Blockage of interleukin-6 signaling with 6-amino-4-quinazoline synergistically induces the inhibitory effect of bortezomib in human U266 cells

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The transcription factor nuclear factor-kappa B (NF-κB) regulates the transcription of a number of genes involved in a variety of cellular responses, including cell survival, inflammation, and differentiation. NF-κB is activated by a variety of stimuli, proinflammatory cytokines, mitogens, growth factors, and stress-inducing agents. Aberrant NF-κB expression is considered to be one of the oncogenic factors of cancer and the constitutive activation of NF-κB is observed in several hematologic disorders [classic Hodgkin's lymphoma, diffuse large B cell lymphoma, and multiple myeloma (MM)], and the modulation of NF-κB activation is emerging as a promising novel anticancer therapeutic strategy.

Therefore, we focused on the regulation of NF-κB activation in MM. When U266 cells were treated with 6-amino-4-quinazoline, an NF-κB activation inhibitor, we determined that it most effectively blocked the interleukin (IL)-6-induced activation of MAPK and JAK/STAT pathways among different signaling inhibitors. The results of the luciferase assay indicated that 6-amino-4-quinazoline inhibited NF-κB activation with diminished NF-κB protein bound to NF-κB DNA binding sites. In addition, 6-amino-4-quinazoline suppressed the production of IL-6, which affected MM cell proliferation. Furthermore, combined treatment with bortezomib and 6-amino-4-quinazoline effectively inhibited the IL-6 and soluble IL-6R-induced activation of STAT3 and extracellular signal-regulated kinase phosphorylation. Our data showed that the inhibition of NF-κB activation abrogated MM cell proliferation induced by the IL-6 pathway, and might represent a promising therapeutic strategy for the treatment of MM. *Anti-Cancer Drugs* 19:777–782 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Multiple myeloma (MM) is a malignancy of differentiated B-lymphocytes characterized by the accumulation of clonal plasma cells in the bone marrow, accounts for 10% of all hematologic cancers, and remains an incurable hematological malignancy [1].

The interaction of MM cells with bone marrow stromal cells activates the production of proinflammatory cytokines, growth factors, and antigenic factors [2]. Among these, interleukin (IL)-6 is important to pathogenesis and the disease progression of MM [3,4], and it promotes MM cell growth and prolongs survival in a paracrine and autocrine manner [5]. IL-6 activation subsequently activates downstream targets, including antiapoptotic proteins and a variety of cytokines [6]. IL-6 transcription is stimulated via the activation of nuclear factor-kappa B (NF-κB) in MM cells, which triggers the proliferation of MM cells and protects against apoptosis from anticancer drugs [7,8].

NF-κB is inactive under normal conditions. Upon activation, NF-κB undergoes phosphorylation and ubiquitination-dependent degradation by the 26S proteasome. p50/65 NF-κB is then translocated to the nucleus and binds to a specific DNA sequence [9]. NF-κB regulates the expression of genes involved in apoptosis, tumorigenesis, and inflammation [10]. The constitutive activation of NF-κB has been observed in MM [11], and the aberrant activation of NF-κB induces the expression of antiapoptotic genes, including Bcl-2, c-FLIP, and XIAP, thereby resulting in the disruption of apoptotic signaling, and also rescues MM cells from apoptosis [8,12].

Some anti-MM agents, including dexamethasone, thalidomide, and bortezomib, inhibited NF-κB activity [13]. Recently, the cell-permeable specific inhibitor of NF-κB activity, SN-50, inhibited the nuclear translocation of NF-κB, resulting in the inhibition of NF-κB activation and triggered apoptosis in MM cells [14]. However, the specific

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mechanisms underlying the activity of NF-κB inhibitors in MM remain unknown. In the process of screening candidate antimyeloma agents among several cell signal transduction inhibitors, we have chosen 6-amino-4-quinazoline because of its prominent NF-κB inhibitory effects.

In this study, we demonstrated the effects of 6-amino-4-quinazoline (an NF-κB inhibitor) in U266 cells. 6-amino-4-quinazoline effectively inhibited IL-6-induced MAPK and JAK/STAT pathways, inhibited NF-κB activation via the abrogation of nuclear translocation, and also suppressed the secretion of IL-6. Soluble IL-6 receptor (sIL-6R) was shown to potentiate the IL-6 signal pathways and partially negated the inhibitory effects of bortezomib. Combined treatment with bortezomib and 6-amino-4-quinazoline, however, effectively inhibited STAT3 and extracellular signal-regulated kinase (ERK) phosphorylation in U266 cells treated with exogenous IL-6 and sIL-6R as compared with bortezomib treatment alone. These suggest that inhibitors targeting NF-κB abrogate MM cell proliferation.

