

NS-398 enhances the efficacy of bortezomib against RPMI8226 human multiple myeloma cells

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Abstract. Bortezomib is commonly used in treating multiple myeloma (MM). However, a number of patients develop resistance to bortezomib over time. Cox-2 is overexpressed in MM cells and contributes to apoptosis resistance and MM development. In the present study, RPMI8226 MM cells were treated with the Cox-2 inhibitor NS-398 to investigate whether it enhanced the effect of bortezomib on MM. The results showed that NS-398 and bortezomib acted synergistically to inhibit growth, arrest the cell cycle at the G₁ phase and to induce the apoptosis of MM cells. NS-398 inhibited the NF-κB p65 protein levels and the expression of various NF-κB target genes, including cyclin D1, c-Myc, survivin and Bcl-2. In conclusion, NS-398 enhanced the efficacy of bortezomib against MM cells *in vitro* and this was associated with the inhibition of NF-κB signaling. These findings suggest that the combined use of NS-398 and bortezomib may constitute a promising novel treatment protocol for MM patients.

Introduction

Multiple myeloma (MM) is a B-cell malignancy characterized by the clonal proliferation of neoplastic plasma cells in the bone marrow (BM). MM is an incurable disease (1). Bortezomib (Velcade®) was developed as an anticancer drug that acts by inhibiting the 26S proteasome complex and exhibiting a strong cytotoxic effect against MM cells (2-4). Nevertheless, numerous MM patients who initially respond well, inevitably develop resistance to this drug.

Previous studies have demonstrated that MM cells become refractory to bortezomib possibly due to their increased constitutive NF-κB activity, which is further increased when

cultured with MM patient-derived bone marrow stromal cells (BMSCs). In addition, increased NF-κB activity in the MM RPMI8226 cell line has been correlated with a decreased bortezomib sensitivity *in vitro* (5,6).

Prostaglandin E2 (PGE2) has been shown to upregulate the expression of Cox-2 or to contribute to its direct stabilization (7-9). Upon binding with its cell surface receptors, PGE2 has been shown to exert anti-apoptotic and proliferative effects which may promote carcinogenesis and to a certain extent, abrogate the anti-tumor effects of proteasome inhibition (10). In addition, NS-398 (a Cox-2 highly selective inhibitor) has been demonstrated to inhibit cell growth and induce apoptosis by inhibiting the NF-κB pathway (11). Thus, Cox-2 inhibition is a potentially effective way to enhance the efficacy of bortezomib.

In the present study, the RPMI8226 cell line was used as a model to determine the *in vitro* effects of NS-398 and bortezomib on MM RPMI8226 cells, with the aim of investigating the possible treatment of MM by combined use of NS-398 and bortezomib.

Materials and methods

Cell culture and reagents. RPMI8226 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (Gibco-BRL, Grand Island, NY, USA) and maintained in a 5% CO₂ incubator at 37°C. The highly selective Cox-2 inhibitor, NS-398 (Sigma, St. Louis, MO, USA), and the bortezomib (LC Laboratories, Woburn, MA, USA) were dissolved in dimethyl sulfoxide (DMSO; Sigma) and stored at -20°C. The two drugs were diluted in culture medium (10⁻⁴-10⁻² mol/l) with <0.1% DMSO immediately prior to use. The study was approved by the Ethics Committee of Fujian Medical University, Fuzhou, China.

Cell growth assay. RPMI8226 cells were seeded in triplicate at a density of 5x10³ cells/well in 96-well plates and 180 μl RPMI-1640 medium containing various concentrations of bortezomib and/or NS-398. The cells were cultured for 24, 48 or 72 h. Subsequently, 20 μl of MTT substrate (5 mg/ml) was added to each well, and the plates were returned to the standard cell incubator for an additional 4 h. The supernatants were then carefully removed and 200 μl DMSO was

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added to each well. After the insoluble crystals were completely dissolved, colorimetric analysis was performed at a wavelength of 570 nm. The inhibition rate was calculated as follows: $100\% - (\text{individual OD value} / \text{control OD value})\%$. Each independent experiment was performed three times. The formula presented by Jin (12) was calculated as: $Q = Ea + b / (Ea + Eb - Ea \times Eb)$. Q was the combination index; Ea + b represented the inhibition rate of the combined drug (A+B); and Ea and Eb represented the inhibition rates of A and B, respectively. When the Q value ranged between 0.85 and 1.15, the combined drug effect was a simple 'arithmetic' sum. A Q value of >1.15 indicated a synergic effect, while a Q value of <0.85 indicated an antagonistic effect.

