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Bmf is upregulated by PS-341-mediated cell death of glioma cells through JNK phosphorylation

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Abstract Malignant glioma is resistant to the induction of apoptosis, resulting in a subsequent failure of chemotherapy in clinical treatment strategies. Downregulation of bcl-2 and bcl-xl expression in glioblastoma cells can induce apoptosis. BH3-only proteins, which include Bmf, are essential initiators of stress-induced cell death and apoptosis. Whether PS-341 regulates expression of BH3-only proteins in glioblastoma cells during the procedure of apoptosis is unclear. This study was designed to investigate the effects of PS-341 on glioma cell death and its possible signaling pathway. Our results demonstrate that Bmf is upregulated by PS-341 in A172 and T98G cells, and Bmf has a crucial role in PS-341-mediated cell death. In addition, we found that expression of Bmf is regulated by JNK phosphorylation.

Keywords Glioma · PS-341 · Bmf · Cell death · JNK

Introduction

Ubiquitin-dependent proteolysis is essential for the regulation of cellular proliferation and apoptosis [1]. When the ubiquitin–proteasome pathway is inhibited, cell growth and death are affected directly [2]. PS-341 (Bortezomib, Velcade) is a highly specific dipeptidyl boronic acid inhibitor of the 26 s proteasome [3, 4]. It has extensive anti-tumor activity in malignant cells of the colon [5], prostate [6], lung [7], and pancreas [8, 9], as well as in human glioblastoma multiforme [10]. There are four main hypotheses regarding the mechanism by which induction of malignant cellular apoptosis occurs via PS-341: (1) the inhibition or inactivation of nuclear factor-kB (NF-kB) [11, 12]; (2) the stabilization of p21 and p27 [13, 14]; (3) the stabilization of p53 and up-regulation of Bid or an accumulation of BAX [15–17]; and (4) the JNK pathway [10, 18, 19].

The Bcl-2 family is the key regulator for apoptosis, which is induced by stress signals [20, 21]. This family contains 1–4 Bcl-2 homology (BH) regions, which include a transmembrane region (TM) in the C-terminal. Both the third BCL-2 homology (BH3) and the fourth BCL-2 homology (BH4) domain are functionally relevant to apoptosis.

The BCL-2 family mainly consists of two subfamily proteins: (1) pro-apoptotic proteins, which include a downstream mediator of apoptosis BAX, BAK and BH3-only proteins (Bim, Hrk, Puma, NOXA, Bmf, Bad, Bik and Bid) and (2) pro-survival proteins BCL-2, Bcl-xL, BCL-w, Mcl-1 and A1. BH3-only proteins are essential initiators of stress-induced cell death and apoptosis [22]; the BH3 domain can insert into a hydrophobic groove of the pro-survival protein [23]. BH3-only proteins trigger apoptosis indirectly through Bax or Bak by neutralizing all relevant pro-survival proteins and allowing activation of Bax and Bak to proceed [24]. The binding affinities of BH3 proteins with pro-survival proteins are different. Bim and Puma have high relative binding affinities with all pro-survival proteins. Bmf and Bad selectively bind to BCL-2, BCL-xL and BCL-w. Noxa selectively binds to A1 and Mcl-1 [25].

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Malignant glioma is the most invasive primary tumor in the brain. Even with complete removal of the tumor and the combination of radiotherapy and chemotherapy treatments, the average survival time is <12 months from the date of diagnosis. Bcl-2 and bcl-xl are extensively expressed in most human glioma cells [26]. Glioblastomas are resistant to the induction of apoptosis, with subsequent failure of chemotherapy in clinical strategies. Apoptosis can be induced by the downregulation of bcl-2 and bcl-xl expression in glioblastoma cells [27]. Some research suggests that Bim, Noxa and Bik are upregulated or accumulated during apoptosis, induced by PS-341 in some cell lines [28] [29–31]. Whether PS-341 regulates the expression of BH3-only proteins in glioblastoma cells during apoptosis is unclear. The present study finds that Bmf is upregulated by PS-341 in A172 and T98G cells, and Bmf has a crucial role in PS-341-mediated apoptosis. In addition, we find the expression of Bmf is affected by JNK phosphorylation.

