Bortezomib Relieves Immune Tolerance in Nasopharyngeal Carcinoma via STAT1 Suppression and Indoleamine 2,3-Dioxygenase Downregulation

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Abstract

Radiotherapy is the primary treatment for nasopharyngeal carcinoma (NPC). Patients with intermediate and advanced stage NPC receiving only radiotherapy have limited survival, so newer immunotherapeutic approaches are sought. The major impediment to better clinical outcomes is tumor immune tolerance. Indoleamine 2,3-dioxygenase (IDO), an IFN-γ-inducible enzyme, is a major inducer of immune tolerance during tumor development; therefore, inhibition of the IDO pathway is an important modality for cancer treatment. We show that bortezomib, a proteasomal inhibitor, inhibited the pathways leading to STAT1 and IRF-1 activation, both of which are necessary for IDO expression. Bortezomib downregulated IFN-γ-induced IDO expression via inhibition of STAT1 phosphorylation and nuclear transliteration, thereby suppressing STAT1-driven IDO transcription in NPC cells. Bortezomib also promoted IkB-α phosphorylation-ubiquitation, which released NF-κB from IkB-α. However, the released NF-κB could not enter the nucleus to conduct its biological effects and accumulated in the cytoplasm. Negative feedback inhibited the transcription of NF-κB, which is important for activating IRF-1 expression. IDO expression is regulated by two important transcription factor binding sites, ISREs, which bind STAT1 and IRF-1, and GASs, which binds STAT1. Bortezomib up-regulated IRF-1 protein by inhibiting its proteasome-dependent degradation, but it also inhibited STAT1 phosphorylation, which directly inhibited the activation of GAS and indirectly inhibited the activation of ISRE, which needs both STAT1 and IRF-1. These discoveries provide a mechanism for the antitumor action of bortezomib and have implications for the development of clinical cancer immunotherapies for preventing and treating NPC.

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Introduction

Nasopharyngeal carcinoma (NPC) is an Epstein–Barr virus (EBV)-associated malignancy with high prevalence in South China and Southeast Asia (1). At present, radiotherapy is still the main treatment for this disease, but the 5- to 10-year survival rate of patients with intermediate and advanced stages who receive only radiotherapy is only 40% (2). Moreover, the disease relapse rate is relatively high, which is associated with poor survival from recurrent or metastatic disease (3). Therefore, development of novel therapeutic strategies against NPC is clearly needed.

The development of therapeutic strategies that block CTLA-4 or PD-1 have had success treating a subset of melanoma patients, as have had other immunotherapies (4, 5). Immunotherapeutic strategies aimed at boosting antitumor immunity are also promising candidates for the treatment of NPC. Many studies using vaccines targeting EBV and other antigens have focused on reversing the impaired immune response to NPC tumors (6, 7). However, the clinical outcomes of these immunotherapeutic strategies have been less effective than anticipated. Immune tolerance to these tumors is still a major impediment in cancer immunotherapy, requiring more elucidation of the immune tolerance mechanisms involved (8, 9).

Cytokine IFN-γ production from tumor-infiltrating lymphocytes (TIL) in NPC patients is greater than that in healthy controls. Secretion of IFN-γ, a key antitumor cytokine, by activated Th1 cells and natural killer (NK) cells, is a trait of a successful tumor vaccine. However, IFN-γ has the potent ability to induce indoleamine 2,3-dioxygenase (IDO) expression in various kinds of tumors including NPC, and IDO was reported as one of the main factors that contribute to tumor-induced immunosuppressive mechanisms (10–13). IDO-positive cells were found scattered in the tumor tissues from patients with NPC, compared with controls, with...
significantly more IDO activity in the plasma of NPC patients, especially among patients with metastatic cancer (2, 14). These reports provide evidence that IDO is involved in tumor immune evasion of NPC, suggesting that it could be a relevant therapeutic target for NPC.

IDO is responsible for initiating the first, rate-limiting step in tryptophan metabolism in the kynurenine (Kyn) pathway. It can induce immune tolerance by depleting tryptophan locally and producing toxic tryptophan catabolites, such as kyn, which can induce proliferation arrest, inactivation, and apoptosis of T lymphocytes or NK cells (15, 16). Therefore, inhibition of the IDO pathway is emerging as an important modality for cancer treatment. Currently, four IDO inhibitors are under clinical development (17–20). In the present study, we found that bortezomib (Velcade; formerly known as PS-341) is a peptide boronate inhibitor of the proteasome that can downregulate the expression of IDO induced by IFNγ in human NPC cells and therefore may provide potential therapeutic strategies in tumor immunotherapy.

