BRD4 inhibitor nitroxoline enhances the sensitivity of multiple myeloma cells to bortezomib in vitro and in vivo by promoting mitochondrial pathway-mediated cell apoptosis

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Abstract
Background: Multiple myeloma (MM) is the second most common hematological neoplasm. Wide administration of bortezomib significantly improves the survival of MM patients compared with conventional chemotherapy. Bromodomain-containing protein 4 (BRD4) inhibitors also have been demonstrated to retard cell proliferation and induce cellular apoptosis in various cancers. However, it is unclear whether the BRD4 inhibitor nitroxoline plus bortezomib has a synergistic anti-tumor effect on MM.

Methods: Cell viability was determined via 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell cycle and cell apoptosis were assessed via flow cytometry. Protein expression levels were determined via western blotting. The expression of apoptosis-related proteins in xenograft tissue were detected by means of immunohistochemistry.

Results: Treatment with nitroxoline or bortezomib suppressed cell proliferation, and caused G0/G1 phase arrest and apoptosis in H929 and RPMI8226 cells. Furthermore, nitroxoline intensified the retardation of cell proliferation, as well as further enhanced the G0/G1 phase arrest and apoptosis induced by bortezomib in H929 and RPMI8226 cells. The western blot analysis revealed that nitroxoline or bortezomib treatment markedly diminished the levels of Bcl-2 and cyclin D1, and increased the levels of p21, Bax, cleaved PARP and cleaved caspase-3. Combination of these two agents was observed to result in further marked changes on these levels compared with nitroxoline or bortezomib treatment alone. What is more, in the xenograft tumor model, combinative treatment markedly inhibited tumor growth compared with the single drug treatment.

Conclusion: Combination of bortezomib with nitroxoline has a synergistic anti-tumor activity in MM cells and may be a novel treatment method for MM.

Keywords: apoptosis, BRD4 inhibitor, G1 phase cell cycle checkpoint, multiple myeloma

Introduction
Multiple myeloma (MM), the second most common hematological neoplasm, is characterized by clonal proliferation of plasma cells within the bone marrow.1-2 During the past decades, proteasomes have been tested as a new effective target in the treatment of MM3 and the first-generation proteasome inhibitor bortezomib significantly improved therapeutic effect compared with previously used treatments.4,5 In addition, novel agents including monoclonal antibodies and histone deacetylase inhibitors have also been used to treat...
MM, resulting in significant extension of patients’ survival. Bortezomib remains the mainstay of MM treatment and bortezomib resistance is unavoidable. Therefore, it is imperative to develop more efficient strategies to augment the sensitivity of bortezomib and reverse drug resistance.

The bromodomain-containing protein 4 (BRD4) is the most studied member of the bromodomain and extraterminal domain (BET) protein family, which can recognize acetylated-histones and activate downstream gene expression via recruiting transcription factors. Previous studies have demonstrated that deregulation of the BRD4 protein took an important part in tumorigenesis, including in the development of prostate, colorectal, pancreatic, lung and breast cancer. And BRD4 inhibitors, such as JQ1, SF1126 and SF2523, have been demonstrated to exert an anti-tumor effect on various types of tumors. Furthermore, Guo et al. demonstrated that the BET inhibitor I-BET151 had a beneficial effect in MM treatment by inhibiting the BRD4-mediated signaling pathway. In addition, Jiang et al. reported that nitroxoline acted as a BRD4 inhibitor. However, the pharmacological effects of nitroxoline in MM remain unclear.

Our study aimed to explore the synergistic effects of nitroxoline and bortezomib on cell proliferation, cell cycle progression and apoptosis in MM. We also investigated the molecular mechanism by which nitroxoline and bortezomib combatted against MM. The combination of nitroxoline with bortezomib may be a novel treatment for MM.

Material and methods

Reagents and antibodies
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Aldrich (Merck KGaA). Nitroxoline was kindly provided by Dr Cheng Luo as a gift, while bortezomib was obtained as a pure substance from Millennium Pharmaceuticals. Antibodies against B-cell lymphoma 2 (Bcl-2) (ab182858), Bcl-2-associated X protein (Bax) (ab32503), cleaved poly (ADP-ribose) polymerase (PARP) (ab74290), cyclin D1 (ab134175), p21 (ab109199) and GAPDH (ab128915) were obtained from Abcam, while the antibody against cleaved caspase-3 (cat. no. 9664), anti-rabbit HRP secondary antibody (cat. no. 7074) was purchased from Cell Signaling Technology, Inc. RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific, Inc. (Gibco).

