RESEARCH PAPER

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# Bortezomib induces apoptosis and growth suppression in human medulloblastoma cells, associated with inhibition of AKT and NFκB signaling, and synergizes with an ERK inhibitor

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**Keywords:** bortezomib, medulloblastoma, AKT, apoptosis, ERK inhibitor, proliferation, NFκB, brain tumor

Medulloblastoma is the most common brain tumor in children. Here, we report that bortezomib, a proteasome inhibitor, induced apoptosis and inhibited cell proliferation in two established cell lines and a primary culture of human medulloblastomas. Bortezomib increased the release of cytochrome *c* to cytosol and activated caspase-9 and caspase-3, resulting in cleavage of PARP. Caspase inhibitor (Z-VAD-FMK) could rescue medulloblastoma cells from the cytotoxicity of bortezomib. Phosphorylation of AKT and its upstream regulator mTOR were reduced by bortezomib treatment in medulloblastoma cells. Bortezomib increased the expression of Bad and Bak, pro-apoptotic proteins, and $p21^{Cip1}$ and $p27^{Kip1}$, negative regulators of cell cycle progression, which are associated with the growth suppression and induction of apoptosis in these tumor cells. Bortezomib also increased the accumulation of phosphorylated IκBα, and decreased nuclear translocation of NFκB. Thus, NFκB signaling and activation of its downstream targets are suppressed. Moreover, ERK inhibitors or downregulating ERK with ERK siRNA synergized with bortezomib on anticancer effects in medulloblastoma cells. Bortezomib also inhibited the growth of human medulloblastoma cells in a mouse xenograft model. These findings suggest that proteasome inhibitors are potentially promising drugs for treatment of pediatric medulloblastomas.

## Introduction

Medulloblastomas are the most common cerebellar tumors in childhood, which are thought to arise primarily from neural stem or precursor cells of cerebellar external germinal layer.[1] Medulloblastomas account for approximately 20% of all pediatric central nervous system tumors and 40% of all posterior fossa tumors.[2] The etiology of medulloblastomas is still unclear, although several signaling pathways that control cell proliferation are thought to be involved in disease progression. Deregulation of the Sonic hedgehog and Wingless pathways have been linked to the development of medulloblastomas.[3,4] The phosphoinositide-3-kinase (PI3K)/AKT cell survival pathway is activated in many cancers, and elevated phosphorylation of AKT at Ser473, which is linked to proliferation of medulloblastoma cells, is observed in human medulloblastoma samples.[5,6] The activity of STAT (signal transducer and activator of transcription) proteins, particularly STAT3, is frequently elevated in a variety of solid tumors and hematological malignancies.[7] STAT3 is found to be constitutively activated in medulloblastomas.[8] Extracellular signal-regulated kinases (ERK)/mitogen-activated protein kinases (MAPK) also contributate to the progression and metastasis of the tumors in certain cases.[6,9,10]

Proteasomes are large proteolytic complexes that are responsible for ubiquitin-mediated protein degradation and maintenance of homeostasis in eukaryotic cells. Since tumor cells are more sensitive to proteasome inhibitors than normal cells, the inhibition of proteasome activity has become a promising target for cancer treatment.[11,12] Bortezomib is a highly selective and reversible inhibitor of the 26S proteasomes, and inhibits ubiquitin-dependent proteolysis. Bortezomib has been used to treat relapsed and refractory multiple myeloma in front-line therapy.[13] Currently, bortezomib is undergoing clinical trials for various other forms of cancer.[14] The key biological activities of bortezomib as an anticancer agent are to induce apoptosis and inhibit cell proliferation in different kinds of tumors. Bortezomib induces apoptosis via multiple pathways, including the caspase-8 mediated extrinsic death-receptor pathway, the caspase-9 mediated intrinsic mitochondrial apoptotic pathway and endoplasmic reticulum stress-response pathway.[13,15,16] Inhibition of cell proliferation by