Bortezomib was approved by the FDA for the treatment of multiple myeloma and has been evaluated for the treatment of solid tumors (21, 22). Bortezomib combined with the HDAC inhibitor SAHA synergistically induced the killing of NPC cells. The major mechanism of cell death is reactive oxygen species–driven caspase-dependent apoptosis. In vivo, the bortezomib/SAHA combination potently induces apoptosis and suppresses the growth of NPC xenografts in nude mice, which provides the basis from which to progress to clinical testing of this drug combination regimen in patients with NPC (23). Bortezomib can pharmacologically sensitize tumor cells to the lytic effects of dendritic cell (DC)–activated immune effector cells (24). However, the mechanism remains to be fully characterized. Here, we demonstrate that bortezomib downregulated the expression of the IDO induced by IFNγ in NPC cells, primarily by inhibiting the JAK/STAT1 signaling pathway. It throws light on the mechanism by which immune evasion affects the response to treatment of NPC and may have implications for the development of a clinical immunotherapeutic strategy for NPC.

Materials and Methods

**Chemicals and reagents**

IFNγ was purchased from Sigma-Aldrich. Bortezomib was purchased from LC Laboratories. Vectors (pGL3-Enhancer, pRL-TK, and pNF-kB-luc) and dual-luciferase assay kit were purchased from Promega. Protein A/G Sepharose, the polyclonal rabbit antibody to human IDO, and mAbs to STAT1, phospho-(Y701), and p-IκBα (ser32) are products of Santa Cruz Biotechnology Inc. MAbs to β-actin, NF-kB p65, IRF1, IκB-α, and p-IκB-α (ser32) are products of Cell Signaling Technology. SYBR Premix ExTaq II is a product of TaKaRa BIO Inc. The secondary antibody to mouse IgG, conjugated to FITC and DAPI dye, was purchased from Invitrogen.

**Cell lines**

The human NPC cell lines CNE2 and CNE1 were gifts from Sun Yat-sen University (Guangzhou, China) as described previously (25), received on May 2013. The CNE2 line was used from January 2015 to February 2016, and the CNE1 line from July to September 2016. Both cell lines were authenticated by short tandem repeat analysis and passaged for fewer than 6 months before experiments. Vials were thawed and maintained in culture for only several weeks at a time. The CNE2 and CNE1 cell lines were maintained in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated endotoxin-free newborn calf serum (Hyclone), streptomycin (100 μg/mL), and penicillin (100 units/mL) under a humidified 5% CO2 atmosphere at 37°C in a CO2 incubator.

**Cytotoxicity assay**

The cytotoxicity of bortezomib toward the cultured cells was assessed using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays (Sigma Chemical Co.). CNE2 and CNE1 cells were seeded onto 96-well microplates (NunC) at a density of 1 × 10⁴ cells per well and incubated for 24 hours. Cells were then treated with selected concentrations of bortezomib for 24 or 48 hours.

Cells in culture medium alone served as the untreated control. The MTT reagent (5 mg/mL in distilled water) was prepared immediately prior to use. After removing the incubation medium from the wells, cells were washed with PBS, and 10 μL of MTT reagent was added. After incubation for 4 hours at 37°C, MTT reagent in 100 μL of dimethylsulfoxide (DMSO) was added to each well. Surviving cells were then detected by measuring absorbance at 570 nm using a plate reader. The cell viability was expressed as a percentage of the values obtained for the controls.
Western blot analysis

Cells were lysed in cell lysis buffer containing 1% NP-40, 20 mmol/L Tris-HCl (pH 7.6), 0.15 mol/L NaCl, 3 mmol/L EDTA, 3 mmol/L EGTA, 1 mmol/L phenylmethylsulfonyl fluoride, 20 mg/mL aprotinin, and 5 mg/mL leupeptin. Lysates were cleared by centrifugation and denatured by boiling in Laemmli buffer. Equal amounts of protein samples were separated on 12% SDS–polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. Following blocking with 5% non-fat milk at room temperature for 2 hours, membranes were incubated with the primary antibody at 1:1,000 dilution overnight at 4°C and then incubated with a horseradish

Figure 2.

