Bortezomib sensitizes human glioblastoma cells to induction of apoptosis

by Type I interferons through NOXA expression and Mcl-1 cleavage

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Abstract

Glioblastomas are highly invasive and aggressive primary brain tumors. Type I interferons have significant, pleiotropic anticancer activity. However, through various pathways many cancers become interferon-resistant, limiting interferon's clinical utility. In this study, we demonstrated that the proteasomal inhibitor bortezomib sensitized human glioblastoma cells to the antiproliferative action of interferons, which involved the induction of caspase-dependent apoptosis but not necroptosis. We found that death ligands such as TRAIL (TNF-related apoptosis-inducing ligand) were not involved in interferon/bortezomib-induced apoptosis, although interferon induced TRAIL expression. However, apoptosis was induced through an intrinsic pathway involving increased NOXA expression and Mcl-1 cleavage. Our findings may provide an important rationale for combining type I interferons with bortezomib for glioblastoma therapy.

Keywords: apoptosis; interferon; bortezomib; glioblastoma

Abbreviations: **IFN**, interferon; **IFN α** , interferon alpha; **IFN β** , interferon beta; **ISG**, IFN-stimulated gene; **TRAIL**, TNF-related apoptosis-inducing ligand; **Mcl-1**, Myeloid cell leukemia 1; **qPCR**, quantitative real-time polymerase chain reaction; **DR4**, death receptor 4; **DR5**, death receptor 5; **FADD**, Fas-associated protein with death domain; **DN-FADD**, dominant-negative FADD; **MTT**, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **PARP**, poly (ADP-ribose) polymerase.

Introduction

Glioblastoma (GBM) is the most aggressive primary brain tumor, and among the deadliest forms of human cancer [1, 2]. Despite poor drug delivery across the blood-brain barrier and chemoresistance, chemotherapy plays an important role as an adjuvant to surgical resection in treating GBM. Type I interferons (IFNs) are a family of pleiotropic endogenous proteins and the first human cytokines found to have significant anticancer activity [3]. IFNs inhibit cell proliferation, regulate cellular responses to apoptosis, and modulate angiogenic and immunomodulatory responses by inducing the expression of IFN-stimulated genes (ISGs). Many ISGs have pro-apoptotic activities, including TNF-related apoptosis-inducing ligand (TRAIL), Fas receptor and its ligand (FasL), X-linked inhibitor of apoptosis (XIAP) associated factor 1 (XAF1) that counteracts the action of XIAP, and NOXA [4, 5]. However, IFNs also activate several pro-survival pathways including the NF- κ B pathway that antagonizes apoptosis [6, 7].

The Bcl-2 protein family regulate apoptosis and contain one or more Bcl-2 homology (BH) domains. They can have pro-apoptotic (Bax, Bak, Bid and Bim) or anti-apoptotic activity (Bcl-2, Bcl-xL and Mcl-1) [8]. NOXA belongs to the BH3-only Bcl-2 subfamily with pro-apoptotic activity [9]. NOXA competes with BAK1 or Bim for Mcl-1 binding and triggers the proteasomal degradation of Mcl-1. Various apoptotic stimuli induce NOXA expression and activation promoting Mcl-1 inactivation and degradation, and initiating apoptosis [10-14].

Bortezomib is the first proteasomal inhibitor to receive approval for treating cancer [15, 16]. Bortezomib induces cell growth arrest and apoptosis in human GBM cell lines [17], and sensitizes them to cell death induced by TRAIL (tumor necrosis factor-related apoptosis-inducing ligand)[18]. Bortezomib-induced apoptosis involves NF- κ B inactivation and/or NOXA up-regulation [19]. In the present study we examined whether bortezomib sensitized human GBM cells to type I IFNs.

Materials and Methods

Cell culture and reagents: Human GBM SJG2 and MT330 cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Cellgro), supplemented with 10% fetal bovine serum (FBS, Atlanta biologicals). Reagents used include recombinant human IFN α and IFN β (Intermune and Biogen, respectively), bortezomib (EMD Millipore), necrostatin-1 and staurosporine (Sigma), z-VAD-FMK, z-IETD-FMK and z-LEHD-FMK (BD Biosciences), and anti-TRAIL antibody (Enzo Lifescience).