Materials and methods

Cell line and cell culture

Human multiple myeloma U266 cells were obtained from the American Type Culture Collection (Rockville, Maryland, USA) and maintained in RPMI-1640 medium (Gibco-BRL, Gaithersburg, Maryland, USA) supplemented with sodium pyruvate, essential vitamins, L-glutamine, 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) (GIBCO, Grand Island, New York, USA). They were cultured in a highly humidified atmosphere of 5% CO_2 and 95% air at 37°C. All experiments were conducted using cells in the logarithmic growth phase.

Reagents

Recombinant human IL-6 and sIL-6R were purchased from R&D Systems (Minneapolis, Minnesota, USA), rehydrated in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin, and stored as a stock solution at −20°C.

The specific proteasome inhibitor, bortezomib (Velcade; formerly known as PS-341), was generously provided by Janssen Korea Ltd (Seoul, Korea). PD98059, LY294002, SP600125, AG490, JAK2 inhibitor II, 6-amino-4-(4-phenoxyphenylethylamino) quinazoline (NF-κB activation inhibitor), SB203580, and SN50 were purchased from Calbiochem Corporation (San Diego, California, USA). These agents were dissolved in dimethylsulfoxide as a stock solution, stored at −80°C, and subsequently diluted with serum-free RPMI 1640 before use.

Western blot analysis

The cells were treated with the indicated reagents for the indicated time period, washed once in ice-cold PBS, and resuspended in lysis buffer (50 mmol/l Tris–HCl (pH 7.4), 150 mmol/l NaCl, 1% NP-40, Na-deoxycholate 0.25%, 1 mmol/l EDTA, 1 mmol/l NaF, 1 mmol/l Na_3VO_4, 1 mmol/l phenylmethylsulfonyl fluoride, aprotinin, leupeptine, and pepstatin 1 μg/ml). The protein concentration of lysate was measured, 30 μg of cytoplasmic protein extracts were boiled for 5 min and the proteins were resolved in 10% SDS–PAGE and electrotransferred to polyvinylidene difluoride membranes (Millipore, Bedford, Massachusetts, USA). The membranes were blocked in Tris-buffered saline containing 0.05% Tween 20 and 5% nonfat dry milk for 1 h at room temperature and incubated with the appropriate primary antibody for 2 h. Immunoreactive proteins were detected using horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories Inc., Pennsylvania, USA) and an enhanced chemiluminescence reagent (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA). Antibodies for the following proteins were used in this study; Phospho-STAT1, phospho-STAT3, phospho-ERK, STAT1, STAT3, ERK (Cell Signaling Technology, Beverly, Massachusetts, USA), and β-actin (Sigma, Michigan, USA) antibodies were used.

Luciferase assay

The PathNet pTRH1-NF-κB-Luc reporter construct (packaged) was purchased from System Biosciences (Mountain View, California, USA). The NF-κB reporter construct (pTRH1-NF-κB-Luc) harbors sequences of transcriptional factor recognition elements $(GGGAC\ TTTCC)_4$. The transcriptional activity of NF-κB was measured as described in the manufacturer's instructions. Briefly, 5×10^5 cells were grown for 24 h in RPMI 1640 containing 10% FBS. After the cells had been washed twice with PBS, they were transfected with an appropriate concentration of packaged pTRH1-NFκB-Luc reporter construct in serum-free Dulbecco's modified Eagle's medium with 8 μg/ml polybrene for 24 h. After transfection, the culture media were replaced with complete RPMI-1640 medium without polybrene but with serum and antibiotics. Infected U266 cells were treated with the indicated reagents for the indicated time period and lysed, and the luciferase activity was assessed with a Luciferase Assay Kit (Applied Biosystems, Bedford, Massachusetts, USA). Cell lysates of 50 μl were added to the luminometer tube and then measured with a Luminescence Counter VICTORTM Light (Perkin Elmer, New Jersey, USA). Each assay was conducted in triplicate.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assays were conducted with IL-6 and a sIL-6R kit (R&D Systems, Minneapolis, Minnesota, USA). The cells were treated with the indicated reagents for the indicated time period, and the cell-free supernatants were harvested. These samples were measured for IL-6, and soluble IL-6R levels in