Bortezomib downregulated the IFNγ-induced expression of IDO in a dose-dependent manner, but bortezomib did not inhibited the activity of IDO. CNE2 and CNE1 cells were treated with reagents as indicated, and IDO expression was detected by Western blotting; β-actin served as the loading control. A, CNE2 and CNE1 cells were treated with different concentrations of IFNγ for 24 hours. B, CNE2 and CNE1 cells were treated with IFNγ (100 U/mL) for the indicated times. C, CNE2 and CNE1 cells were pretreated with various concentrations of bortezomib for 2 hours and then treated with IFNγ (100 U/mL) for 24 hours. Similar results were obtained in three independent experiments. D, CNE2 cells were pretreated with or without IFNγ (100 U/mL) for 12 hours, and then we changed the culture media before cells were treated with or without 2 μmol/L bortezomib for 3 hours. The Kyn contents in cell culture media were measured by HPLC. The arrow indicates the HPLC peaks of Kyn in the samples. **, P < 0.001 and #, P > 0.05.
peroxidase–conjugated secondary antibody at 1:5,000 dilution for 1 hour at room temperature. Specific immune complexes were detected using western blotting plus chemiluminescence reagent (Life Science, Inc.).

High performance liquid chromatography analysis of kynurenine
Cells were plated into 12-well plates. When the cell density reached 80%, cells were stimulated with or without IFNγ for 12 hours, then we changed the culture media before cells were treated with or without bortezomib for 3 hours. Cell culture media were collected, and 1/5 volume of trichloroacetic acid was added to precipitate the protein. The collected supernatant was then analyzed by high performance liquid chromatography (HPLC; Waters 1525-2487-717). Briefly, 20 μL of the sample were injected into a Phenomenex Gemini C18 chromatographic column (250 mm x 4.60 mm, 5 μm) and eluted with KH2PO4 buffer (1 mmol/L KH2PO4, pH 4.0) containing 20% methanol at a flow rate of 1.0 mL/min. The Kyn content was detected using an ultraviolet detector at 360 nm for excitation and 366 nm for emission. The retention time was previously determined with standard solutions (Sigma-Aldrich).

Immunoprecipitation
To assess the combination of IκB-α and NF-kB, cells were treated with or without bortezomib and IFNγ for 24 hours, then washed twice with ice-cold PBS, and harvested at 4°C in immunoprecipitation lysis buffer [50 mmol/L HEPES, pH 7.5, 150 mmol/L NaCl, 2 mmol/L EDTA, 0.5% NP-40, 10% glycerol, 1 mmol/L NaF, 1 mmol/L Na3VO4, 1 mmol/L dithiothreitol, 1 mmol/L 4-(2-aminoethyl) benzenesulfonyl fluoride, leupeptin (1 μg/mL), aprotinin (1 μg/mL), and pepstatin (1 μg/mL)]. Equal amounts of protein were immunoprecipitated using NF-kB p65 mAb, and the immune complexes were bound to protein A/G Sepharose. The beads were washed with lysis buffer and subjected to Western blotting with IκB-α mAb.

Confocal microscopy for STAT1 and NF-kB
Cells were grown on chamber slides. After 12 hours of cultivation, cells were stimulated with or without bortezomib and IFNγ. Cells were fixed in 4% paraformaldehyde for 30 minutes, blocked with goat serum 30 minutes at 37°C, and then incubated with STAT1 and NF-kB p65 mAbs at 1:100 for 1 hour at 37°C. Slides were washed with PBS and incubated with a secondary antibody to mouse IgG, conjugated to FITC at 1:1,000 for 45 minutes at 37°C. After washed by PBS, cells were incubated with DAPI (10 μg/mL) for 10 minutes to visualize cell nuclei. Samples were examined with confocal laser scanning microscopy (Zeiss) to analyze nuclear translocation of STAT1 and NF-kB.

Transient transfections and reporter genes assay
To examine the effect of bortezomib on STAT1-dependent transcriptional activity, we used 7 × GAS sequence GAS7 (consensus TTC/ANNG/TAA) and 4 × ISRE (IFN-stimulated response elements) sequence ISRE4 (consensus AGTTTCNN-TTNC/T; ref.26) cloned upstream of the luciferase reporter gene of pGL3-Enhancer. The coding strand of GAS7 plus MluI and XhoI sequences is 5'-CGACCGGTTCAGGAAATTCCAGGAAATTACA-GTAATTACGTAATTCCAGTAATTCTGTAATTCTGTAACCTG-AGCCG-3', and the coding and template strands of GAS7 and ISRE4 were synthesized by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd. and annealed to double strands. The double strands of GAS7 and ISRE4 were digested with MluI and XhoI and inserted into pGL3-Enhancer. For measuring the activation of GAS, ISRE, and NF-κB, cells were transfected with 0.2 μg DNA/cm² per plasmid and lipofectamine 2000 reagent (Gibco BRL) according to the manufacturer’s instructions.