Flow cytometric analysis of apoptosis and the cell cycle. RPMI8226 cells were seeded in quadruplicate at a density of 5×10^6 cells/well in 6-well plates and 3 ml of RPMI-1640 medium containing various concentrations of bortezomib and/or NS-398. Subsequent to culturing for 48 h, the cells were collected, washed twice with ice-cold phosphate-buffered saline (PBS) and fixed with 70% ethanol at 4°C overnight. Following washing with PBS, the cells were incubated in 0.5 ml PBS containing 50 µg/ml RNase A for ~30 min at 37°C. Propidium iodide (PI) was then added (Keygen, Nanjing, China) to a final concentration of 50 µg/ml and incubated for 30 min on ice in the dark. The resultant cell suspension was then subjected to flow cytometry (Becton-Dickinson, Franklin Lakes, NJ, USA). The percentage of the apoptotic cells (sub-G1) and the cells in the G0/G1, S and G2/M phases was calculated using CellQuest software (Becton-Dickinson).

Cox activity assay. The activity of Cox-2 was measured using the COX Activity Assay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. The cells were collected at 4°C. Cell pellets were then homogenized in the cold buffer provided with the kit and centrifuged at $10,000 \times g$ at 4°C for 10 min. Then, 10 µl of supernatant was added into each well of a 96-well plate and mixed with 150 µl assay buffer and 10 µl heme. The supernatant samples, which were boiled for 5 min, were matched with each well as the controls. The 96-well plate was carefully agitated for 1 min and incubated at 37°C for 5 min, then 20 µl colorimetric substrate and arachidonic acid were added to each well. The plate was carefully agitated again and incubated at 37°C for 5 min. Colorimetric analysis was performed at a wavelength of 570 nm.

Western blot analysis. The cells were collected and lysed at 4°C for 30 min. Once the protein concentration had been determined using the Lowry method, equal amounts of protein were separated on 6-15% SDS-PAGE gels. The protein was transferred to polyvinylidene fluoride (PVDF) membranes, which were blocked with 5% skimmed milk in TBST (tris-buffered saline solution containing 0.1% Tween-20) at 4°C overnight, and then incubated with antibodies for NF-κB, Cox-2, c-Myc, Bcl-2, survivin, cyclin D1 and GAPDH (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 2 h at room temperature. Following washing with TBST, the membranes were incubated with horseradish peroxidase (HRP)-labeled secondary antibodies for 1 h at 37°C and then developed using the enhanced chemiluminescence (ECL) reagent kit (Beyotime, Jiangsu, China).

Statistical analysis. Data were expressed as the mean ± standard deviation (SD) and the Student's t-test was used to determine the significance of the differences between the drugs and controls. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

NS-398 and bortezomib act synergistically to inhibit the growth of RPMI8226 MM cells. An MTT assay was performed to evaluate the effects of NS-398 and bortezomib on the growth of the RPMI8226 cells. The results showed that 20 µM NS-398 inhibited RPMI8226 cell growth with inhibition rates of 33.7, 41.9 and 66.1% following 24-, 48- and 72-h treatments, respectively. Moreover, exposure of the cells to a 2.5-160 µM range of NS-398 for 48 h inhibited cell proliferation in a dose-dependent manner. The half maximal inhibitory concentration (IC_{50}) of NS-398 for 48 h was 44.1 µM (Fig. 1A and B). Similarly, bortezomib inhibited RPMI8226 cell growth in a dose- and time-dependent manner. The IC_{50} of bortezomib for 48 h was 48.4 nM (Fig. 1C and D). The combination index was calculated to determine whether there was synergy between NS-398 and bortezomib in the inhibition of MM cell growth. The results showed that following treatment of the cells with 2, 10, 50 and 250 nM of bortezomib for 48 h, the inhibitory rate of cell growth was 6.7, 15.1, 52.3 and 91.2%, respectively, while the inhibitory rate was 41.9% following treatment with 20 µM NS-398. When 20 µM NS-398 was combined with these concentrations of bortezomib, the inhibitory rate of cell growth was increased to 58.7, 76.9, 91.4 and 97.7%, respectively ($P < 0.05$), and the combined index was 1.28, 1.52, 1.26 and 1.03, respectively (Fig. 1E). These results demonstrate that NS-398 and bortezomib act synergistically by inhibiting the growth of RPMI8226 MM cells. To better characterize the synergistic action between the two drugs, 50 nM bortezomib and 20 µM NS-398 were used in the subsequent experiments.