Plasmid constructs: The coding sequence for dominant negative FADD was amplified from an SJG2 cDNA library and cloned into the Kpn I and Xho I sites in pcDNA4.0 TO/Myc-HIS. The primers used for plasmid constructing are DN-FADD (forward: 5'-CGGGGTACCACCATGGACTTCGAGGCGGGGGCG-3' and reverse 5'-CCGCTCGAGGGACGCTTCGGAGGTAG-3').

Cell viability and apoptosis assays: GBM cells (5×10^3 cells per well of 96-well plates) were exposed to 1000 IU/ml interferon for 24 hr, and then treated with varying bortezomib concentrations for an additional 48 hr. Fifty μ g of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) was added to each well and incubated at 37 °C for 4.5 hours. Oxidized MTT was solubilized by adding 100 μ L of 1% sodium dodecyl sulfate (SDS) in 0.01 N HCL, plates were incubated at 37 °C overnight, and absorbance determined at 570 nm on an ELISA plate reader (Bio-Rad, Modal 550). For determining apoptosis, GBM cells (5×10^5 cells per 10-cm plate) were exposed to 1000 U/ml IFN for 48 hr, and then treated with 5 nM bortezomib for an additional 24 hr. Cells were harvested with trypsin, washed twice with ice-cold PBS, stained with propidium iodide (PI) and Annexin V at 22°C for 15 min, and analyzed on a flow cytometer (Accuri, Model C6).

Quantitative real time PCR (qPCR): Total RNA was isolated from GBM cells using RNeasy[®] Mini Kit (Qiagen). qPCR was performed with 40 ng RNA using iScript[™] One-Step RT-PCR kit with SYBR[®] Green (Bio-Rad) as previously described [20], using the following primers: TRAIL (forward: 5'-

GAGCTGAAGCAGATGCAGGAC-3' and reverse 5'-TGACGGAGTTGCCACTTGACT-3'), DR4 (forward: 5'-GGGTCCACAAGACCTTCAAGT-3' and reverse: 5'-TGCAGCTGAGCTAGGTACGA-3'), DR5 (forward: 5'-AGACCCTTGTGCTCGTTGTC-3' and reverse: 5'-TTGTTGGGTGATCAGAGCAG-3'), Fas (forward: 5'-GTGGACCCGCTCAGTACG-3' and reverse: 5'-TCTAGCAACAGACGTAAGAACCA-3'), FasL (forward: 5'-TGGGGATGTTTCAGCTCTTC-3' and reverse: 5'-TGTGCATCTGGCTGGTAGAC-3'), FADD (forward: 5'-CCGAGCTCAAGTTCCTATGC-3' and reverse 5'-AGGTCTAGGCCGCTCTGC-3'), and GAPDH (forward: 5'-TGTTGCCATCAATGACCCCTT-3' and reverse: 5'-CTCCACGACGTACTCAGCG-3').

Caspase-3 activity assay: GBM cells were treated with 10 nM bortezomib for 8 hr in the presence or absence of 1000 U/ml IFN β . Cells (4×10^6) were lysed in 100 μ l of cell lysis buffer from caspase-3 colorimetric activity assay kit (Millipore) at 4°C for 10 min, and the cytosolic extract was prepared by centrifugation (10,000xg for 5 min). Cytosolic proteins (100 μ g) was mixed with 20 μ l of 5x assay buffer and 10 μ l Ac-DEVD-pNA solution and incubated for 2 hr at 37°C. Absorbance at 405 nm was determined on an ELISA plate reader. As a control, samples were preincubated with the caspase-3 inhibitor Ac-DEVD-CHO for 10 min at 22°C prior to the addition of the Ac-DEVD-pNA solution.