accordance with the manufacturer's instructions. A standard, diluted sample of 100 µl was added to each well and the microplates were incubated for the indicated time at room temperature (18–25°C). Each of the wells was aspirated and washed four times in washing buffer. A conjugate of 200 µl was then added to each well and the microplate was incubated for 2 h at room temperature. The aspiration/wash process was repeated and 200 µl of substrate solution was added to each well, followed by 20 min of incubation at room temperature. A stop solution of 50 µl was added to each well and the optical density of each well was determined within 30 min using a microplate reader set at 450 nm.

Statistical analysis

The statistical significance of difference observed in experimental versus control cells was determined via the Student's *t*-test. The minimal level of significance was *P* less than 0.05.

Results

Effects of various signal transduction inhibitors including 6-amino-4-quinazoline on the interleukin-6-induced activation of MAPK and JAK/STAT3 pathways

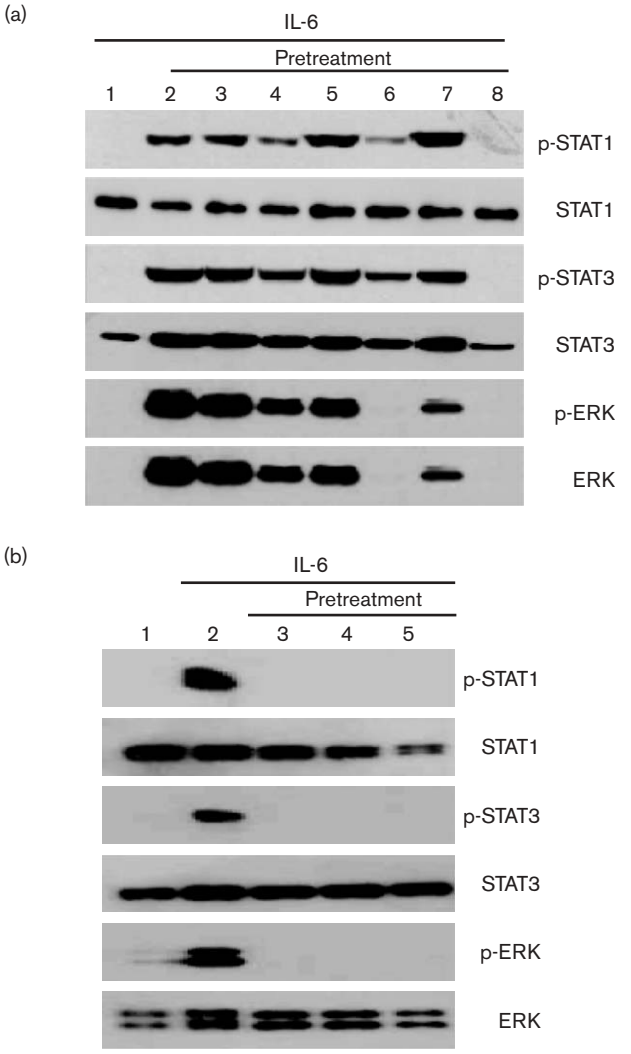
To determine the effect of various signal transduction inhibitors on the IL-6 cell signal pathways in MM cells, U266 cells were pretreated with indicated inhibitors for 1 h after cell starvation with serum-free media and was then treated for 15 min with recombinant human IL-6 (5 ng/ml). PD98059 and SB203580 (MAPK inhibitors) inhibited STAT1, STAT3, and ERK phosphorylation. LY294002 (PI3K/AKT inhibitor) and SP600125 (JNK inhibitor) partially inhibited ERK phosphorylation.

6-amino-4-quinazoline, an inhibitor of NF-κB activation, most effectively inhibited the phosphorylation of STAT1, STAT3, and ERK among a variety of signal transduction inhibitors. This showed that NF-κB activation plays a critical role in the IL-6-induced cell signal pathways. Therefore, the inhibition of NF-κB activation abrogated IL-6-induced MAPK and JAK/STAT pathways (Fig. 1a). We attempted to determine whether 6-amino-4-quinazoline is effective at lower concentrations. 6-amino-4-quinazoline at 500 nmol/l rather than 2 µmol/l completely inhibited the phosphorylation of STAT1, STAT3, and ERK (Fig. 1b), thereby indicating that 6-amino-4-quinazoline at very low concentrations could block IL-6-induced cell signal pathways.