Figure 3.
Bortezomib inhibited STAT1 phosphorylation and nuclear translocation. A, CNE2 cells were treated with or without 2 μmol/L bortezomib for 2 hours and then treated with IFNγ (100 U/mL) for 5 or 15 minutes. The phosphorylation of STAT1 and total STAT1 was detected by Western blotting with a pSTAT1-Y701 mAb and a STAT1 mAb, respectively; β-actin served as the loading control. Similar results were obtained in three independent experiments. Values were compared with the control, and statistically significant values with P < 0.05 are marked with (*). B, CNE2 cells were grown on chamber slides and were pretreated with or without 2 μmol/L bortezomib for 2 hours, followed by treated with or without IFNγ (100 U/mL) for 30 minutes. Immunofluorescence and confocal microscopy were performed as described in the Materials and Methods section. Scale bar, 20 μm.
Transfection efficiency was normalized by cotransfection with pRL-TK. Transcriptional activity was determined by a luminometer, using a dual-luciferase assay kit. Results are displayed as the ratio between the activity of the reporter plasmid and pRL-TK.

Quantitative real-time PCR

To achieve better quantification of NF-κB p65 mRNA expression, NF-κB p65 mRNA was measured by quantitative real-time PCR. CNE2 (2 x 10⁵) cells were pretreated with 2 μmol/L bortezomib for 2 hours, followed by treatment with 100 U/mL IFNγ for 24 hours. Total mRNA was extracted with TRIZOL reagent. The first strand of cDNA was generated from 2 μg total RNA using oligo-dT primer and Superscript II Reverse Transcriptase (GIBCO BRL). Quantitative real-time PCR was run on an iCycler (Bio-rad) using validated primers for β-actin, NF-κB p65, and SYBR Premix Ex Taq II (Takara) for detection. The cycle number when the fluorescence first reached a preset threshold (Ct) allowed the quantification of the specific template concentration. Transcripts of the housekeeping gene β-actin in the same incubations were used for internal normalization. The primer pairs used in the subsequent quantitative real-time PCR reactions were as follows: NF-κB p65, forward 5’-CTGCAGTTTGATGATCTGCAGTTTGATGAT-3’ and reverse 5’-TAGGCGAGTTATAGCCTCAG-3’; β-actin, forward 5’-TGGCAACCAGCAATGGA-3’ and reverse 5’-CTAAGTCATAGTCCGCCTAGAAGCA-3’.

Statistical analyses

All values were reported as mean ± SEM of three independent experiments unless otherwise specified. Data were analyzed by two-tailed unpaired Student t test between two groups and by one-way ANOVA followed by a Bonferroni correction for multiple comparison involved. These analyses were performed using GraphPad Prism Software Version 5.0 (GraphPad Software Inc.). P < 0.05 was considered statistically significant.

Results

Cytotoxicity of bortezomib in NPC cells

An MTT enzyme assay was used to determine the cytotoxicity of bortezomib in NPC CNE2 and CNE1 cells. Bortezomib could suppress the proliferation of cells in a concentration-dependent manner. At 2 μmol/L, the survival rate of cells was about 80%, whereas when cells were exposed to 4 μmol/L bortezomib, the majority of CNE2 and CNE1 cells died (Fig. 1). Therefore, in subsequent experiments, we applied bortezomib at a concentration of 2 μmol/L, which did not affect the survival rate of the NPC cells significantly.

Bortezomib downregulated IFNγ-induced IDO, but did not inhibit its activity

First, the effect of IFNγ on IDO expression was investigated. IFNγ significantly enhanced IDO expression in dose- and

Figure 4.