NS-398 promotes bortezomib-induced apoptosis and the cell cycle arrest of RPMI8226 MM cells. To analyze the synergistic action between NS-398 and bortezomib, apoptosis and the cell cycle of the RPMI8226 cells treated by these two drugs were examined. As shown in Fig. 2, following treatment of the RPMI8226 cells with 50 nM bortezomib and 20 µM NS-398, the rate of apoptosis was 22.4 and 19.6%, respectively. The combination of the bortezomib and NS-398 treatments led to an apoptotic rate of 61.2%, which was higher than the sum of the rate when the cells were treated with each drug alone. In addition, Table I shows the percentage of cells arrested in the G0/G1 phase as 47.5 ± 3.8 and $41.6 \pm 3.3\%$ following the bortezomib and NS-398 treatments, respectively. These results were higher than that of the control ($25.4 \pm 3.3\%$; $P < 0.05$). The combined drug treatment led to a higher percentage of cells being arrested in the G0/G1 phase. Taken together, these data suggest that NS-398 promotes bortezomib-induced apoptosis and the cell cycle arrest of MM cells.

NS-398 inhibits bortezomib-induced Cox-2 activity in RPMI8226 MM cells. As shown in Fig. 3, NS-398 completely suppressed the increased Cox-2 activity induced by bortezomib in the RPMI8226 cells (Fig. 3). To investigate whether