6-amino-4-quinazoline-inhibited nuclear factor-kappa B activation induced by interleukin-6 and soluble interleukin-6 receptor

We investigated the effects of 6-amino-4-quinazoline on IL-6-mediated NF-κB activation because 6-amino-4-quinazoline is an NF-κB inhibitor. When U266 cells transfected with cloned DNA harboring the luciferase gene containing NF-κB-binding sequence were treated with either IL-6 or a combination of IL-6 and sIL-6R, luciferase activity was increased. When the U266 cells were treated with 6-amino-4-quinazoline, NF-κB activation by IL-6 was inhibited in a dose-dependent manner.

Fig. 1



Effects of various signal transduction inhibitors on the interleukin (IL)-6-induced activation of MAPK and JAK/STAT3 pathways. (a) U266 cells pretreated with various signal transduction inhibitors for 1 h and then stimulated with IL-6 (5 ng/ml) for 15 min. Whole-cell extracts of 30 µg were analyzed by western blot for phosphorylated STAT1, STAT3, total STAT1, STAT3, phosphorylated ERK, and total extracellular signal-regulated kinase (ERK). Lane: 1, control; 2, 5 ng/ml IL-6; 3, 25 ng/ml soluble IL-6 receptor, 5 ng/mL IL-6; 4, 10 µmol/l PD98059, 5 ng/ml IL-6; 5, 20 µmol/l LY294002, 5 ng/ml IL-6; 6, 20 µmol/l JAK inhibitor II, 5 ng/ml IL-6; 7, 20 µmol/l SP600125, 5 ng/ml IL-6; and 8, 2 µmol/l 6-amino-4-quinazoline, 5 ng/ml IL-6. (b) 6-Amino-4-quinazoline blocked IL-6-induced activation of MAPK and JAK/STAT3 pathways. U266 cells were treated with 0.5, 1, and 2 µmol/l of 6-amino-4-quinazoline for 1 h and then stimulated with IL-6 (5 ng/ml) for 15 min, and whole-cell extracts were prepared. Then, 30 µg of whole-cell extracts were analyzed by western blot for phosphorylated STAT1, STAT3, total STAT1, STAT3, phosphorylated ERK, and total ERK. Lane: 1, control; 2, 5 ng/ml IL-6; 3, 0.5 µmol/l 6-amino-4-quinazoline, 5 ng/ml IL-6; 4, 1 µmol/l 6-amino-4-quinazoline, 5 ng/ml IL-6; and 5, 2 µmol/l 6-amino-4-quinazoline, 5 ng/ml IL-6.

The addition of sIL-6R to IL-6, however, partially overcame the inhibitory effects of 6-amino-4-quinazoline (Fig. 2a and b).

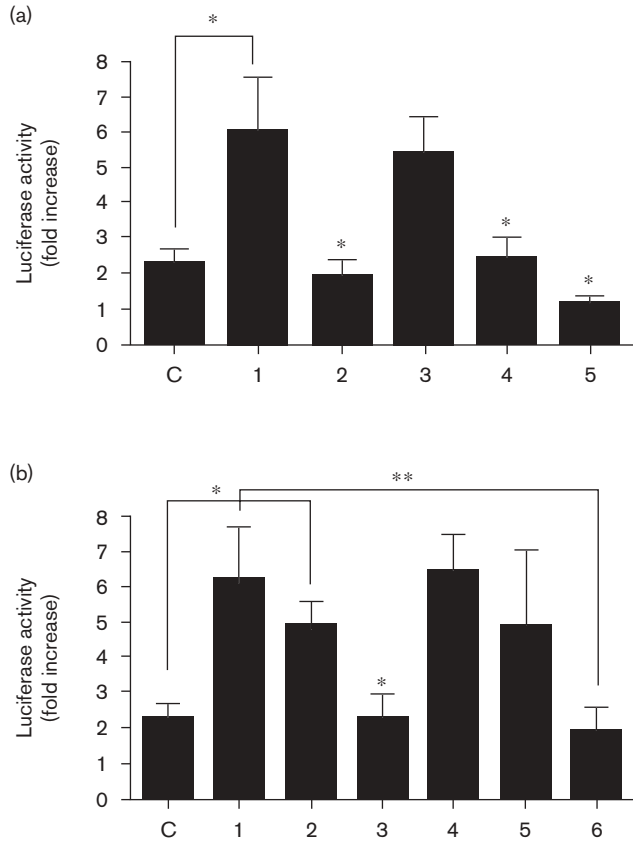
Effect of various signal transduction inhibitors on the secretion of interleukin-6 and soluble interleukin-6 receptor