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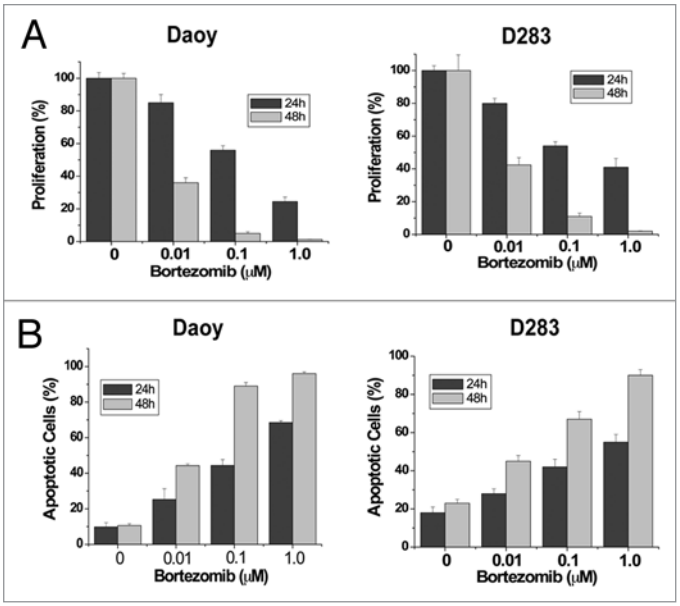


**Figure 1.** Bortezomib inhibited cell proliferation and induced apoptosis in Daoy and D283 medulloblastoma cells. (A) Effect of bortezomib on proliferation of Daoy and D283 cells. Cells were treated with 0, 0.01, 0.1 or 1 μM bortezomib for 24 and 48 h, and cell proliferation was evaluated by MTS assay as described in Methods. Bortezomib induced apoptosis (B) in Daoy and D283 cells after 24 and 48 h treatment. Apoptotic cells represented Annexin V-FITC positive and PI and Annexin V-FITC double-positive cells as determined by flow cytometry. Each experiment was performed in triplicate or duplicate and repeated twice independently. Each bar graph represents the mean, and the error bars represent ± SD.

bortezomib is through accumulation of the growth inhibitory molecules $p21^{Cip1}$ and $p27^{Kip1}$, which are cyclin-dependent kinase inhibitors and are degraded by proteasomes.[15]

Constitutive activation of the Nuclear FactorκB (NFκB) signal transduction pathway has been identified as a frequent event promoting tumor progression of hemopoietic and solid malignancies.[17] Recently, it has been reported that inhibition of NFκB signaling reduces growth of medulloblastoma in vivo.[18] Bortezomib inhibits the NFκB pathway through its inhibitory effects on degradation of ubiquitinated inhibitor κB (IκB), which binds to and sequesters NFκB in the cytoplasm, and blocks its nuclear localization and binding to the promoters of target genes.[19,20]

In the present study, we show that bortezomib induces apoptosis and inhibits cell proliferation in medulloblastoma cells. Bortezomib increases the release of cytochrome *c* to cytosol and activates the caspase cascade. Activities of AKT and mTOR are reduced by bortezomib. Expression of pro-apoptotic proteins (Bad and Bak), and negative regulators in cell cycle ($p21^{Cip1}$ and $p27^{Kip1}$) is increased, associated with induction of apoptosis and inhibition of cell proliferation. In addition, NFκB signaling is inhibited by bortezomib via inhibiting degradation of IκB. Bortezomib also synergizes in its anticancer effects on these tumor cells with ERK inhibitors. Our findings suggest the potential use of bortezomib in the treatment of medulloblastomas.