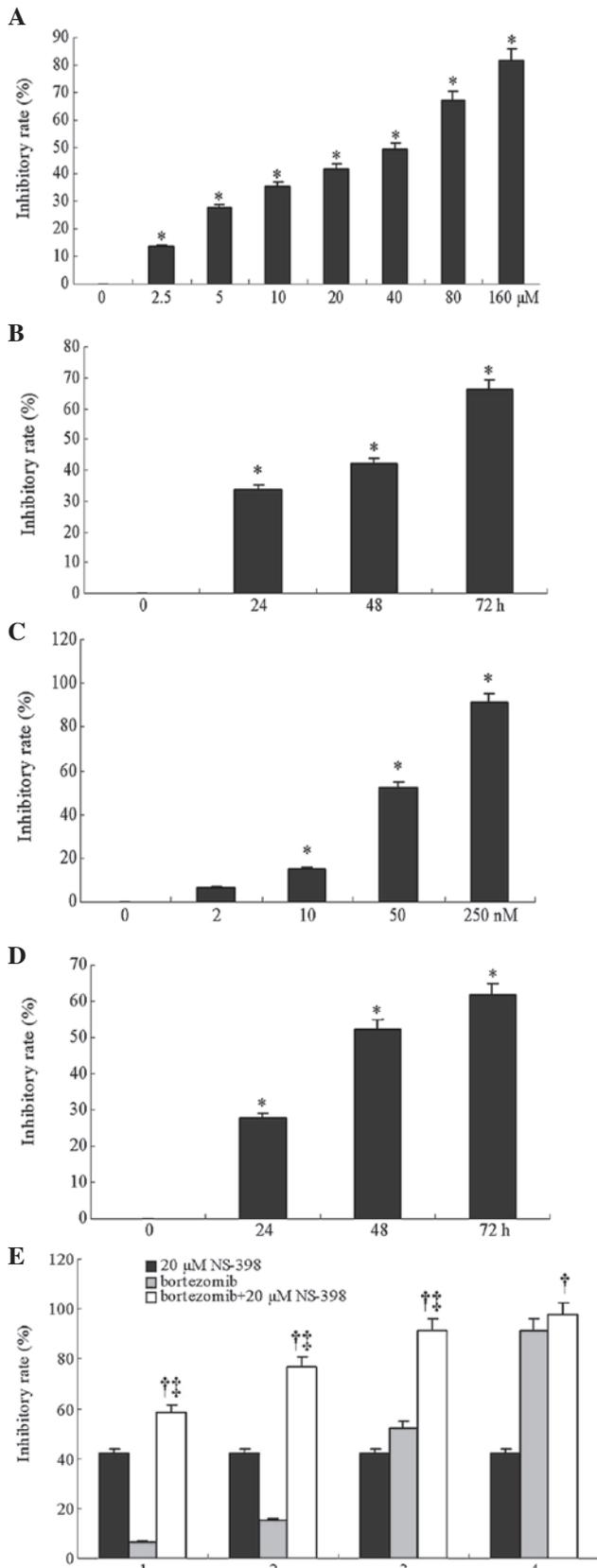


Figure 1. NS-398 and/or bortezomib induce cytotoxicity in RPMI8226 cells. Cells were treated with (A) various concentrations of NS-398 for 48 h; (B) 20 nM NS-398 for varying time periods; (C) various concentrations of bortezomib for 48 h; (D) 10 nM bortezomib for varying time periods; and (E) various concentrations of bortezomib for 48 h. 1, 2 nM; 2, 10 nM; 3, 50 nM; 4, 250 nM bortezomib and 20 nM NS-398. An MTT assay was performed as described in Materials and methods. The data are expressed as the mean ± SD. *P<0.05 vs. control; †P<0.05 vs. treatment with 20 nM of NS-398; ‡P<0.05 vs. treatment with bortezomib.

Table I. NS-398 and/or bortezomib-induced cell cycle arrest in RPMI8226 cells.

Treated types	G0/G1 (%)	S (%)	G ₂ /M (%)
Control	25.4±3.4	51.3±3.5	23.3±1.5
NS-398	41.6±3.3 ^a	38.6±2.7 ^a	19.8±1.4
Bortezomib	47.5±3.8 ^a	29.9±2.2 ^a	22.6±1.3
Combination	70.1±5.4 ^b	10.6±1.6 ^b	19.2±1.4

^aP<0.05 vs. control; ^bP<0.05 vs. either NS-398 or bortezomib alone. Cell cycle distribution of RPMI8226 cells following treatment with NS-398 (20 μM), bortezomib (50 nM) and the two drugs combined (50 nM bortezomib and 20 μM NS-398) for 48 h. The data were expressed as the mean ± SD of 3 independent experiments.

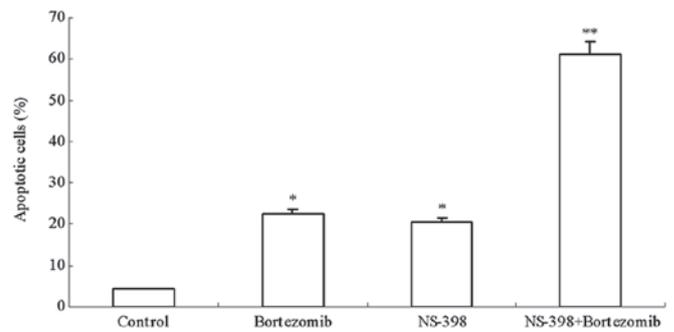


Figure 2. NS-398 and/or bortezomib induce apoptosis in RPMI8226 cells. Cells were treated with NS-398 (20 μM), bortezomib (50 nM) or the two drugs combined (20 μM NS-398 and 50 nM bortezomib) for 48 h. Subsequent to the indicated times, the RPMI8226 cells were fixed with ethanol and stained with PI, then sub-G1 was analyzed using flow cytometry. The sub-G1 was defined as apoptosis. Experiments were performed in duplicates and the data are expressed as the mean ± SD. *P<0.05 vs. control; **P<0.05 vs. either NS-398 or bortezomib alone. PI, propidium iodide.