As the activation of NF-κB is mediated by IL-6 (Fig. 2a and b), we further attempted to determine whether NF-κB activation might influence the production of IL-6 and sIL-6R in U266 cells. U266 cells were treated with a variety of cell signal transduction inhibitors, including 6-amino-4-quinazoline, after cell starvation. IL-6 secretion was inhibited by a variety of cell signal transduction inhibitors. Among them, 6-amino-4-quinazoline was the most potent IL-6 secretion inhibitor. The secretion of sIL-6R remained unaffected by any of the signal transduction inhibitors. These results indicated that the release of IL-6, and not sIL-6R, was regulated by NF-κB (Fig. 3a and b).

Combined treatment of bortezomib and 6-amino-4-quinazoline in U266 cells inhibited interleukin-6 and soluble interleukin-6 receptor-induced MAPK and JAK/STAT pathway

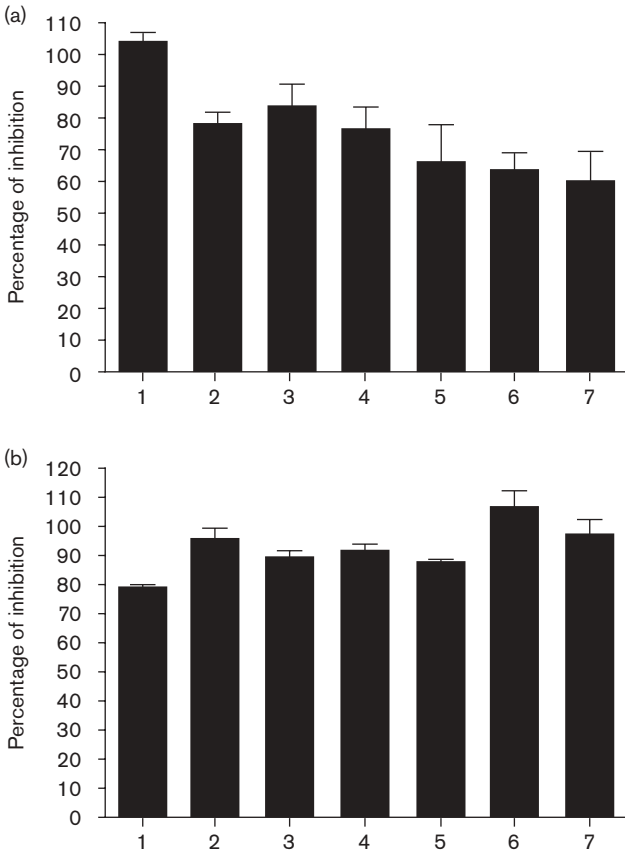
In an effort to determine whether 6-amino-4-quinazoline potentiated the proapoptotic effects of bortezomib, U266 cells were starved for 24 h and treated with either a