## Results

**Bortezomib inhibits cell proliferation and induces apoptosis in Daoy and D283 medulloblastoma cells.** To characterize the effects of bortezomib on cell proliferation in medulloblastoma, we performed dose-response and time-course studies in Daoy and D283 cells, established human medulloblastoma cell lines. Cells were treated with increasing concentrations of bortezomib (0, 0.01, 0.1, 1.0 μM) for 24 and 48 h. Control cells were treated with the vehicle ($H_2O$) only. Bortezomib markedly inhibited proliferation of both Daoy and D283 (**Fig. 1A**) cells in a dose- and time-dependent manner. To investigate whether bortezomib has direct killing effects on medulloblastoma tumor cells, Daoy and D283 cells were treated with either vehicle or bortezomib (0, 0.01, 0.1, 1.0 μM) for 24 and 48 h. Apoptotic cells included both Annexin V-positive (early stage) cells, and Annexin V and propidium iodide double-positive (late stage) cells, which were defined by flow cytometry (**Fig. 1B**). Bortezomib markedly induced apoptosis of Daoy and D283 cells in a dose- and time-dependent manner. At as low as 0.01 μM, bortezomib has showed the killing effects on these tumor cells. Experiments were performed in culture medium containing 2% serum. Effects of bortezomib in medulloblastoma cells do not depend on serum concentrations (data not shown).

**Apoptosis induced by bortezomib via activation of caspase cascade in medulloblastoma cells.** Activation of caspase-3, a critical mediator of apoptosis,[21] resulted in cleavage of poly (ADP-ribose) polymerase (PARP), which is known to help cells to maintain their viability.[22] To further confirm that the cell death induced by bortezomib is apoptosis, immunoblotting analyses were employed to detect the activation of caspase-3 and cleavage of PARP in total cell lysate after 24 h bortezomib treatment. Bortezomib increased the cleaved caspase-3 (active form of caspase-3) and cleaved PARP (inactive form of PARP) levels in Daoy and D283 cells (**Fig. 2A**).

Caspase-3 is activated by cleaved caspase-9 (active form), which activation is dependent on the release of cytochrome *c* from mitochondria to cytoplasm.[23] To confirm whether caspase-3 is activated by caspase-9, we examined expression of cleavaged and total caspase-9 in Daoy cells after bortezomib treatment. Results from immunoblotting assays (**Fig. 2B**) showed that both cleavaged forms of 35 kD and 37 kD caspase-9 were increased by bortezomib treatment. To analyze whether bortezomib increases the release of cytochrome *c* from mitochondria to cytoplasm, we isolated the cytosol after 24 h of bortezomib treatment in Daoy cells. Amount of cytochrome *c* in cytosol or total lysate was determined by immunoblotting assays with specific antibody (**Fig. 2C**). Bortezomib increased the release of cytochrome *c* to cytoplasm. Therefore, bortezomib induced apoptosis in these cells via cytochrome *c*/caspases/PARP cascade.

**Inhibitor of caspases rescues medulloblastoma cells from cell death induced by bortezomib.** Since bortezomib induced apoptosis of human medulloblastoma cells through activation of caspase cascade, we anticipate that the inhibitors of caspases should block the effects of bortezomib. To prove this hypothesis, we employed Z-VAD-FMK, a general inhibitor of caspases.

Daoy cells were treated with 30 μM Z-VAD-FMK and 0.1 or 1.0 μM bortezomib together for 24 h. Apoptotic cells were assayed by flow cytometry with Annexin-V staining. Z-VAD-FMK treatment could significantly block the killing effects of bortezomib on these cells, even in much higher concentration, 1.0 μM (**Fig. 2D**).

**Expression of $p21^{Cip1}$ and $p27^{Kip1}$ was increased by bortezomib in medulloblastoma cells.** Bortezomib inhibited cell proliferation in Daoy and D283 medulloblastoma cells (**Fig. 1A**). To investigate whether bortezomib affected the expression of regulatory proteins involved in cell cycle, immunoblotting analyses were performed for $p21^{Cip1}$ and $p27^{Kip1}$, the cyclin-dependent kinase inhibitors for cell cycle. After 24 h bortezomib treatment, expression of $p21^{Cip1}$ and $p27^{Kip1}$ was tremendously increased by bortezomib in Daoy and D283 cells (**Fig. 3A**). These results correlated with the inhibition of cell proliferation induced by bortezomib in these cells. Degradation of both $p21^{Cip1}$ and $p27^{Kip1}$ depends on ubiquitin-proteasome system.[24,25] Since bortezomib inhibits proteasome function, the amount of $p21^{Cip1}$ and $p27^{Kip1}$ should be increased after bortezomib treatment.