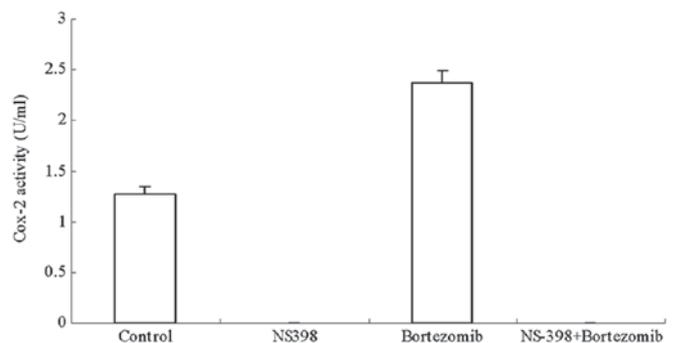


Figure 3. NS-398 and/or bortezomib inhibit Cox-2 activity in RPMI8226 cells. Cells were treated with NS-398 (20 μM), bortezomib (50 nM) or NS-398 (20 μM) + bortezomib (50 nM) for 48 h. Subsequent to the indicated time, the cells were collected and the Cox-2 activity was measured. Experiments were performed in duplicate and the data are expressed as the mean ± SD.

this was due to the downregulation of Cox-2 expression caused by NS-398 treatment, a western blot analysis was performed to investigate the protein level of Cox-2. It was observed that NS-398 had no obvious effect on the high Cox-2 protein

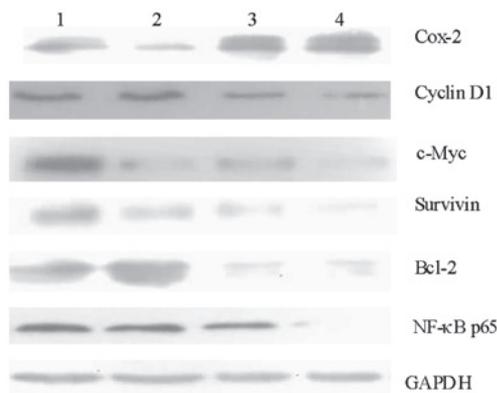


Figure 4. NS-398 and/or bortezomib inhibit NF- κ B signaling in RPMI8226 cells. Cells were treated with NS-398, bortezomib or a combination of the two drugs for 48 h. The protein levels of NF- κ B, Bcl-2, c-Myc, Cox-2, cyclin D1 and survivin were then detected by western blot analysis. GAPDH served as a loading control. Lanes 1, untreated cells (control); 2, cells treated with NS-398 (20 μ M); 3, cells treated with bortezomib (50 nM); 4, cells treated with NS-398 (20 μ M) and bortezomib (50 nM).

level in the RPMI8226 cells treated with bortezomib (Fig. 4), suggesting that NS-398 inhibits the bortezomib-induced Cox-2 activity in RPMI8226 cells without modulating the expression of Cox-2.

NS-398 promotes bortezomib-induced downregulation of NF- κ B signaling in RPMI8226 MM cells. It is well-known that bortezomib inhibits NF- κ B activity in MM cells. Therefore, we examined the activity of NF- κ B and the expression of its downstream targets, including Bcl-2, c-Myc, cyclin D1 and survivin in RPMI8226 MM cells. Western blot analysis showed that the levels of NF- κ B p65, a marker of the activation of NF- κ B signaling, as well as those of Bcl-2, c-Myc, cyclin D1 and survivin, were significantly decreased upon combined treatment with NS-398 and bortezomib, compared with the untreated control or treatment with NS-398 or bortezomib alone. These results demonstrate that NS-398 and bortezomib act synergistically to inhibit NF- κ B signaling in RPMI8226 MM cells.