Fig. 2



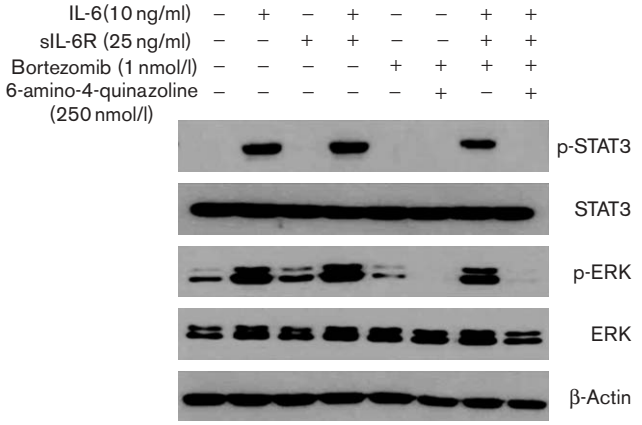
6-Amino-4-quinazoline inhibited the nuclear factor-kappa B (NF-κB) activation induced by interleukin (IL)-6 and soluble IL-6 receptor (sIL-6R). U266 cells were transfected transiently with an NF-κB luciferase reporter plasmid construct. Transfected U266 cells were treated with 6-amino-4-quinazoline for 6 h after serum starvation, and then treated with combined IL-6 and sIL-6R and either IL-6 or sIL-6R alone for 16 h. Luciferase activity was expressed as *n*-fold increase ± standard deviation. (a) Lane: 1, 10 ng/ml IL-6 alone; 2, 6-amino-4-quinazoline 1 μmol/l alone; 3, 10 ng/ml IL-6, 500 nmol/l 6-amino-4-quinazoline; 4, 10 ng/ml IL-6, 1 μmol/l 6-amino-4-quinazoline; 5, 10 ng/ml IL-6, 2 μmol/l 6-amino-4-quinazoline. (b) Lane: 1, 10 ng/ml IL-6; 2, 10 ng/ml IL-6/ 25 ng/ml sIL-6R; 3, 1 μmol/l 6-amino-4-quinazoline alone; 4, 10 ng/ml IL-6/ 25 ng/ml sIL-6R, 500 nmol/l 6-amino-4-quinazoline; 5, 10 ng/ml IL-6/ 25 ng/ml sIL-6R, 1 μmol/l 6-amino-4-quinazoline; and 6, 10 ng/ml IL-6/25 ng/ml sIL-6R, 2 μmol/l 6-amino-4-quinazoline.

Fig. 3



Effect of various signal transduction inhibitors on interleukin (IL)-6 and soluble IL-6 receptor (sIL-6R) secretion. (a) IL-6 secretion: 6-amino-4-quinazoline most effectively suppressed IL-6 secretion of U266 cells among several signal transduction inhibitors. (b) sIL-6R secretion: sIL-6R secretion was not suppressed by signal transduction inhibitors. U266 cells treated with various signal transduction inhibitors for 24 h after serum starvation and then the cell culture supernatants were collected. IL-6 and sIL-6R expression levels were measured via enzyme-linked immunosorbent assay. The data were expressed as the mean ± SE of three independent experiments. Lane: 1, 20 μmol/l LY294002; 2, 10 μmol/l PD98059; 3, 10 μmol/l SB203580; 4, 20 μmol/l AG490; 5, 20 μmol/l JAK inhibitor II; 6, 5 μmol/l SN50; and 7, 2 μmol/l 6-amino-4-quinazoline.

Fig. 4



Combined treatment of bortezomib and 6-amino-4-quinazoline in U266 cells inhibited interleukin (IL)-6 and soluble IL-6 receptor (sIL-6R)-induced MAPK and JAK/STAT pathways. U266 cells were starved for 24 h, then treated with a combination of bortezomib and 6-amino-4-quinazoline or bortezomib alone for 2 h and stimulated with combined IL-6 and sIL-6R or either IL-6 or sIL-6R alone for 15 min, after which the whole-cell extracts were prepared. Then, 30 μg of whole-cell extracts were analyzed via western blotting for phosphorylated STAT3, total STAT3, phosphorylated extracellular signal-regulated kinase (ERK), total ERK, and β-actin.

combination of bortezomib and 6-amino-4-quinazoline or with bortezomib alone for 2 h, and stimulated with a combination of IL-6 and sIL-6R or either agent alone for 15 min. sIL-6R alone exerted no appreciable effect on STAT3 and ERK phosphorylation, but surprisingly, combined treatment with IL-6 and sIL-6R potentiated STAT3 and ERK phosphorylation as compared with IL-6 alone in the U266 cells. Bortezomib alone reduced IL-6-induced STAT3 and ERK phosphorylation to some degree. Surprisingly, combined treatment with bortezomib and 6-amino-4-quinazoline resulted in a complete blockage of IL-6-induced MAPK and JAK/STAT pathways. These findings indicated that 6-amino-4-quinazoline potentiated the proapoptotic effects of bortezomib in U266 cells, and could overcome drug resistance in MM cells (Fig. 4).