Materials and methods

Reagents

PS-341 (bortezomib) was purchased from Millennium pharmaceuticals (Cambridge, MA, USA), and dissolved in DMSO at −20°C with a stock concentration of 10^{-2} M, using fresh dilutions with medium for each experiment. Anti-Bmf antibodies were purchased from ProSci Co. (Poway, CA, USA). Anti-14-3-3, anti-Bim, and anti-p-JNK antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-BAD and anti-Bid antibodies were purchased from Cell Signaling Inc. (Cell Signaling, Beverly, MA) and MBL (Nagoya, Japan), respectively. The JNK inhibitor SP600125, p38 inhibitor SB203580, and ERK inhibitor PD98059 were purchased from Calbiochem (Calbiochem, San Diego, CA) and stored at −20°C. The DePsipher™ Kit (Catalog # 6300-100-K) was purchased from Trevigen, Inc.

Cell culture

Human T98G and A172 glioma cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). T98G cells were grown in DMEM supplemented with 10% fetal calf serum (FCS) and 10% non-essential amino acid (NEAA). A172 cells were grown in RPMI 1640 supplemented with 10% FCS. All cells were incubated at 37°C in 5% CO_2. To evaluate viability, cells were mixed with the same volume of 0.4% trypan blue solution and immediately examined by light microscopy.

Clonogenic assay

Cells (3×10^3) were seeded onto 60-mm plates, cultured for 24 h and treated with or without 100 nM PS-341. On day 9, colonies (>25 cells) were counted after cultured cells were fixed in methanol and stained with Giemsa. A survival fraction was defined as the number of colonies divided by the number of plated cells.

MTT assay

Cells were cultured in 96-well plates at 2.0×10^3 cells/well and the growth inhibitory effect of PS-341 (50–200 nM) was measured at 48 h with 3-(4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide (MTT) dye absorbance according to the protocol provided by Roche Molecular Biochemicals (Basel, Switzerland).

pEGFP-Bmf transfection

An isolated full-length human Bmf clone was sequenced and subcloned into a pEGFP expression vector (Clontech, CA, USA). The pEGFP-Bmf (3 mg/10 cm^2) or vehicle was transfected using LipofectAMINE 2000 (Life and Technologies, MD, USA). The efficiency of transfection was achieved at 30–50% for both cells. At 4–12 h after transfection, cells were treated as indicated and harvested to evaluate apoptosis.

DNA fragmentation assay

As described previously [32], low-molecular weight genomic DNA extracted with lysis buffer (0.5% Triton X-100, 10 nM EDTA and 10 mM Tris–HCl, pH 7.4) was treated with 400 mg/ml of RNase A and Proteinase K for 1 h at 37°C, isopropanol precipitated and run on 1% agarose gels. The gels were stained with 1 mg/ml of ethidium bromide.

FACS analysis

Fluorescence-activated cell sorting (FACS) based on DNA fragmentation was performed to determine the percentage of apoptotic cells after treatment with PS-341. A172 and T98G cells were cultured on 6-well plates (Corning Life Sciences, Corning, NY) at a density of 1×10^5 cells per well. After 24 h, the cells were treated with PS-341 at different concentrations in serum-free medium. The same volume of DMSO was used as a control. The cells were harvested at 48 h after treatment and washed once with PBS. The cells were then dispersed in 1 ml of membrane permeable propidium iodide (50 μg/ml) (Biosure, Grass Valley, CA) and incubated for 20–30 min in the dark at

4°C. The cells were sorted on a FACS machine (FACSCalibur, Becton-Dickinson, Franklin Lakes, NJ) and the DNA content was analyzed based on the red fluorescence of propidium iodide at 488 nm. Data are presented in a dot plot graph and FACS histogram.

Activity assay for caspase-3

The activity assay for caspase-3 (colorimetric) was performed using a kit (Sigma, St. Louis, MO) per the manufacturer's instructions. The cells were cultured and treated with PS-341 as described above. The harvested cells were transferred to 96-well plates and treated with the caspase-3 peptide substrate conjugated with *p*-nitroaniline (Ac-DEVD-*p*NA) and the release of the *p*-nitroaniline by caspase-3 was measured on a microplate reader at 405 nm. The assay was carried out in four independent samples in triplicate. Data are quantitatively represented as the percentage activity of caspase-3.

Total RNA isolation and RT-PCR

Total RNA of cells was extracted with TRIZOL (Invitrogen, Inc. USA). The indicated cDNA were amplified from 2 ng of total RNA using M-MLV Reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with oligo dT and the platinum Quantitative RT-PCR Thermoscript one-step system (Life Technologies). The cDNA products were checked on a 2% agarose gel and confirmed by nucleotide sequencing. The following primers were used for RT-PCR: Bmf: 5′-atggagccatctcagtgtgtg-3′ and 5′-ccccgttcctgttctctt ct-3′; GAPDH: 5′-cgaccactttgtcaagctca-3′ and 5′-aggggtcta catggcaactg-3; Bim: 5′-ctgcagatatgcgcccagagat-3′ and 5′-cac caggcggacaatgtaacg-3′; Bid: 5′-gcatgtcaacagcgttccta-3′ and 5′-ggaacctgcacagtggaaat-3′; Bad: 5′-gcggatccgccaccatgttcc agatcccagag-3′ and 5′-gcggatccgctcactgggaggggcggagct tc-3′; BAX: 5′-ggaactgatcagaaccatca-3′ and 5′-tcagcccat cttcttccaga-3′. Specificity of amplified PCR fragments was confirmed by DNA sequence analysis.