Cell culture and treatments
The human MM cell lines H929 and RPMI8226 (American Type Culture Collection) were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C with 5% CO2.

RPMI8226 and H929 cells were treated with 0, 0.25, 0.50, 1.00, 2.00, 4.00, 8.00 and 16.00 μM nitroxoline for 24h, and the cell viability was determined via MTT assay. Subsequently, RPMI8226 cells were treated with 0, 1, 2, 4, 8, 16, 32 and 64 nM bortezomib, H929 cells were treated with 0, 0.125, 0.25, 0.5, 1, 2, 4 and 8 nM bortezomib for 24h, and then cell viability was determined via an MTT assay. Dimethyl sulfoxide (DMSO) was used in the control group at the same dilution as the corresponding treatment in the nitroxoline and bortezomib alone groups. Finally, RPMI8226 cells were treated with 0.5 μM nitroxoline and 5.0 nM bortezomib for 24h, while H929 cells were treated with 0.25 μM nitroxoline and 1.00 nM bortezomib for 24h, and then the cell cycle distribution and cell apoptosis were examined via flow cytometry.

MTT cytotoxicity assay
Cell viability was measured using an MTT assay. Briefly, the H929 and RPMI8226 cells were seeded into 96 well plates at a density of 1.5 × 10^4 cells/well for 12h. Next, the cells were treated with different concentrations of nitroxoline and bortezomib for 24h. A final concentration of 0.5 mg/ml MTT was then added to each well and incubated for an additional 4h at 37°C. Cells were adhered to a 96-well plate via centrifugation at 2000 g for 10 min at 25°C. The supernatant was then discarded after centrifugation, and 150 μl/well DMSO (Sigma Aldrich; Merck KGaA) was added to dissolve the solid residue. Finally, the absorbance at 570nm was determined using a microplate reader (DNM 9602; Perlong Medical Equipment Co., Ltd.). All experiments were performed at least in triplicate.
**Cell cycle assay**

For the assessment of cell cycle progression, the H929 and RPMI8226 cells were seeded at a density of \(2.5 \times 10^5\) cells/ml in six-well plates, and treated with different concentrations of nitroxoline and/or bortezomib for 24h. Next, the H929 and RPMI8226 cells were fixed with 75% ethanol overnight. Propidium iodide (PI; Sigma-Aldrich; Merck KGaA) was then used to stain the DNA of samples for 15 min. Subsequently, flow cytometry was conducted with an Epics XL flow cytometer (Beckman Coulter, Inc.) to determine the cell cycle progression, and data were analyzed using Flowjo software (version 7.6; FlowJo, LLC). All experiments were performed at least in triplicate.

**Apoptosis assay**

The H929 and RPMI8226 cells were seeded at a density of \(2.5 \times 10^5\) cells/ml in six-well plates, and treated with different concentrations of nitroxoline and/or bortezomib for 24h. Cell apoptosis was then assessed using an Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit (BD Biosciences). Briefly, the cells were stained with Annexin V-FITC and PI in binding buffer for 15 min, and the apoptotic cells were then detected using a FACScalibur flow cytometer (BD Biosciences). The results were analyzed using CXP software (version 2.1; Beckman Coulter, Inc.). All experiments were performed at least in triplicate.

**Western blotting**

Following the different treatments, the cells were lysed in lysis buffer as previously described, and then the cell lysates were separated via SDS-PAGE (10–18% gel). Proteins were transferred onto nitrocellulose membranes (Pall Corporation), and the membranes were then blocked with 5% non-fat milk in Tris-buffered saline/Tween 20 (consisting of 50 mM Tris-HCl, pH 8.0, 10 mM NaCl and 0.1% Tween 20) for 2h at room temperature. Subsequently, the membranes were incubated overnight at 4°C with anti-cyclin D1 (dilution 1:3000), anti-p21 (dilution 1:1000), anti-Bax (dilution 1:1000), anti-Bcl2 (dilution 1:1000), anti-cleaved caspase-3 (dilution 1:500), anti-cleaved PARP (dilution 1:1000) and anti-GAPDH (dilution 1:3000) primary antibodies. The membranes were then incubated with antirabbit HRP secondary antibody (1:20,000, cat. no. 7074, Cell Signaling Technology, Inc.) for 2h at 25°C. Visualization was achieved using SuperSignal West Pico chemiluminescent Substrate (Pierce; Thermo Fisher Scientific, Inc.) and Aplegen (Omega Lum G).