Cell lysates and immunoblotting: Cells were harvested by trypsinization and lysed in Pierce IP lysis buffer containing 25mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EDTA, 1% NP-40 and 5% glycerol. To determine cytochrome C release, mitochondrial and cytoplasmic lysates were prepared as previously described [21]. Cell lysates were analyzed on SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore) and immunoblotted with primary antibodies (cleaved caspase-3, caspase-8 and -9 from Pierce; and PARP, GAPDH, c-Myc, cytochrome C, Bcl-2, Bcl-xL, Bak, Bax, Bim, NOXA, and Mcl-1 from Santa Cruz), followed by IRDye 800CW goat anti-mouse IgG or IRDye680 goat anti-rabbit IgG. Blots were visualized on an Odyssey Infrared Imaging System (LI-COR Biosciences).

RNA interference: NOXA expression in SJG2 cells was knocked down by transient transfection of NOXA siRNA (Santa Cruz; sc-37305) using Lipofectamine 2000.

Data analysis: All quantitative data represent at least three independent experiments performed in duplicate, at least, and presented as mean $\pm$ S.D. Data were analyzed by two-tailed Student's *t* test, and considered significant at *p* values <0.05 .

Results

Bortezomib sensitizes glioblastoma cells to type I IFNs. We previously found that SJG2 and MT330 GBM cells were relatively insensitive to the antiproliferative effect of IFN [22]. We first tested the activity of bortezomib alone on GBM cell viability by exposing SJG2 and MT330 cells to varying concentrations of bortezomib (from 0 to 20 nM) for 72 hr. Bortezomib resulted in a dose-dependent inhibition of cell proliferation with the greatest effect obtained at highest concentration (20 nM) in SJG2 and MT330 (Fig. 1A) cells, but cells showed a slight sensitivity at low concentrations (<5 nM). GBM cells were then treated with 1000 U/ml Type I IFNs for 72 hr in the presence or absence of bortezomib. While IFN α/β treatment alone resulted in a slight inhibition of cell proliferation, treatment with bortezomib markedly sensitized GBM cells to the antiproliferative action of both IFN α and IFN β even at low doses of bortezomib (1 to 5 nM) (Fig. 1A). These data suggest that bortezomib enhances the antiproliferative action of IFNs in GBM cells.

Combining IFN β with bortezomib induces apoptosis but not necroptosis. Type I IFNs induce apoptosis, which is antagonized by an IFN-induced cell survival pathway [23, 24], but also reportedly induce necroptosis [25]. To determine the role of apoptosis and necroptosis in the marked antiproliferative effect of IFN and bortezomib, we performed MTT assays in the presence of the necroptosis inhibitor, necrostatin-1, and apoptosis inhibitor, Z-VAD-fmk (Z-VAD). IFN β was used, because it had a slightly more potent antiproliferative effect than IFN α . As a positive control for apoptosis, cells were treated with staurosporine, which induced marked cell death in SJG2 and MT330 (Fig. 1B) cells. Cell death in both GBM cell lines induced by staurosporine and the IFN β /bortezomib combination was markedly inhibited by Z-VAD (apoptosis inhibitor), but not by necrostatin-1 (necroptosis inhibitor) (Fig. 1B). These results strongly suggest that IFN β /bortezomib combination induces apoptosis in GBM, and this is largely responsible for the loss of GBM cell viability.

GBM cells stained with annexin V and propidium iodide, and early-stage (annexin+/propidium-) and late-stage (annexin+/propidium+) apoptosis determined by flow cytometry. The IFN β /bortezomib combination enhanced early and late apoptosis in both GBM cells (Fig. 1C). The cleavage of poly (ADP-ribose) polymerase (PARP) and caspases are associated with apoptosis. While treatment with the IFN β /bortezomib combination markedly increased the cleavage of PARP in both GBM cells as compared to vehicle-treated control cells, treatment with bortezomib or IFN β alone showed little or no detectable PARP cleavage (Fig. 2A). Moreover, in SJG2 cells treated with the IFN β /bortezomib combination, marked cleavage of caspase-8, -9 and -3 (Fig. 2B) was observed. The increased caspase-3 activity in SJG2 cells treated with the IFN β /bortezomib combination or staurosporine, was significantly inhibited by addition of the caspase-3 specific inhibitor Ac-DEVD-CHO (Fig. 2C). Moreover, the enhanced antiproliferative effect of the IFN β /bortezomib combination was inhibited by inhibitors for caspase-3 inhibitor (z-VAD-FMK), caspase-8 (z-IETD-FMK) and caspase-9 (z-LEHD-FMK) (Fig. 2D). These data indicate that the IFN β /bortezomib combination induced caspase-dependent apoptosis in GBM cells.