Western blotting

Cells were harvested by adding 200 μl of RIPA buffer [100 mM NaCl, 2 mM EDTA, 1 mM PMSF, 1% NP-40 and 50 mM Tris–HCl (pH 7.2)] and crushed by ultrasonic wave. After being boiled for 3 min, the lysates were fractionated by 10–15% SDS–PAGE gels and then transferred to PVDF membranes (Millipore, Bedford, MA). Membranes were immersed in 5% bovine serum albumin (BSA, Sigma), incubated with P-JNK, Bim, Bmf, Bid and BAD primary antibodies, respectively, and incubated with the corresponding secondary antibodies (Amersham or MBL) for 1 h at room temperature. Membranes were developed by a standard enhanced chemiluminescence (ECL) method based on the manufacturer's protocol (Amersham).

Flow cytometry assay of ΔΨm disruption

About 1×10^6 cells per sample were harvested by centrifugation at 500*g* for 5 min at room temperature. One ml of DePsipher^{TM} solution to 1 ml of 1× prewarmed Reaction Buffer was added (final concentration 5 mg/ml). After being vortexed, cells were re-suspended in 1 ml of diluted DePsipher^{TM} solution. Samples were incubated at 37°C, 5% CO_2 for 20 min, and then washed 2 times in 1× Reaction Buffer with centrifugation at 500×*g* between each wash. Resuspended cells were analyzed quickly by flow cytometry (488 nm argon laser).

Small interfering RNA transfection

Transfection with small interfering RNA (siRNA) was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The 21-nt duplex siRNAs for Bmf (target sequence: 5′-AAAGG TGTCATGCTGCCTTGT-3′) and scrambled siRNAs (random: 5′-ATACTCTATCTGCACGCTGAC-3′) were synthesized by Dharmacon. Cells were plated at a density of 2×10^5 cells/60 mm dish 1 day before transfection. At 24 h after transfection, cells were collected for the trypan blue exclusion assay, flow cytometry assay and the evaluation of Bmf expression.

Statistical analysis

The data were expressed as the mean ± SD. Statistical differences between two groups were calculated using Student's *t* test. One-way ANOVA was used to determine statistical differences between multiple groups using SPSS, version 10.1 (San Rafael, CA). $P < 0.05$ was considered statistically significant.

Results

PS-341 induces growth arrest and apoptosis of glioma cells

We examined the effect of PS-341 on growth arrest of glioma cell lines A172 (P53wt) and T98G (mt), measured by the MTT assay. As shown in Fig. 1A, the percentage of cell survival decreases gradually when the concentration of PS-341 increases from 5 to 625 nM. The IC50 of A172 and T98G was ~142 and 77 nM, respectively. The trypan blue exclusion assay was used to detect cell death. The results showed that PS-341 induced cell death in a dose- and