Bortezomib promoted IκB-α phosphorylation and inhibited combination of IκB-α with NF-κB. A, CNE2 cells were pretreated with or without 2 μmol/L bortezomib for 2 hours and then treated with or without IFNγ for 24 hours. IκB-α protein in cell lysate was detected by Western blotting; β-actin served as the loading control. Similar results were obtained in three independent experiments. Values were compared with the control, and statistically significant values with P < 0.05 are marked with (*). B, CNE2 cells were treated with or without 2 μmol/L bortezomib for 2 hours and then treated with IFNγ (100 U/mL) for 5, 15, and 30 minutes. The phosphorylation of IκB-α and total IκB-α was detected by Western blotting with p-IκB-α(ser32) and IκB-α mAbs, respectively; β-actin served as the loading control. Similar results were obtained in three independent experiments. C, CNE2 cells were pretreated with or without 2 μmol/L bortezomib for 2 hours and then treated with or without IFNγ for 12 hours; total proteins in the cell lysate were subjected to immunoprecipitation with an NF-κB p65 mAb. The IκB-α combined with NF-κB p65 in immune complex were detected by Western blotting with an IκB-α antibody (top). The membrane was stripped of antibodies and immunoblotted with an NF-κB p65 antibody (bottom).
time-dependent manners, from 4 hours at 20 U/mL through 8 hours at 100 U/mL (Fig. 2A and B). We then explored the influence of bortezomib on IDO expression (Fig. 2C). Bortezomib reduced IDO induction (from 0.25 μmol/L) and almost completely inhibited the expression of IDO at a concentration of 2 μmol/L. We also examined the effect of bortezomib on IDO activity (Fig. 2D) and found that while IFNγ increased the concentration of Kyn when compared with the control group, the concentration of Kyn did not change when bortezomib and IFNγ were added together, suggesting that bortezomib did not inhibit the activity of IDO.

Bortezomib inhibited STAT1 phosphorylation and nuclear translocation

IFNγ induces IDO expression through the activation of STAT1. STAT1 phosphorylation at residue Y701 is required for its translocation to the nucleus, where it acts as an active transcription factor (27). Therefore, we asked whether bortezomib interfered with STAT1 Y701 phosphorylation and inhibited its nuclear translocation. Stimulation of cells with IFNγ for 5 minutes alone resulted in a rapid increase in tyrosine phosphorylation of STAT1, and this increase was remarkably inhibited by bortezomib treatment (Fig. 3A). We further studied whether bortezomib inhibited STAT1 nuclear translocation. Results in untreated cells showed that STAT1 was localized exclusively in the cytoplasm, and bortezomib did not alter basal subcellular localization of STAT1. In contrast, treatment of cells with IFNγ for 30 minutes induced a significant nuclear translocation of STAT1, but pretreatment of cells with bortezomib for 2 hours markedly reduced IFNγ-induced nuclear translocation of STAT1 (Fig. 3B). Taken together, these results suggested that bortezomib blocked IFNγ-induced expression of IDO and that this block might be a result of inhibition of phosphorylation and nuclear translocation of STAT1.

IRF-1 phosphorylation and release of NF-κB by bortezomib

IRF-1 is an important factor for IFNγ-induced IDO expression, and the IRF-1 promoter region contains both GAS elements and NF-κB binding motifs. Normally, NF-κB is complexed with IκB-α. When IκB-α is phosphorylated, it is degraded by proteasomes, releasing the NF-κB subunit to conduct its biological effects (28). Stimulation of cells with bortezomib resulted in a rapid increase in phosphorylation of IκB-α (Fig. 4A). Further studies demonstrated that phosphorylated IκB-α was partially degraded (Fig. 4B, black arrow). To determine whether IκB-α released NF-κB after it degradation, NF-κB p65 was immunoprecipitated, and an IκB-α mAb was used to detect IκB-α by Western blotting. After treatment with bortezomib, the amount of IκB-α complexed with NF-κB decreased, which revealed that bortezomib promoted the release of NF-κB from IκB-α (Fig. 4C).

NF-κB translocation unaffected, but IRF-1 upregulated by bortezomib

Because IFNγ can induce the expression of NF-κB and promote its nuclear translocation, we determined whether bortezomib interfered with NF-κB nuclear translocation by confocal microscopy with a NF-κB p65 mAb. NF-κB was localized exclusively in the cytoplasm of untreated cells, and bortezomib did not alter basal subcellular localization of NF-κB p65. In contrast, treatment of cells with IFNγ for 30 minutes induced a significant nuclear translocation of NF-κB p65. Pretreatment of cells with bortezomib for 2 hours did not increase IFNγ-induced nuclear translocation of NF-κB, which was unexpected, given that bortezomib increased the amount of free NK-κB available to be translocated (Fig. 4). Together, this suggested that the released NF-κB from IκB-α could not enter the nucleus to conduct its biological effects (Fig. 5A).