The IFN β /bortezomib combination induced apoptosis independent of the extrinsic pathway.

Caspase-dependent apoptosis can be induced through extrinsic and intrinsic pathways. The extrinsic pathway involves the interaction of death ligands and their receptors at the cell surface. Since the death ligand, TRAIL, is an ISG [26-28], we examined whether death ligands and their receptors are involved in the apoptosis induced by the IFN β /bortezomib combination treatment. As expected, TRAIL gene expression in SJG2 cells was significantly induced by IFN β as determined by qPCR, but this induction was markedly inhibited by bortezomib treatment (Fig. 3A). In contrast, bortezomib alone slightly increased the gene expression of two TRAIL receptors, DR4 and DR5, whose expression was unaffected by IFN β treatment (Fig. 3A). To determine whether TRAIL is involved in apoptosis induced by the IFN β /bortezomib combination, we examined the effect of anti-TRAIL antibody that blocks TRAIL and receptor interaction. While anti-TRAIL significantly blocked cell death induced by TRAIL peptide alone

or in combination with bortezomib, anti-TRAIL had no effect on cell death induced by the IFN β /bortezomib combination, indicating that this apoptosis pathway was TRAIL-independent (Fig. 3B). The extrinsic pathway of caspase-dependent apoptosis is mediated by FADD (Fas-Associated protein with Death Domain). FADD expression was not affected by IFN β alone or in combination with bortezomib in SJG2 cells (data not shown). Moreover, expression of a dominant-negative FADD construct had no significant effect on cell death induced by the IFN β /bortezomib combination (Fig. 3C), indicating that the extrinsic death pathway is not involved in apoptosis induced by the combination of IFN β and bortezomib.

Combination of IFN β and Bortezomib induces apoptosis via intrinsic pathway and involves stimulating the expression of NOXA and the cleavage of Mcl-1. Intrinsic apoptosis is characterized by the release of cytochrome C from mitochondria into cytoplasm. IFN β /bortezomib combination treatment in SJG2 cells increased cytochrome C release into cytoplasm when compared to either agent alone (Fig. 3D). To define whether other apoptotic proteins play a role in this pathway, we immunoblotted for the expression of Bcl-2 family members: anti-apoptotic Bcl-2, Bcl-xL and Mcl-1, pro-apoptotic Bak and Bax, and the BH3-only proteins Bim and NOXA. IFN β treatment had no effect on the levels of the anti-apoptotic Bcl-2 members, Bcl-2, Bcl-xL and Mcl-1 (Fig. 3E). Bortezomib slightly decreased Bcl-2 expression, but increased the expression and cleavage of Mcl-1 (Fig. 3E), which was abolished by the caspase inhibitors, Z-VAD, Z-IETD and Z-LEHD (Fig. 3F). Neither IFN β or bortezomib affected the expression of pro-apoptotic Bak and Bax (Fig. 3E). However, IFN β and bortezomib alone and in combination induced Bim cleavage (Fig. 3E) and stimulated the expression of NOXA at both the protein (Fig. 3E) and mRNA levels (Fig. 4A). To determine the role of NOXA in IFN β /bortezomib-induced apoptosis, NOXA expression was knocked down using siRNA. Partial NOXA knockdown was confirmed by qPCR analysis (Fig. 4B) and immunoblotting (Fig. 4C). Most interestingly, Mcl-1 cleavage induced by the IFN β /bortezomib combination was inhibited to a similar extent by NOXA siRNA (Fig. 4C), and

blocked cell death in both SJG2 and MT330 cells (Fig. 4D) indicating NOXA is a key mediator of apoptosis induced by the IFN β /bortezomib combination.