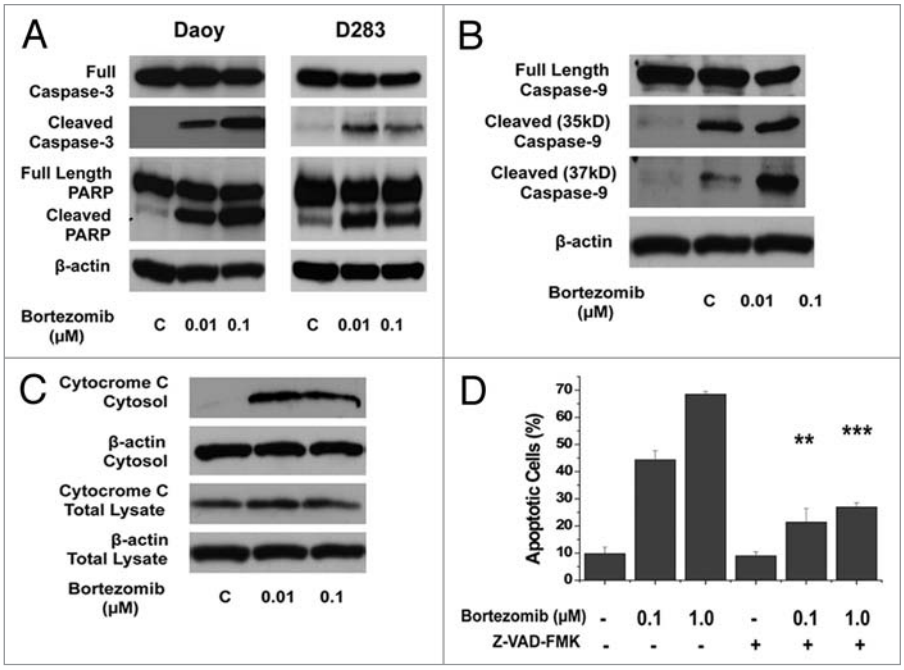


**Figure 2.** Bortezomib activated cytochrome *c*/caspase cascade, and caspase inhibitor blocked the effects of bortezomib on medulloblastoma cells. (A) Expression of full-length or cleaved caspase-3 and PARP in Daoy and D283 cells was analyzed by immunoblottings with total cell lysate after 24 h bortezomib treatment. Anti-β-actin monoclonal antibody was used as a loading control. (B) Expression of full-length or cleaved caspase-9 in Daoy cells was assayed by immunoblottings after 24 h bortezomib treatment. (C) Expression of cytochrome *c* in cytosol and total cell lysate was determined by immunoblottings after 24 h bortezomib treatment in Daoy cells. (D) Z-VAD-FMK, caspase inhibitor, rescued Daoy cells from cytotoxicity of bortezomib. Cells were treated with 30 μM Z-VAD-FMK and bortezomib (0.01 and 0.1 μM) respectively, or combination for 24 h. Apoptotic cells were assayed by flow cytometry with Annexin-V staining. **$p < 0.01$; ***$p < 0.001$.

**Effects of bortezomib on proteins involved in regulation of apoptosis in medulloblastoma cells.** Bcl-2 family proteins have a key role in survival of normal and tumor cells. Members of Bcl-2 family regulate the cytochrome *c*/caspases/PARP pathway by controlling cytochrome *c* release through modulating mitochondrial outer membrane permeabilization.[26] Thus, we investigated the effects of bortezomib on the expression of certain Bcl-2 family members in Daoy and D283 cells. After 24 h treatment with bortezomib, immunoblotting assays (**Fig. 3B**) showed that bortezomib increased the expression of pro-apoptotic proteins, Bad and Bak, in both Daoy and D283 cells. Anti-apoptotic protein Bcl-2 was only decreased in D283 cells and Bcl-$x_L$ was not affected by bortezomib treatment.