Discussion

Many human leukemias and lymphomas involve the constitutive activation of NF-κB in the nucleus [11,15,16]. Persistent NF-κB is critical to cell proliferation, resistance to apoptosis, and tumor formation. NF-κB is considered to modulate the survival and growth of MM cells [7,8]. The adhesion of MM cells to bone marrow stromal cells triggered by NF-κB activation has been shown to induce the paracrine secretion of cytokines [17]. Many cytokines are involved in the growth of tumor cells and the progression of disease, including IL-6 [3,4], insulin-like growth factor [18], vascular endothelial growth factor [19], and tumor necrosis factor (TNF) [20]. Among them, IL-6 is considered to be the most important and best characterized growth stimulatory factor for myeloma cells *in vitro* and *in vivo*. We determined that exogenous IL-6 activated STAT1 and STAT3 and ERK phosphorylation in U266 cells.

Recently, bortezomib (PS-341, Velcade) demonstrated potent activity in both preclinical and clinical studies. Bortezomib, a proteasomal inhibitor, blocked TNF-α-induced NF-κB activation via the inhibition of phosphorylation and degradation of IκB-α [21,22]. Similarly, the efficacy of the targeted NF-κB inhibitor and IκB kinase inhibitor in MM cells was reported earlier. SN50 [14] and PS1145 [13,23] inhibited NF-κB activation and induced apoptosis in MM cells via the activation of proapoptotic proteins. As the signaling pathways involved in targeting NF-κB have yet to be fully elucidated, we assessed the effect of 6-amino-4-quinazoline, a known NF-κB inhibitor, in U266 cells.

We determined that 6-amino-4-quinazoline blocked IL-6-induced MAPK and JAK/STAT pathways most effectively among a variety of signal inhibitors. Even low doses of 6-amino-4-quinazoline completely blocked STAT1, STAT3, and ERK phosphorylation. This shows that NF-κB performs a crucial function in the IL-6-mediated cell signal pathway and NF-κB modulation is a useful target for the development of novel therapeutic agents.

IL-6 is induced by proinflammatory cytokines (TNF-α, IL-1β, and IL-6) modulated by NF-κB (data not shown). Therefore, NF-κB activation may trigger the production of IL-6 in MM cells, and the secretion of IL-6 is induced by IL-6, thereby indicating the autocrine stimulatory loop. On the basis of our results, we attempted to determine whether IL-6 or sIL-6R or a combination of IL-6 and sIL-6R activated NF-κB and 6-amino-4-quinazoline inhibited NF-κB activation. IL-6 or a combination of IL-6 and sIL-6R, but not sIL-6R alone, activated NF-κB, and 6-amino-4-quinazoline inhibited NF-κB activation. The addition of sIL-6R to IL-6 partially negated the inhibitory effects of 6-amino-4-quinazoline. This indicates that sIL-6R potentiates the IL-6 pathway.

Serum IL-6 and sIL-6R levels in MM patients are correlated with disease stages and prognosis [24,25]. Our results indicated that a variety of signal transduction inhibitors suppressed IL-6 secretion, including 6-amino-4-quinazoline, but sIL-6R secretion was not affected by any of the signal transduction inhibitors. This suggested that the regulation of sIL-6R secretion is not associated with the MAPK, JAK/STAT, or NF-κB pathways. We also assessed the effects of a combination of bortezomib and 6-amino-4-quinazoline on IL-6-induced cell signal

pathways, because bortezomib was shown to inhibit NF-κB activation. A combination treatment of bortezomib and 6-amino-4-quinazoline completely inhibited IL-6-induced STAT-3 and ERK phosphorylation as compared with bortezomib alone, thereby suggesting that 6-amino-4-quinazoline potentiated the proapoptotic effect of bortezomib in MM.

In summary, 6-amino-4-quinazoline effectively inhibited IL-6-induced MAPK and JAK/STAT pathways, NF-κB activation, and IL-6 secretion. In addition, 6-amino-4-quinazoline was shown to increase the proapoptotic effect of bortezomib in U266 cells, and completely blocked the STAT-3 and ERK phosphorylation induced by IL-6 and sIL-6R. In conclusion, targeted therapies for the inhibition of NF-κB can provide another form of novel MM therapy and can be used either alone or coupled with existing agents to overcome drug resistance and improve treatment outcomes.

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