Discussion

Experimental studies have shown induction of apoptosis by type I IFNs in tumor systems including brain tumors. In this study, we examined the effect of the combination of type I IFNs and the proteasome inhibitor bortezomib in the induction of apoptosis in GBM cells *in vitro*. In addition, we found that the mechanism by which IFN β /bortezomib-induced apoptosis involved up-regulation of NOXA and the cleavage of Mcl-1. Type I IFNs exert their anti-tumor effects by inhibiting cell proliferation and inducing cell death through apoptosis [26, 29]. Type I IFN was reported to induce cell death through necroptosis in macrophages during parasitic infection [25], and induced necroptosis when FADD was deficient or disabled by phosphorylation, or when caspases were inactivated [30]. By using inhibitors of apoptosis and necroptosis, we revealed that the antiproliferative effect of IFN β /bortezomib in GBM cells involved apoptosis instead of necroptosis. TRAIL is a promising anticancer agent, and its sensitivity can be enhanced by bortezomib, which is consistent with previous studies in GBM cells [18]. However, while TRAIL expression was markedly reduced by bortezomib treatment, we found that inactivating TRAIL with anti-TRAIL antibody did not affect IFN β /bortezomib-induced cell death, indicating that TRAIL was not directly involved in this pathway.

Bcl-2 family proteins are apoptosis regulators that govern mitochondrial outer membrane permeabilization [8]. All pro-apoptotic Bcl-2 proteins contain a BH3 domain which is necessary for dimerization with other Bcl-2 proteins and is crucial for their apoptotic activity. NOXA is a BH3-only protein, which mediates apoptosis induced by pro-apoptotic agents, including bortezomib [12] and IFNs [10, 11]. We found that IFN induced NOXA expression, which was augmented by bortezomib treatment in GBM cells. Consistent with previous findings [31], NOXA also appeared to promote the Mcl-1 degradation, since silencing NOXA expression by siRNA interference inhibited to induction of apoptosis and Mcl-1 degradation by the IFN β /bortezomib combination. Our data strongly support that NOXA/Mcl-1 axis plays a key role in IFN β /bortezomib-induced apoptosis. In addition, IFN β /bortezomib induced the cleavage of another BH3-only protein, Bim, which might trigger a positive feedback amplification of

apoptotic signaling [32]. In the present study, we demonstrated that up-regulation of NOXA/Mcl-1 cleavage mediated the apoptosis induced by the combination of IFN β /bortezomib in GBM cells. Moreover, the combination treatment lowered the effective doses of agents needed to reduce cell viability, and thus possibly reducing toxic side-effects. Taken together, our results provide a rationale for the combinational therapy of type I IFNs and bortezomib for GBM.

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Figure legends

Figure 1. Bortezomib sensitizes GBM cells to apoptosis induced by type I IFNs, but not necroptosis.

(A) SJG2 and MT330 GBM cells were treated with bortezomib at indicated concentrations in the absence or presence of 1,000 U/ml IFN α or IFN β for 72 hr, and then subjected to MTT assays and the data presented as relative to vehicle-treated cells. (B) GBM cells were pretreated with 10 μ g/ml z-VAD-FMK (z-VAD) or 10 μ g/ml necrostatin 1 (Nec) in the presence of 1,000 U/ml IFN β for 24 hr, and then exposed to 10 nM bortezomib (Bor) for an additional 24 hr or 1 μ M staurosporine (St) for 1 hr, subjected to MTT assays and the data presented as relative to vehicle-treated cells. (C) GBM cells were cultured in the presence of 1,000 U/ml IFN β for 2 days and then exposed to 5 nM bortezomib (Bort) for an additional 24 hr. Apoptosis was analyzed by flow cytometry of propidium iodide and annexin V stained cells [33], and data presented relative to vehicle control cells. *, $p < 0.05$ and **, $p < 0.01$ by Student's t test.

Figure 2. IFN β /bortezomib-induced apoptosis is caspase dependent.