**Bortezomib inhibits phosphorylation of AKT and mTOR in Daoy and D283 cells.** To investigate effects of bortezomib on common signaling pathways, we examined the levels of total and phosphorylated AKT, ERK1/2 and STAT3 proteins in Daoy and D283 cells after 24 h bortezomib treatment (**Fig. 4A**). Phosphorylated AKT at Ser473 was reduced by bortezomib in both Daoy and D283 cells and phosphorylated STAT3 was only reduced in Daoy cells. However, phosphorylation of ERK1/2 was increased in both cell lines. Total proteins of ERK and STAT3 were not affected by bortezomib. Total protein of AKT in D283 cells was slightly decreased.

To confirm whether de-phosphorylation of AKT (Ser473) influenced the downstream targets of AKT signaling, the activity of Mammalian Target of Rapamycin (mTOR) was investigated after 24 h treatment with bortezomib. mTOR is a positive regulator for cell cycle progression and cellular proliferation, and is activated by AKT via phosphorylation of mTOR at Ser2448.[27] We found that mTOR phosphorylation at Ser2448 was inhibited by bortezomib (**Fig. 4B**) in Daoy and D283 medulloblastoma cells.

AKT and mTOR are involved in the control of a wide variety of cellular processes such as transcription, translation, cell cycle and apoptosis. To further elucidate whether inhibition of AKT or mTOR signaling by bortezomib is correlated with increased expression of Bak, Bad, $p21^{Cip1}$ and $p27^{Kip1}$, we employed triciribine, an inhibitor of AKT and RAD001, an inhibitor of mTOR. Daoy cells were treated with 5 μM triciribine or RAD001 for 30 h. Then, total cell lysate was prepared and immunoblotting assays were performed. Pro-apoptotic Bak was increased by both triciriine and RAD001 and Bad was only increased by RAD001 (**Fig. 4C**). The increased Bak and Bad may contribute to release of cytochrome *c* from mitochondria and induction of apoptosis by bortezomib. $p27^{Kip1}$ was increased by both inhibitors (**Fig. 4C**). However, $p21^{Cip1}$ was only slightly increased by these two inhibitors (**Fig. 4C**). Though both $p21^{Cip1}$ and $p27^{Kip1}$ are