We then determined whether bortezomib affected expression of the IRF-1 protein, because IRF-1 is important for IFNγ-induced expression of IDO and is directly upregulated by NF-κB. Results showed that IFNγ significantly upregulated IRF-1 expression. However, whereas bortezomib alone could not induce the expression of IRF-1, the amount of the IRF-1 protein in the group treated with both bortezomib and IFNγ was significantly increased compared with the group treated with IFNγ alone (Fig. 5B). This may be the result of bortezomib inhibiting the degradation of IRF-1 protein.
Bortezomib blocked IFNγ-induced activation of GAS, ISRE, and NF-κB

We demonstrated that tyrosine phosphorylation of STAT1 was inhibited by bortezomib. Therefore, we examined whether the inhibitory effect of bortezomib can block transcriptional activation of STAT1. The reporter gene plasmids pGL3-Enhancer-GAS7-luc, pGL3-Enhancer-ISRE4-luc, and pNF-κB-luc were transfected into CNE2 cells. Transfection efficiency was normalized by cotransfection with pRL-TK. It demonstrated that dual-Glo-Luciferase analysis revealed that IFNγ significantly enhanced the activity of pGL3-Enhancer-GAS7-luc, pGL3-Enhancer-ISRE4-luc, and pNF-κB-luc, whereas bortezomib markedly inhibited the activity of pGL3-Enhancer-GAS7-luc, pGL3-Enhancer-ISRE4-luc, and pNF-κB-luc (Fig. 6A–C). These data suggested that bortezomib downregulated IFNγ-induced expression of IDO by blocking STAT1 phosphorylation, which drives activation of the GAS and ISRE. The transcriptional activation of NF-κB may be inhibited by the negative feedback induced by the increased NF-κB proteins released from IκBα, as shown by quantitative real-time PCR (Fig. 6D), demonstrating that bortezomib can downregulate the production of NF-κB mRNA significantly.

Discussion

It was reported that the promoter of IDO gene contains a series of putative transcription factor binding sites, including one GAS sequence, two ISRE regions, and two AP-1 binding sites (29, 30). As an important transcriptional factor for the IFNγ-induced expression of IDO, STAT1 is phosphorylated by JAK first, then STAT1 dimerizes and translocates to the nucleus, where it binds GAS to activate IDO and IRF-1 gene expression directly. But STAT1 also contributes indirectly by inducing production of IRF-1, whose promoter region contains both GAS elements and NF-κB binding motifs, and both elements are important for IRF-1 expression. The IRF-1 protein then binds to ISRE-1 and ISRE-2 elements in the IDO regulatory region to activate IDO gene expression (31, 32).

In the present study, we demonstrated that bortezomib did not inhibit the activity of IDO in CNE2 cells, but it downregulated the expression of IDO induced by IFNγ in a dose-dependent manner. Dual-Glo-Luciferase analysis revealed that cells treated with IFNγ significantly induced transcriptional activity of the reporter plasmids pGL3-Enhancer-GAS7, pGL3-Enhancer-ISRE4, and pNF-κB-luc, suggesting the importance of GAS, ISRE, and NF-κB in IFNγ-induced expression of IDO. Furthermore, our results showed that bortezomib significantly inhibited IFNγ-induced transcriptional activation of the reporter plasmids pGL3-Enhancer-GAS7, pGL3-Enhancer-ISRE4, and pNF-κB-luc. This phenomenon could be explained by the fact that bortezomib inhibited STAT1 phosphorylation and nuclear translocation, which was important and enough for STAT1 binding GAS to activate IDO expression. Unlike GAS, however,

Figure 6.