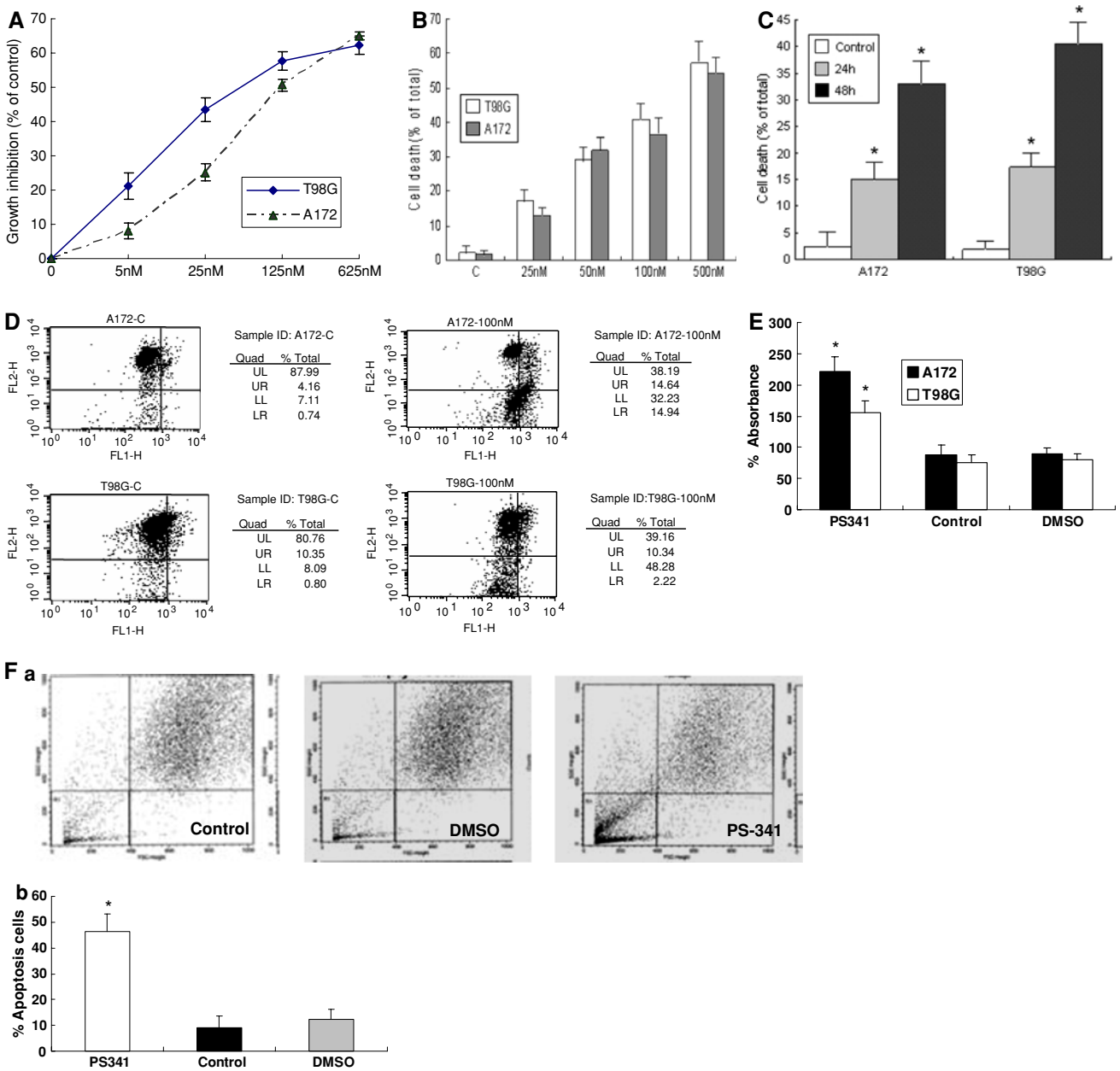

Fig. 1 PS-341 induces growth arrest and cell death in glioma cells. **A** MTT assay of A172 and T98G cells in the presence or absence of PS-341 at 5, 25, 125 and 625 nM. Representative results are shown; each point represents the mean ± SD. Data are representative of three separate experiments. **B** Trypan blue exclusion assay. The dead cells of A172 and T98G were counted at 48 h after treatment with an indicated concentration of PS-341. **C** Indicated time cell death rate was checked by trypan blue exclusion assay. *$P < 0.05$ versus control group. **D** Effects of PS-341 on the ΔΨm disruption of glioma cells by flow cytometry. **E** Assay of caspase-3 activities after PS-341 treatment. *$P < 0.05$ versus control group. **F** Representative results of flow cytometry for apoptotic cell death (a) and summary of four independent experimental results (b). *$P < 0.05$ versus control group

time-dependent manner (Fig. 1B); 28% of A172 cells and 33% of T98G cells were induced by 100 nM PS-341 after 24 h, and 37% of A172 and 41% of T98G cell death was observed at 48 h, respectively (Fig. 1C). Mitochondrial membrane potential (ΔΨm) disruption is an early prerequisite step toward programmed cell death [33]. Treatment with 50 nM PS-341 causes a distinct ΔΨm disruption, which in A172 cells is 36.94% and in T98G cells is 33.09% (Fig. 1D).

To further determine the mode of cell death, caspase-3 activity assay was conducted. As shown in the results, there was a significant increase ($P < 0.01$) in caspase-3 activity

after treatment with PS-341 (100 nM). A 2.5-fold and twofold increase was observed in the activity of mature caspase-3 in A172 and T98G cells treated with PS-341 at 100 nM, respectively. We saw no significant difference in caspase-3 activity between parental cells and cells treated with DMSO (Fig. 1E).