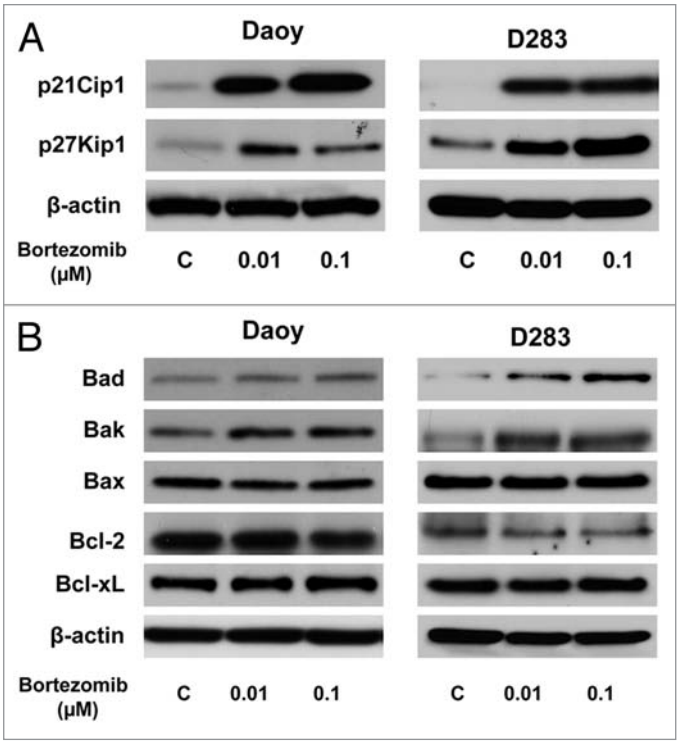


**Figure 3.** Effects of bortezomib on regulatory proteins in cell cycle progression and apoptosis in Daoy and D283 cells. (A) Effects of bortezomib on $p21^{Cip1}$ and $p27^{Kip1}$, negative regulators for cell growth. Levels of $p21^{Cip1}$ and $p27^{Kip1}$ were determined by immunoblottings after 24 h bortezomib treatment. (B) Effects of bortezomib on regulatory proteins of apoptosis. Pro-apoptotic proteins, Bad, Bak and Bax, and anti-apoptotic proteins, Bcl-2 and Bcl-$x_L$, were detected with specific antibodies by immunoblottings after 24 h bortezomib treatment.

downstream targets of AKT/mTOR,[28] increased $p21^{Cip1}$ induced by bortezomib in medulloblastoma cells depends on inhibition of protesome function.

**ERK inhibitor increases the anticancer effects of bortezomib in medulloblastoma cells.** ERK/MAPK cascades are key signaling pathways involved in the regulation of normal cell proliferation, survival and differentiation. Aberrant regulation of ERK/MAPK cascades contribute to cancer and other human diseases.[29] **Figure 4A** showed that bortezomib increased the phosphorylation of ERK1/2 after bortezomib treatment in medulloblastoma cells. Since this increased activities of ERK1/2 may attenuate the inhibiting effects of bortezomib on proliferation and survival of medulloblastoma cells, we tested whether the combination of bortezomib and ERK inhibitors had the synergic effects. We used ERK inhibitor, 3-(2-Aminoethyl)-5-[(4-ethoryphenyl)methylene]-2,4-thiazolidinedione, from EMD Chemicals, Inc. This inhibitor is a cell-permeable and reversible thiazolidinedione compound, and preferentially binds to ERK2 with $IC_{50} \geq 25$ μM and prevents its interaction with protein substrates. After Daoy cells were treated with 10 nM bortezomib and 25 μM ERK inhibitor respectively, or combination of both drugs for 24 h and 48 h, cell proliferation was evaluated by MTS assays as described in Materials and Methods. The results (**Fig. 5A**, left) showed that the combination of both bortezomib and ERK inhibitor exhibited much stronger inhibition in cell proliferation than any single reagent in Daoy cells. We also determined the effects of combination on induction of apoptosis in Daoy cells. Tumor cells were treated with 10 nM and 40 nM bortezomib only, or with addition of 25 μM ERK inhibitor for 24 h. Then, both attached and detached cells were collected and analyzed by flow cytometry with PI/Annexin V staining. Data (**Fig. 5A**, right) showed that addition of ERK inhibitor to bortezomib treatment significantly induced higher rates of apoptosis than one of either bortezomib treatment only or ERK inhibitor only.

**ERK1/2 siRNA transfectants increases the anticancer effect of bortezomib on medulloblastoma cells.** To further confirm that the effects of inhibiting ERK1/2 activities on bortezomib function in medulloblastoma cells, ERK1/2 small interfering RNA (siRNA) was transfected into Daoy cells. After 24 h ERK1/2 siRNA transfection, bortezomib (40 and 100 nM) was added to the cells. Then, cell proliferation was determined after 24 h incubation. Partial knockdown of ERK1/2 in Daoy cells increased inhibition of cell growth induced by bortezomib (**Fig. 5B**).