**In vivo human plasmacytoma xenograft model**

All experimental protocols were approved by Animal Ethics Committee of The First Affiliated Hospital of the Fourth Military Medical University (No. IACUC-20160905). A xenograft tumor model was established as previously described. Briefly, 24 female BALB/c nude mice (16–20 g; 4–6 weeks) were obtained from Shanghai Laboratory Animal Center. Female BALB/c nude mice were housed at 22 ± 2°C room with a 12-h light/12-h dark cycle, a relative humidity of 40–60%, and had free access to food and water. RPMI8226 cells (1×10⁷ per mouse) were injected subcutaneously into the right flanks of nude mice in 100 μl serum-free RPMI-1640 medium. Seven days after tumor cell injection, the mice were divided into four groups \((n = 3\) per group), as follows: saline control, bortezomib (0.6 mg/kg) or nitroxoline (60 mg/kg) treatment alone, and combination of bortezomib (0.6 mg/kg) and nitroxoline (60 mg/kg) treatment groups. Nitroxoline (60 mg/kg) was administered via intraperitoneal injection three times per 7 days, while bortezomib (0.6 mg/kg) was administered via intravenous injection twice per 7 days for 14 days. The volume of the tumor was measured every 3 days for 21 days and was calculated as follows: Volume \((\text{mm}^3) = (\text{long diameter of the tumor} \times \text{short diameter of the tumor})^2/2\). At the end of the experiment (day 21), the mice underwent euthanasia via CO₂ asphyxiation in a chamber (100% CO₂, 9.6 l/min, 10 min) followed by cervical dislocation to confirm death, and the tumors were excised. The tumor samples were then examined using hematoxylin and eosin (HE) staining. Furthermore, a TUNEL assay was performed to detect in situ apoptosis using an In Situ Cell Death Detection Kit, POD (cat. no. 11684817910, Roche, USA) according to the manufacturer’s instructions and immunohistochemical staining was used to assess the Ki-67 expression of tumor tissues using a Ki-67 assay kit (immunohistochemical) (cat. no. MAB-0672, MXB Biotechnology, Fuzhou, China) according to the manufacturer’s instructions.
instructions for quantifying the cell proliferation in the human plasmacytoma xenograft model. All procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996).

Statistical analysis
The results are presented as the mean ± standard deviation. One-way analysis of variance followed by Dunnett’s and Tukey’s post-hoc tests were used to determine the statistical significance of the observed differences, with \( p < 0.05 \) considered statistically significance.

Results
Nitroxoline increases the bortezomib-induced proliferation inhibition in human MM cell lines
In order to investigate the effect of nitroxoline and bortezomib on human MM cell proliferation, H929 and RPMI8226 cells were treated with different concentrations of bortezomib and nitroxoline for 24h, and cell viability was investigated using an MTT assay. As presented in Figure 1(A) and (B), bortezomib or nitroxoline as single treatments significantly inhibited the viability of H929 and RPMI8226 cells compared with the control group, in a concentration-dependent manner. The viability of cells co-treated with bortezomib and nitroxoline was further measured. As presented in Figure 1(C), 5.00 nM bortezomib and 0.50 nitroxoline combination was able to markedly inhibit the viability of H929 and RPMI8226 cells compared with the control group, in a concentration-dependent manner. The viability of cells co-treated with bortezomib and nitroxoline was further measured. As presented in Figure 1(C), 5.00 nM bortezomib and 0.50 nitroxoline combination was able to markedly inhibit the viability of H929 and RPMI8226 cells compared with the control group, in a concentration-dependent manner. The viability of cells co-treated with bortezomib and nitroxoline was further measured. As presented in Figure 1(C), 5.00 nM bortezomib and 0.50 nitroxoline combination was able to markedly inhibit the viability of H929 and RPMI8226 cells compared with the control group, in a concentration-dependent manner.