To accurately detect apoptotic cells, we performed flow cytometry for DNA fragmentation in glioma cells after treatment with PS-341. A representative dot plot for A172 cells after treatment with PS-341 at 100 nM is shown in Fig. 1Fa. A marked increase in the cell population was observed in the column R1 area, which represents apoptotic cells. However, there was no significant difference in the cell population in the column R1 area between parental cells and cells treated with DMSO. The quantitative evaluation of FACS data for DNA fragmentation analysis using Imagepro plus software is shown in Fig. 1Fb. Quantitative analysis demonstrated that 46.5 ± 6.8% of apoptotic cells were found after treatment with PS-341 at 100 nM. Similar results were also observed in T98G cells treated with PS-341 (data not shown).

Bmf is upregulated by PS-341 in glioma cells

We first evaluated whether PS-341 affects the gene expression of pro-apoptotic Bcl-2 family members. RT-PCR was used to determine BH3-only proteins and BAX/BAK in A172 cells (Fig. 2a). Bmf mRNA expression was elevated following PS-341 treatment from 25 to 100 nM. As shown in the results, PS-341 had no obvious effects on mRNA expression of the other BH3-only proteins and BAX. We further examined the expression of pro-apoptotic Bcl-2 family members using a western-blot assay (Fig. 2b). The results also showed that the level of Bmf protein was markedly increased in a dose-dependent manner; between the control and the PS-341 treated group, other anti-apoptotic proteins were not obviously affected.

