis of Raji cells by activating the MAPK signaling pathway.

However, there are limitations of the present study: Among TRAIL-resistant BL, the present study focused on two representative cell lines, CA46 and Raji, which exhibited differential sensitivity profiles. Furthermore, whilst the present study identified the mechanism through which bortezomib sensitized Raji cells, such as DR5 upregulation and caspase-8 activation, the generalizability of this mechanism across other TRAIL-resistant BL cell lines warrants further investigation. Future studies should systematically evaluate this sensitization pathway *in vivo* using xenograft mouse models to validate the therapeutic efficacy of the bortezomib-TRAIL combination for BL treatment.

In conclusion, bortezomib appeared to increase the sensitivity of Raji BL cells to TRAIL through ROS-dependent upregulation of DR5, and to induce apoptosis via the MAPK signaling pathway and the mitochondrial apoptosis pathway with synergistic effects. This combined approach may provide a more effective and less harmful option for the treatment of TRAIL-resistant BL.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

JC, WZ, PY and ML designed the research study. JC, WZ, YY, YZ, SP and XB performed the experiments. JC, WZ, YY, ML and PY analyzed the data. JC and YY confirm the authenticity of all the raw data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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