Effect of nitroxoline and bortezomib on cell cycle-associated protein in human MM cell lines
In order to further elucidate the mechanism underlying the G0/G1 phase arrest, the present study detected the expression levels of cyclin D1 and p21 in H929 and RPMI8226 cells. As presented in Figure 3(A), nitroxoline evidently decreased the level of cyclin D1 and increased the level of p21 in a concentration-dependent manner. In addition, the combination of bortezomib and nitroxoline clearly decreased the level of cyclin D1 and increased the level of p21 compared with those in the bortezomib or nitroxoline alone groups (Figure 3(B)). These results suggested that nitroxoline significantly increased the bortezomib-mediated G0/G1 phase arrest via downregulating the expression of cyclin D1 protein and upregulating the expression of p21 protein in H929 and RPMI8226 cells.

Nitroxoline increases the bortezomib-induced apoptosis in human MM cell lines
The present study further investigated the effect of nitroxoline and bortezomib on human MM cell apoptosis. H929 and RPMI8226 cells were treated with bortezomib and/or nitroxoline for 24h, and then cell apoptosis was investigated using flow cytometry. As presented in Figure 4(A) and (B), bortezomib and nitroxoline induced apoptosis in H929 and RPMI8226 cells. Furthermore, the combination of bortezomib and nitroxoline significantly increased the percentage of apoptotic cells compared with the bortezomib or nitroxoline alone groups. These results indicated that nitroxoline may significantly increase bortezomib-induced cell apoptosis.
Figure 1. Effects of nitroxoline and bortezomib on the proliferation of RPMI8226 and H929 cells. (A) RPMI8226 and H929 cells were treated with various concentrations of nitroxoline (0.00, 0.25, 0.50, 1.00, 2.00, 4.00, 8.00 and 16.00 μM) for 24 h, and the cell viability was determined via MTT assay. (B) Cells were treated with various concentrations of bortezomib (RPMI8226 cells: 0, 1, 2, 4, 8, 16, 32 and 64 nM; H929 cells: 0, 0.125, 0.25, 0.5, 1, 2, 4 and 8 nM) for 24 h, and then cell viability was determined via an MTT assay. DMSO was used in the control group at the same dilution as the corresponding treatment in the nitroxoline and bortezomib alone group. (C) Cells were treated with nitroxoline and/or bortezomib for 24 h (RPMI8226 cells, 0.50 μM and 5.00 nM, respectively; H929 cells, 0.25 μM and 1.00 nM, respectively), and cell viability was determined via an MTT assay. Data are presented as the mean ± standard deviation from three independent experiments. (**p < 0.01, ***p < 0.001 and ****p < 0.0001, versus control group; ###p < 0.001 and ####p < 0.0001 versus nitroxoline alone group; +++p < 0.001 versus bortezomib alone group.)
Effect of nitroxoline and bortezomib on apoptosis-associated protein in human MM cell lines

In addition, a number of apoptosis-associated proteins in H929 and RPMI8226 cells were detected via western blotting. As presented in Figure 5(A), nitroxoline evidently decreased the level of Bcl-2, and increased the levels of Bax, cleaved PARP and cleaved caspase-3 in a concentration-dependent manner. The combination of bortezomib and nitroxoline markedly decreased the level of Bcl-2, and increased the levels of Bax, cleaved PARP and cleaved caspase-3, as compared with those in the bortezomib or nitroxoline alone groups (Figure 5(B)). Overall, these results demonstrated that nitroxoline may significantly increase the cell apoptosis induced by bortezomib via mitochondrial-dependent apoptotic pathways.

Nitroxoline enhances the bortezomib-induced inhibition of xenograft tumor growth in mice in vivo

The present study next investigated the effects of nitroxoline and bortezomib on RPMI8226 cell
The results verified that the combination of bortezomib and nitroxoline enhanced the inhibition of proliferation, and enhanced G0/G1 phase cell cycle arrest and apoptosis in H929 and RPMI8226 cells compared with that in cells treated with a single drug or untreated cells. In addition, it was observed that nitroxoline enhanced the bortezomib-induced G0/G1 arrest by downregulating the expression of cyclin D1 protein and upregulating the expression of p21 protein in the cells. The data also indicated that nitroxoline may enhance bortezomib-induced cell apoptosis via activating the mitochondrial-dependent apoptotic pathway in H929 and RPMI8226 cells. Furthermore, in vivo experiments performed in the present study demonstrated that combination treatment remarkably inhibited the tumor growth and induced cell apoptosis in comparison with the use of single drug treatment in an RPMI8226 xenograft tumor model.