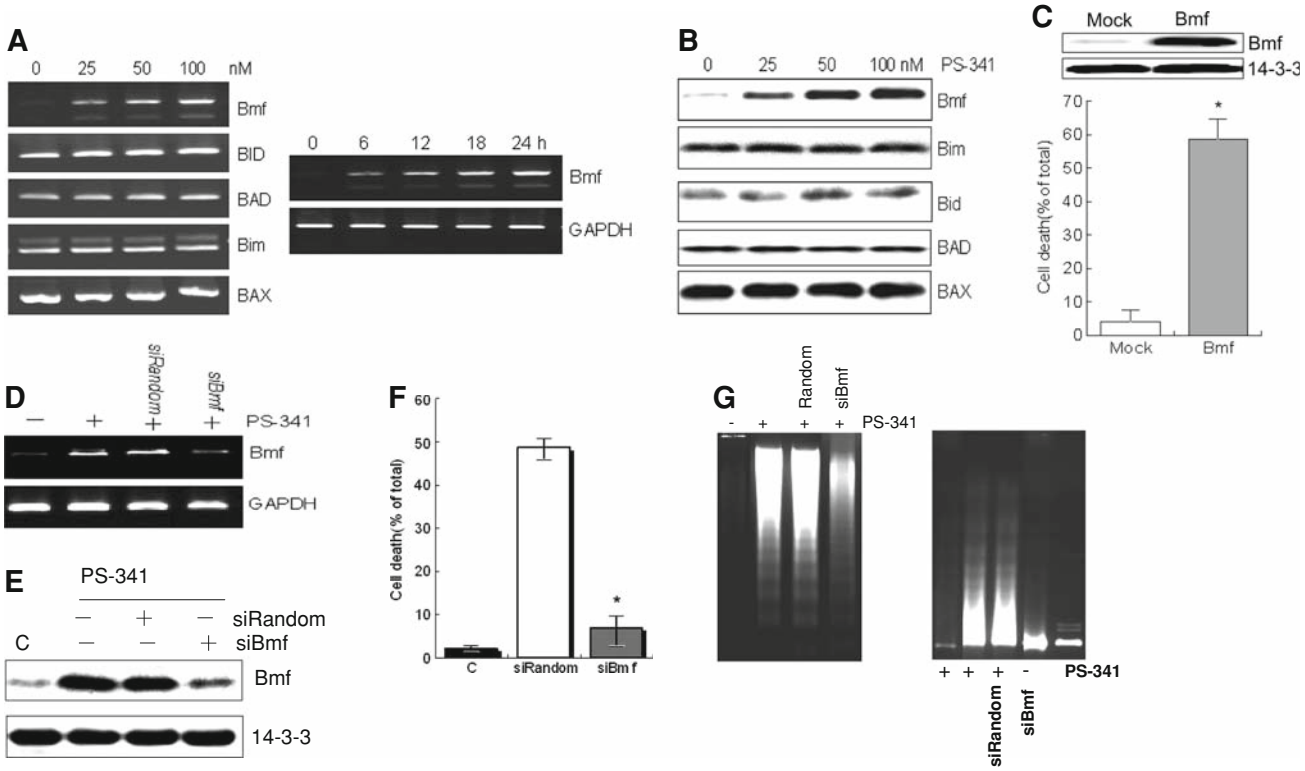

Fig. 2 Role of Bmf in PS-341-mediated apoptosis. **a** Time and dose response of Bmf mRNA induction. A172 cells were cultured with PS-341 for the indicated time or with the indicated doses for 24 h. RT-PCR was performed with the indicated primers and GAPDH was used as an internal control. **b** Protein expression of proapoptosis-related proteins. **c** Bmf overexpression increased cell death in A172 (*upper panel*). $^*P < 0.05$ versus control group. **d** A172 cells (10^5/ml) were incubated for 6 h with 100 nM PS-341 after transfection with scrambled siRNA or siRNA of Bmf; then, Bmf mRNA was evaluated by RT-PCR. **e** Western blotting analysis of Bmf and Bim. **f** A172 cells were incubated with or without PS-341 for 48 h after transfection with siRNA. $^*P < 0.05$ versus control group. **g** DNA fragmentation after A172 cells were transfected with scrambled or Bmf siRNA 36 h. Treatment with PS-341 was shown as "+". **h** FCM of A172 cells with or without 100 nM PS-341 for 48 h after transfection with the indicated siRNA. **i** Representative results of colony assay after cells were transfected twice with scrambled siRNA (*gray*) or siRNA of Bmf (*closed bar*) in the presence or absence of 25 nM PS-341 for 9 days. Colony numbers (>25 cells) were counted. $^*P < 0.05$ compared with scrambled siRNA transfected cells