Effect of bortezomib on IFNγ-induced activation of GAS, ISRE, and NF-κB. CNE2 cells were transfected with pGL3-Enhancer-GAS7-luc (A), pGL3-Enhancer-ISRE4-luc (B), or pNF-κB-luc (C) reporter plasmids and treated with 2 μmol/L bortezomib for 2 hours, followed by treatment with IFNγ (100 U/mL) for 24 hours. Luminescence was measured with a luminometer. pRL-TK plasmids served to correct transfection efficiency. Results are expressed as the ratio between the activity of the reporter plasmid and pRL-TK. *P < 0.05 and **P < 0.001. D) CNE2 cells were pretreated with 2 μmol/L bortezomib for 2 hours, followed by treatment with 100 U/mL IFNγ for 24 hours; NF-κB p65 mRNA was detected by quantitative real-time PCR. *P < 0.05.
activation of ISRE needed both phosphorylated STAT1 and IRF-1. Although bortezomib could upregulate the expression of IRF-1 by inhibiting its degradation, phosphorylated STAT1 was more important than IRF-1. When the phosphorylation of STAT1 was inhibited, the ISRE was inactivated. These results suggested that bortezomib inhibited the IDO expression mainly by interfering with phosphorylation and nuclear translocation of STAT1 to inhibit its binding to GAS and ISRE elements in the IDO regulatory region. Still unclear is the mechanism by which bortezomib inhibited IFNγ-induced transcriptional activation of the reporter plasmid pNFxB-luc.

Bortezomib is the first proteasomal inhibitor to be used therapeutically for treating relapse cases of multiple myeloma and mantle cell lymphoma. Preclinically, it has activity in many hematologic and solid malignancies as a single agent and in combination with other chemotherapy agents in models of multiple myeloma, acute leukemia, and lung and pancreatic cancers (33–35). One of the major mechanisms associated with the anticancer activity of bortezomib is through upregulation of NOXA, which is a proapoptotic protein. NOXA may interact with the antiapoptotic proteins of Bcl-2 subfamily Bcl-XL and Bcl-2, which results in apoptotic cell death of malignant cells. Another important mechanism of bortezomib is through suppression of the NF-kB signaling pathway, resulting in the downregulation of its antiapoptotic target genes (36). The prototypical mechanism of NF-kB activation depends on the signal-induced phosphorylation and ubiquitination of an inhibitory protein called IkB-α, which is subsequently degraded by the proteasome (37).

In this article, we found that bortezomib promoted IkB-α phosphorylation, then the phosphorylated IkB-α was partially degraded after ubiquitination. Further immunoprecipitation demonstrated that bortezomib promoted IkB-α phosphorylation, leading to partial ubiquitination and degradation, and the amount of IkB-α complexed with NF-kB decreased. This revealed that bortezomib enhanced the IkB-α release of NF-kB, which was then free to conduct its biological effects. However, imaging results showed that the group treated with both bortezomib and IFNγ did not have increased NF-kB nuclear translocation compared with the group treated with IFNγ alone, which means that the NF-kB released from IkB-α could not enter the nucleus to conduct its biological effects. The accumulated NF-kB in the cytoplasm was a source of negative feedback that inhibited the transcriptional expression of NF-kB, explaining how bortezomib inhibited IFNγ-induced transcriptional activation of the reporter plasmid pNFxB-luc.

In summary, we demonstrated in NPC cells that bortezomib downregulated IFNγ-induced IDO expression by inhibiting STAT1 phosphorylation and nuclear translocation, which suppressed STAT1-driven transcription of IDO. Although bortezomib can promote IkB-α phosphorylation-ubiquitination to release the NF-kB from IkB-α, the released NF-kB cannot enter into the nucleus to conduct its biological effects. Accumulated NF-kB in the cytoplasm could induce negative feedback inhibition of NF-kB transcription, which then inhibited IRF-1 transcription, because its promoter region contains both GAS elements and NF-kB binding motifs. However, IRF-1 is a protein degraded by the proteasome, and as a proteasome inhibitor, bortezomib can inhibit the degradation of IRF-1 and indirectly increase the amount of IRF-1 protein. Nevertheless, because phosphorylation of STAT1 is important for induction of IDO through both GAS and ISRE sequences, although bortezomib can increase IRF-1 protein concentrations, the inhibition of STAT1 phosphorylation by bortezomib directly inhibited the activation of GAS and indirectly inhibited the activation of ISRE, which binds both STAT1 and IRF-1 (Fig. 7). Although the dose of bortezomib (2 μmol/L) in this in vitro experiment is not suitable for clinical application, due to toxicities such as neuropathy, structurally modified versions that have reduced toxicity may be worth pursuing. In general, this mechanism of antitumor action of bortezomib may have implications for the development of clinical cancer immunotherapy, especially that which combines bortezomib with NPC-associated tumor vaccines, as a promising method for preventing and treating NPC.
Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: G.-M. Jiang
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