**Bortezomib induces the accumulation of phosphorylated IκB and inhibits translocation of NFκB to nucleus.** NFκB is normally bound to inhibitor κBα (IκBα) in the cytosol. Phosphorylation, ubiquitination, and degradation of IκBα by proteasome are required for NFκB translocation to nucleus and activation of the transcription of target genes. To confirm whether bortezomib induce accumulation of phosphorylated IκBα in medulloblastoma cells, immunoblotting assays were employed to detect the expression of p-IκBα after 0.01 and 0.1 μM bortezomib treatment in Daoy cells with different time points (0, 2, 4, 8 and 16 h). Results (**Fig. 5C**) showed that expression of p-IκBα was greatly increased after bortezomib treatment in Daoy cells. Since p-IκBα cannot be degraded by proteasomes, NFκB is sequestered in the cytoplasma and amount of NFκB in the nucleus should be decreased after bortezomib treatment. In order to prove this speculation, we determined the amount of major NFκB subunit, p65/RelA, in the nucleus after bortezomib treatment. Nuclear extract was prepared from Daoy cells after 24 h treatment with 0.01 and 0.1 μM bortezomib and the expression of NFκB (p65/RelA) was analyzed by immunoblottings (**Fig. 5D**). The results showed that expression of NFκB (p65/RelA) in the nucleus was inhibited by bortezomib treatment and the inhibition was in a dose-dependent manner. These data indicate that bortezomib induces the accumulation of p-IκBα, and blocks the translocation of NFκB to the nucleus, resulting in inhibition of NFκB function.

**Bortezomib inhibits cell proliferation and induces apoptosis in a short-term primary culture of human medulloblastomas.** VC312 cells, a primary culture derived from human medulloblastoma specimens,[30] were treated with bortezomib for 48 h at 0, 0.01, 0.1 and 1 μM. Bortezomib inhibited proliferation and induced apoptosis of VC312 cells in dose-dependent manner (**Fig. 6A**). Caspase-3 was activated (cleaved form) and PARP was cleaved (**Fig. 6B**), resulting in induction of apoptosis. Expression of pro-apoptotic proteins, Bad and Bak, was increased and expression of anti-apoptotic proteins, Bcl-2 and Bcl-$x_L$, was

reduced (**Fig. 6B**). Bad and Bak are common targets for bortezomib in both established cell lines and primary culture of human medulloblastomas. Bortezomib inhibited activities of AKT and mTOR (**Fig. 6C**) and slightly increased activity of ERK1/2 in VC312 cells, which is similar to ones in Daoy and D283 cells. Expression of $p21^{Cip1}$ and $p27^{Kip1}$ was increased by bortezomib in VC312 cells (**Fig. 6D**). Thus, bortezomib promotes growth suppression and cell death in both established cell lines and primary culture of medulloblastomas.

**Bortezomib inhibits growth of human medulloblastoma tumor xenografts in mice.** To examine the effect of bortezomib on growth of medulloblastoma cells in vivo, nude mice were inoculated with Daoy cells subcutaneously and were administered bortezomib by intraperitoneal injection (0.5 mg/kg, two times/week). At this dose, no lethal toxicity or weight loss was observed among treated animals. Bortezomib treatment started at 21 d after subcutaneous inoculation of Daoy cells. Bortezomib significantly inhibited the growth of medullobastomas in mice as shown in **Figure 6E** ($p < 0.01$, $n = 11$). Even though bortezomib was only administered two times per week with a lower dose, the inhibitory effect of bortezomib on growth of tumor xenografts was obvious compared with control after 2 week treatment (day 35, **Fig. 6E**).

## Discussion

Medulloblastomas represent a therapeutic challenge because of their prevalence in children and conventional treatment, which includes surgery, chemotherapy and radiation of the craniospinal axis, is often associated with major long-term side effects.[1] Proteasome inhibitors are a novel class of compounds with promising antitumor activity. In this study, we report that bortezomib inhibits cell proliferation and induces apoptosis in human medulloblastoma cells, which are associated with the inhibition of AKT and NFκB signaling. In addition, ERK inhibitors and ERK siRNA can increase the anticancer effects of bortezomib on medulloblastomas.