(A) SJG2 and MT330 cells were cultured in the presence of 1,000 U/ml IFN β for 2 days and then exposed to 10 nM bortezomib (Bort) for an additional 20 hours. Cell lysates were immunoblotted for PARP with GAPDH serving as loading control. (B) SJG2 cells were treated as above and immunoblotted for caspase-8 and -9 and cleaved caspase-3, or (C) assayed for caspase-3 activity, and caspase-3 activity relative to vehicle control presented. (D) SJG2 cells were treated with 10 μ g/ml caspase inhibitors: z-VAD-FMK (pan-caspase), z-IETD-FMK (caspase-8) or z-LEHD-FMK (caspase-9) in the presence of 1,000 U/ml IFN β for 48 hr, then exposed to 5 nM bortezomib (bor) or 1 μ M staurosporine (St) for 24 hr. Cells were subjected to MTT assays and the data presented as relative to vehicle-treated cells. *, $p < 0.05$; **, $p < 0.01$ by Student's t test.

Figure 3. Apoptosis induced by the combination of IFN β and bortezomib through the intrinsic pathway.

(A) SJG2 cells were pretreated with 10 nM bortezomib (Bort) for 1 hr, and then exposed to 1,000 U/ml IFN β for an additional 5 hr. The gene expression was determined by qPCR and data presented as expression relative to GAPDH. (B) SJG2 cells were treated with 1 μ g/ml anti-TRAIL antibody in the

presence of 1,000 U/ml IFN β for 48 hr, and then exposed to 5 nM bortezomib for an additional 24 hr. As a positive control, cells were treated with 5 nM TRAIL or /and bortezomib in the absence or presence of anti-TRAIL antibody for 24 hr. Data are presented as number of viable cells relative to vehicle control. (C), SJG2 cells were transfected with DN-FADD expression vector or empty-vector (EV). At 5 hr after transfection, cells were treated with IFN β and/or bortezomib and subjected to MTT assays. The insert shows DN-FADD expression as determined by Myc immunoblotting. (D,E) SJG2 cells were treated with 1,000 U/ml IFN β for 24 hr and followed by 20 hr bortezomib (10 nM) treatment. Mitochondrial and cytoplasmic fractions were prepared and (D) cytochrome C and (E) Bcl-2 family proteins were detected by western blot, with GAPDH serving as a loading control. Arrow shows the cleaved Mcl-1 band. (F) SJG2 cells were treated with 10 μ g/ml caspase inhibitors in the absence or presence of IFN β and bortezomib treatment. Mcl-1 cleavage (arrow) was detected by immunoblotting with GAPDH as loading control. *, $p < 0.05$; **, $p < 0.01$ by Student's t test.

Figure 4. Combination of IFN β and Bortezomib induces apoptosis via stimulating the expression of NOXA and the cleavage of Mcl-1. (A) SJG2 cells were treated with 10 nM bortezomib (Bort) for 1 hr and then exposed to 1,000 U/ml IFN β for 5 hr. NOXA mRNA level was determined by qPCR. Data are presented as gene expression relative to GAPDH. (B) SJG2 cells were transfected with NOXA siRNA for 30 hours, scrambled RNA was used as control. NOXA expression was determined by qPCR analysis, and presented as gene expression relative to GAPDH. (C) SJG2 cells were transfected with NOXA siRNA or scrambled RNA, and at 5 hr after transfection cells were exposed to 1,000 U/ml IFN β for 24 hr, and then treated with 10 nM bortezomib for 20 hr. NOXA and cleaved Mcl-1 were detected by immunoblotting with GAPDH serving as a loading control. (D) SJG2 and MT330 cells transfected with NOXA siRNA or scrambled RNA were exposed to 1,000 U/ml IFN β for 24 hr and then treated with 10 nM bortezomib for an additional 24 hr. Cells were subjected to MTT assays and data presented relative to vehicle control. *, $p < 0.05$; **, $p < 0.01$ by Student's t test.

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Figure 1

Figure 2

Figure 3

Figure 4