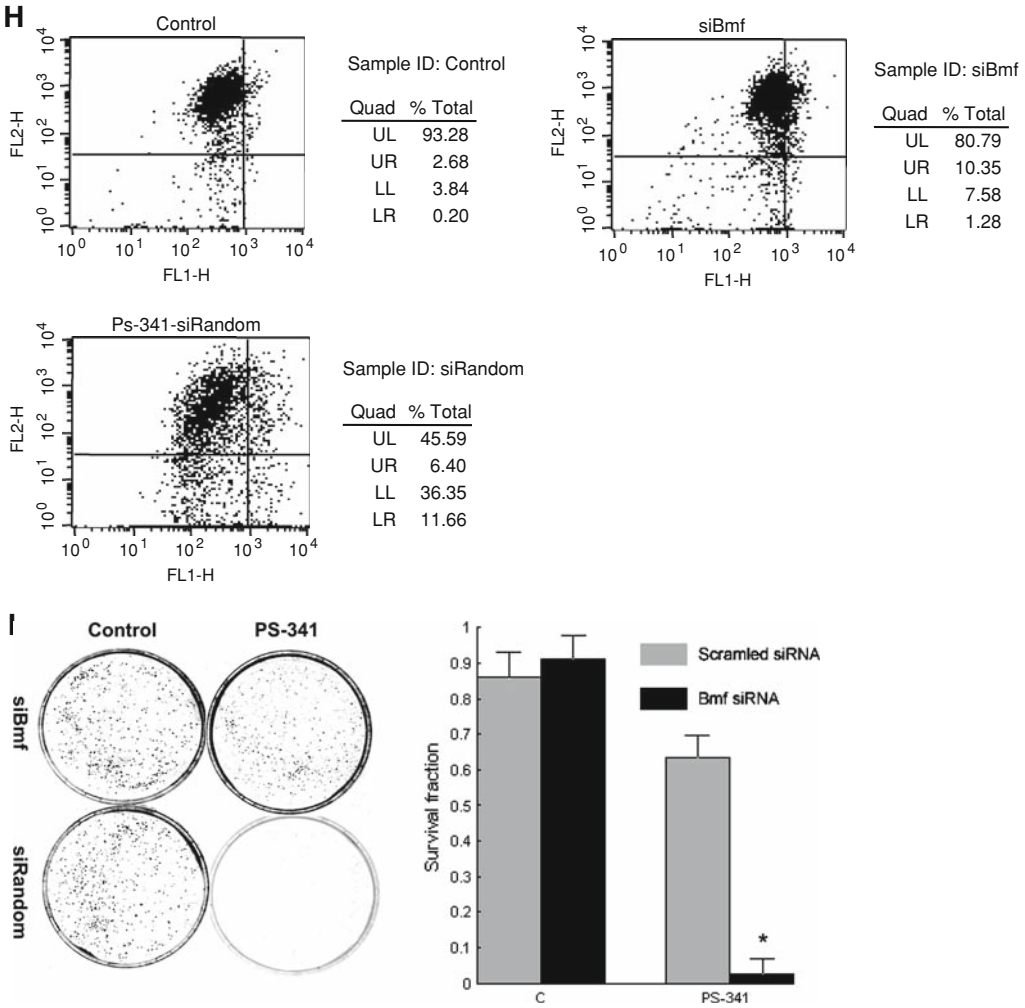

Fig. 2 continued

The role of Bmf in PS-341 mediated cell death in glioma cells

In order to explore whether overexpression of Bmf can induce glioma cell death, a Bmf full-length mRNA clone from A172 cells was isolated and its cDNA fragment was subcloned into a pEGFP expression vector. The data (Fig. 2c) showed that overexpression of Bmf significantly increased cell death. To further evaluate the effects of Bmf in PS-341-mediated apoptosis, siRNA-mediated knockdown of Bmf was used in A172 cells. After transfection with siRNA targeting Bmf, mRNA expression of Bmf induced by PS-341 was reduced significantly in A172 cells. (Fig. 2d). The level of Bmf protein was also decreased accordingly (Fig. 2e). After transfection with siRNA of Bmf or scrambled siRNA, PS-341 was added at 48 h and the trypan blue exclusion assay was conducted (Fig. 2f). The cell death in the si-RNA Bmf group was $6.84 \pm 2.13\%$, whereas it was $48.84 \pm 8.45\%$ in the scrambled siRNA group. Results of DNA fragmentation and loss of $\Delta\Psi_m$ were consistent with that of the trypan blue exclusion assay (Fig. 2g, h). To elucidate whether Bmf knockdown can maintain cell viability after PS-341 treatment, T98G and A172 cells were transfected twice with the indicated siRNAs. The data from the colony assay (Fig. 2i) clearly revealed that Bmf knockdown greatly increased cell viability after treatment with PS-341.

PS-341 activates phosphorylation of JNK and SP600125 neutralized PS-341-mediated cell death

We explored whether PS-341 can induce phosphorylation of JNK in glioma cells. Results from western blotting (Fig. 3a) showed that JNK phosphorylation was induced by PS-341 at both 50 and 100 nM after incubation for 6 or 12 h in A172 cells. The p-JNK expression levels were dose responsive. On the other hand, neither P38 nor the extracellular signal-regulated protein kinase 1/2 (ERK1/2) was

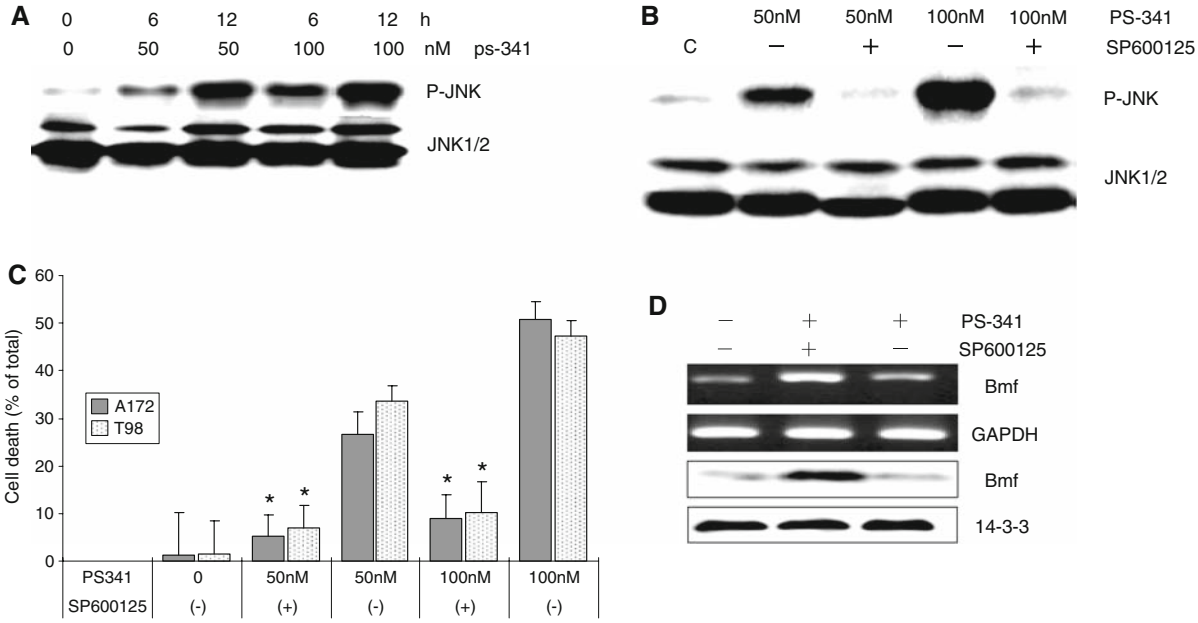

Fig. 3 **a** Western blotting analysis for phospho-JNK of A172 or T98G cells treated with various concentrations of PS-341. **b** Western blot analysis of A172 cells treated with PS-341 with (+) or without (−) SP600125 (20 μM). **c** Trypan blue exclusive assay for A172 and T98G cells treated with PS-341 in the presence (+) or absence (−) of SP600125 (20 μM). $*P < 0.05$ compared with the corresponding SP600125 (−) group. **d** mRNA and protein expression of Bmf affected by activation of JNK