Discussion

Cox-2 is important in the regulation of growth and apoptosis in tumor cells (13,14). Previous studies have indicated that Cox-2 is highly expressed in MM, and its overexpression has often been used as an indicator of poor prognosis (15). Cox-2 knockdown has been shown to induce the growth inhibition and apoptosis of RPMI8226 MM cells (16). NS-398 has been demonstrated to inhibit growth and induce the apoptosis of MM cells through Cox-2-dependent and -independent pathways (17). A previous study showed that the combination of bortezomib and aspirin, a classical Cox inhibitor, acted synergistically to inhibit proliferation and induce the apoptosis of human colorectal cells (10). However, to the best of our knowledge, no studies have been reported on the synergistic effects between NS398 and bortezomib in MM cells.

In the present study, NS-398 and bortezomib inhibited RPMI8226 cell growth in a time- and dose-dependent manner. When 20 μ M NS-398 was combined with various concentra-

tions (2-250 nM) of bortezomib, the combined index ranged between 1.26 and 1.52, with the exception of 1.03, when a combination of 20 μ M of NS-398 and 250 nM bortezomib was used, suggesting that this combination had synergistic growth inhibitory effects on the RPMI8226 cells.

To elucidate the mechanisms by which NS-398 enhanced the cytotoxicity of bortezomib in the MM RPMI8226 cells, we first examined apoptosis induction. Our data showed that the apoptotic rate of the combined group was significantly higher than that of either NS-398 or bortezomib treatment alone. This is in agreement with a previous study which indicated that NS-398 enhanced the growth inhibition of MM cells by dexamethasone or thalidomide treatment, and that the synergic effect was mainly mediated by the increase of apoptosis (18). In addition, we showed that NS-398 promotes bortezomib-induced cell cycle arrest of MM cells at the G0/G1 phase.

The sensitization of RPMI8226 cells to bortezomib was thought to be associated with the decreased activity of Cox-2. Our data indicated that NS-398 was able to completely inhibit Cox-2 activity, i.e., not only the endogenous activity in RPMI8226 cells, but also the enhanced activity induced by the bortezomib treatment. Notably, we found that NS-398 had no effects on Cox-2 expression, which was in agreement with a previous study (10). Thus, our results indicate that the NS-398-mediated inhibition of Cox-2 activity may increase the cytotoxicity of bortezomib in the MM cells.

Cox-2 inhibitors are known to act synergistically with chemotherapeutic agents in numerous cancer cells by down-regulating the NF- κ B target genes, such as Bcl-2, c-Myc and survivin (19-22). In the present study, we found that several downstream targeted genes of NF- κ B signaling, including Bcl-2, c-Myc, cyclin D1 and survivin, were highly expressed in the RPMI8226 cells, indicating that the activation of NF- κ B signaling contributes to the development of myeloma. Following the combined treatment with NS-398 and bortezomib, the expression of these NF- κ B target genes was significantly decreased. These data indicate that the inhibition of the NF- κ B pathway is essential to the synergistic effect of NS-398 and bortezomib on the MM cells.

Bortezomib is used in MM patients based on the inhibition of NF- κ B activity by the prevention of the proteasomal degradation of I κ B α . However, a previous study indicated that bortezomib-induced cytotoxicity in MM cells is not associated with the inhibition of NF- κ B activity (23). This raised a question with regard to whether the NF- κ B pathway remains a major therapeutic target of MM. Thus, we investigated the activity of NF- κ B in the RPMI8226 MM cells. Western blot analysis indicated that the level of NF- κ B p65 in cells treated with a combination of bortezomib and NS-398 was significantly lower than in cells treated with bortezomib or NS-398 alone. Taken together, these results show that NF- κ B signaling is involved in the synergistic action between NS-398 and bortezomib against MM cells.

In summary, it was found that NS-398 and bortezomib acted synergistically to inhibit cell growth, arrest the cell cycle at the G1 phase and induce the apoptosis of MM cells. NS-398 enhanced the efficacy of bortezomib against MM cells *in vitro* and this was associated with the inhibition of NF- κ B signaling. These findings suggest that the combined use of

NS-398 and bortezomib may constitute a promising novel treatment protocol for MM patients.

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