The disorder of the cell cycle is usually a characteristic of human tumors. Thus, cell cycle arrest plays an active role in cell proliferation inhibition caused by anti-cancer drugs. A number of studies have reported that bortezomib may induce cell cycle arrest in various types of tumor cells lines, including colon cancer, ovarian cancer and MM cells. Furthermore, indole-3-carbinol has been demonstrated to enhance the bortezomib-induced G2/M phase arrest in OVCAR3 and OVCAR5 cells. In HT29 and HCT116 cells, the combination of vorinostat and bortezomib induced G2/M phase arrest. The results of the present study demonstrated that treatment with nitroxoline or bortezomib shifted the cell cycle towards G0/G1 phase in H929 and RPMI8226 cells, as observed via flow cytometric analysis. The combination of bortezomib and nitroxoline significantly increased G0/G1 phase cell cycle arrest as compared with the bortezomib or nitroxoline alone study.
The transition from G0/G1 to S phase is regulated by cyclin D1 and p21 proteins. In order to further investigate the molecular mechanism underlying the G0/G1 phase cell cycle arrest induced by nitroxoline and bortezomib in H929 and RPMI8226 cells, the present study assessed the expression levels of cyclin D1 and p21 proteins. The data revealed that nitroxoline markedly decreased the level of cyclin D1 and increased the level of p21 in a concentration-dependent manner. In addition, the combination of bortezomib and nitroxoline markedly decreased the level of cyclin D1 and increased the level of p21 when compared with the bortezomib or nitroxoline alone groups. These findings provided evidence that the addition of nitroxoline enhanced the bortezomib-induced inhibition of H929 and RPMI8226 cell proliferation, through inducing G0/G1 phase arrest via downregulating the expression of cyclin D1 protein and upregulating the expression of p21 protein.

Apoptosis has a balancing role in cell proliferation and cell death. Previous studies have reported that the majority of drugs exert their...
anti-cancer effects 

Figure 5. Effects of nitroxoline and bortezomib on apoptosis-related protein of H929 and RPMI8226 cells. (A) RPMI8226 cells were treated with 0, 0.5, 2, 5 \( \mu \)M nitroxoline for 24 h, H929 cells were treated with 0, 0.25, 1, 3 \( \mu \)M nitroxoline for 24 h. Bcl-2, Bax, cleaved PARP and cleaved caspase-3 protein expression levels were assessed via western blotting. (B) Bcl-2, Bax, cleaved PARP and cleaved caspase-3 protein expression levels were assessed via western blotting in RPMI8226 cells treated with 0.5 \( \mu \)M nitroxoline and 5.0 nM bortezomib for 24 h, and in H929 cells treated with 0.25 \( \mu \)M nitroxoline and 1.00 nM bortezomib for 24 h. GAPDH was used as the internal control. Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; PARP, poly (ADP-ribose) polymerase

In conclusion, the results of the present study verified that nitroxoline augmented the hindrance of cell proliferation, and strengthened the G0/G1 phase cell cycle arrest and apoptosis induced by bortezomib in H929 and RPMI8226 cells. Furthermore, nitroxoline was observed to enhance the bortezomib-induced G0/G1 arrest via down-regulating cyclin D1 protein expression and upregulating p21 protein expression. The data also indicated that nitroxoline may enhance the bortezomib-induced cell apoptosis via the mitochondrial apoptotic pathway. Importantly, the dose of nitroxoline and bortezomib used in vitro is achievable clinically because the concentration of the combination of nitroxoline and bortezomib used in H929 and RPMI8226 did not exceed blood drug peak concentration in patients’ clinical application. However, there is a limitation in our present study. The dosages of nitroxoline and bortezomib in vivo are higher than those which have been readily implemented in clinical treatment and our study lacked the evaluation of adverse drug reactions. But nitroxoline cotreatment with bortezomib did not result in additional toxicity in treated animals. We have confirmed that nitroxoline as BRD4 inhibitor could enhance the sensitivity of bortezomib in multiple myeloma cells. Therefore, it provides the basis for the combinative administration of bortezomib.
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Author contribution
XQC, YPS and CL conceived the experiment. GL and YHZ drafted the manuscript. GL, YHZ, LX performed the cell experiment and statistical analysis. JF and HLT performed the immunohistochemistry. All authors critically reviewed the manuscript and approved the final version of this manuscript.

Availability of data and materials
Data, samples or materials will be made available upon request by communicating with Xie-Qun Chen.

Conflict of interest statement
The author(s) declare that there is no conflict of interest.

Consent for publication
All authors have read, understood and approved the authorship agreement.

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