phosphorylated in A172 and T98G cells (data not shown). We further examined whether JNk phosphorylation plays a critical role in PS-341-induced glioma cell death. SP600125 was utilized to block phosphorylation of JNK. As shown in Fig. 3b, 20 μM SP600125 can almost abrogate JNK phosphorylation. Glioma cells were cultured with PS-341 (25 or 125 nM), either alone or in combination with SP600125 (20 μM) for 48 h, and apoptosis was measured by the trypan exclusion assay (Fig. 3c). The cell death rate of T98G treated with PS-341 was 33.57 ± 4.54% (50 nM) and 47.31 ± 6.12% (100 nM), respectively. Interestingly, when SP600125 was added to the culture, only about 6.88 or 10.17% cell death was observed after treatment with PS-341. The results from A172 cells are consistent with that of T98G cells and suggest that the JNK inhibitor, SP600125, effectively blunted cell death mediated by PS-341 in glioma cells. Phosphorylation of JNK plays a critical role in glioma cell apoptosis induced by PS-341.

SP600125 blunts PS-341 induced upregulation of Bmf

As described in the results, PS-341 markedly increased levels of Bmf expression in glioma cells. However, when phosphorylation of JNK was blocked by SP600125, a selective inhibitor of JNK, expression of Bmf induced by PS-341 was blunted (Fig. 3d). These results suggest that PS-341 increased Bmf expression via activation of the phosphorylation of JNK. The kinetics study of p-JNk protein induction revealed that p-JNK protein began to increase at 6 h, which was prior to the Bmf protein expression (data not shown).

Discussion

In the present study, PS-341-induced cell death in A172 and T98G cells was observed. Although some studies have shown the effects of PS-341 on glioma cells, the precise mechanisms needed further elucidation. Previous studies indicated that Bcl-2 family members were regulated by PS-341 in many cell lines [33]. In the present study, we found that Bmf was upregulated by PS-341 in glioma cells. Additionally, phosphorylation of JNK was also observed after PS-341 treatment. Inhibition of JNK phosphorylation blunted the upregulation of Bmf induced by PS-341. These results showed that, at least in part, glioma cell death induced by PS-341 occurs via the JNK-Bmf pathway.

Bmf (Bcl-2 modifying factor) is related to Bim, a component of the myosin V motor complex. In normal cells, the BH3-only proteins are sequestered in motor complexes that interact with the cytoskeleton [34]. Exposure of cells to stress such as anoikis [34], UV radiation [35], and some histone deacetylase (HDAC) inhibitors [36] can cause the release of Bmf from sites of sequestration by dissociating it from myosin V motor complexes, thereby

allowing the apoptotic pathway to occur. In order to check Bmf function in glioma cell death mediated by PS-341, a Bmf expression vector was transfected into A172 cells. As found in the results, the cell death rate was greatly elevated. siRNA gene silencing of Bmf reduces the cell death, which further indicated that Bmf may have a crucial role in PS-341-mediated cell apoptosis. Our results were consistent with previous studies that reported that PS-341 induced glioma cell apoptosis via JNK pathway [10]. The kinetics of Bmf and JNK expression induction revealed that a 100 nM PS-341 treatment initiated increased Bmf expression at 12 h, measured by western blotting, whereas phosphorylation of JNK was markedly expressed at 6 h. This may suggest that Bmf was phosphorylated by activated JNK and then dissociated from myosin V motor complexes. This hypothesis is supported by previous research [35]. The fact that the JNK inhibitor, SP600125, blocked Bmf expression mediated by PS-341 also lends support to this hypothesis.

In summary, our data shed light on the role of Bmf in PS-341-induced glioma cell death. Although previous studies have shown that the proteasome inhibitor PS-341 causes cell growth arrest, apoptosis and blocking NF-κB in glioma cells, our data may represent some additional mechanisms for effects of PS-341 in glioma treatment. Our findings favor future clinical trials involving PS-341 as an adjuvant or in combination with other therapies in glioma cells.

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