AKT, a serine/threonine kinase, is a central mediator of the PI3K pathway with multiple downstream effectors that influence key cellular processes, including proliferation, cell survival and metabolism.[31] Constitutive activation of the PI3K/AKT pathway has been demonstrated in many malignancies, including medulloblastoma.[5,6,32,33] mTOR is a key kinase acting downstream of AKT and has an important role in cell proliferation and apoptosis in tumor cells, consequently targeting proteins that regulate these processes.[34,35] Therefore, AKT/mTOR signaling has emerged as a new target for development of anticancer agents. Bortezomib inhibits activities of AKT and mTOR in human medulloblastoma cells, which is associated with the induction of apoptosis and growth suppression in these cells.

NFκB, a sequence-specific transcription factor, is known to be involved in the process of tumorigenesis based on extensive evidence.[36] The major regulatory pathway for NFκB signaling is via IκB proteins, which bind and retain NFκB in the cytoplasma.[36] When IκB proteins are phosphorylated by IκB kinase, IκB proteins are degraded by the ubiquitin-protesome system, resulting in release of NFκB and its transportation to nucleus. Bortezomib inhibits the function of proteasomes and causes the accumulation of phosphorylated IκB in medulloblastoma cells. AKT also promotes cell survival via activation of NFκB by increasing degradation of IκB.[37] Since bortezomib inhibited AKT activities in medulloblastoma cells, this effect should further increase the function of IκB. Thus, NFκB is sequestered by IκB in the cytoplasma and translocation of NFκB to the nucleus is blocked. Consequently, activation of NFκB downstream genes is inhibited by bortezomib in these tumor cells.

The cellular levels of $p21^{Cip1}$ and $p27^{Kip1}$, cyclin-dependent kinase inhibitors, are subject to control by the ubiquitin-proteasome system.[38,39] Since bortezomib inhibited proteasome function and caused the accumulation of ubiquitin-conjugated proteins in medulloblastoma cells (data not shown), expression of $p21^{Cip1}$

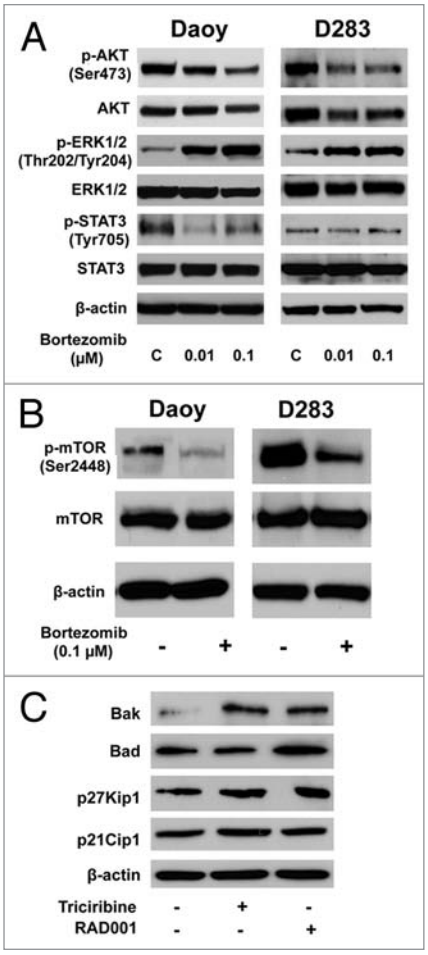


**Figure 4.** Bortezomib inhibited phosphorylation of AKT and mTOR in medulloblastoma cells. (A) Total protein was isolated from Daoy and D283 cells incubated with 0.01 and 0.1 μM bortezomib for 24 h. Immunoblotting analyses for phosphorylated and total proteins of AKT, ERK and STAT3 were performed with specific antibodies. (B) Immunoblotting assays were performed for phosphorylated and total mTOR after 24 h bortezomib treatment. (C) Effects of AKT inhibitor, triciribine and mTOR inhibitor, RAD001, on Bak, Bad, $p21^{Cip1}$ and $p27^{Kip1}$. Daoy cells were treated with 5 μM triciribine and RAD001 for 30 h and total protein was isolated from these cells. Expression of Bak, Bad, $p21^{Cip1}$ and $p27^{Kip1}$ was detected by immunoblotting assays.

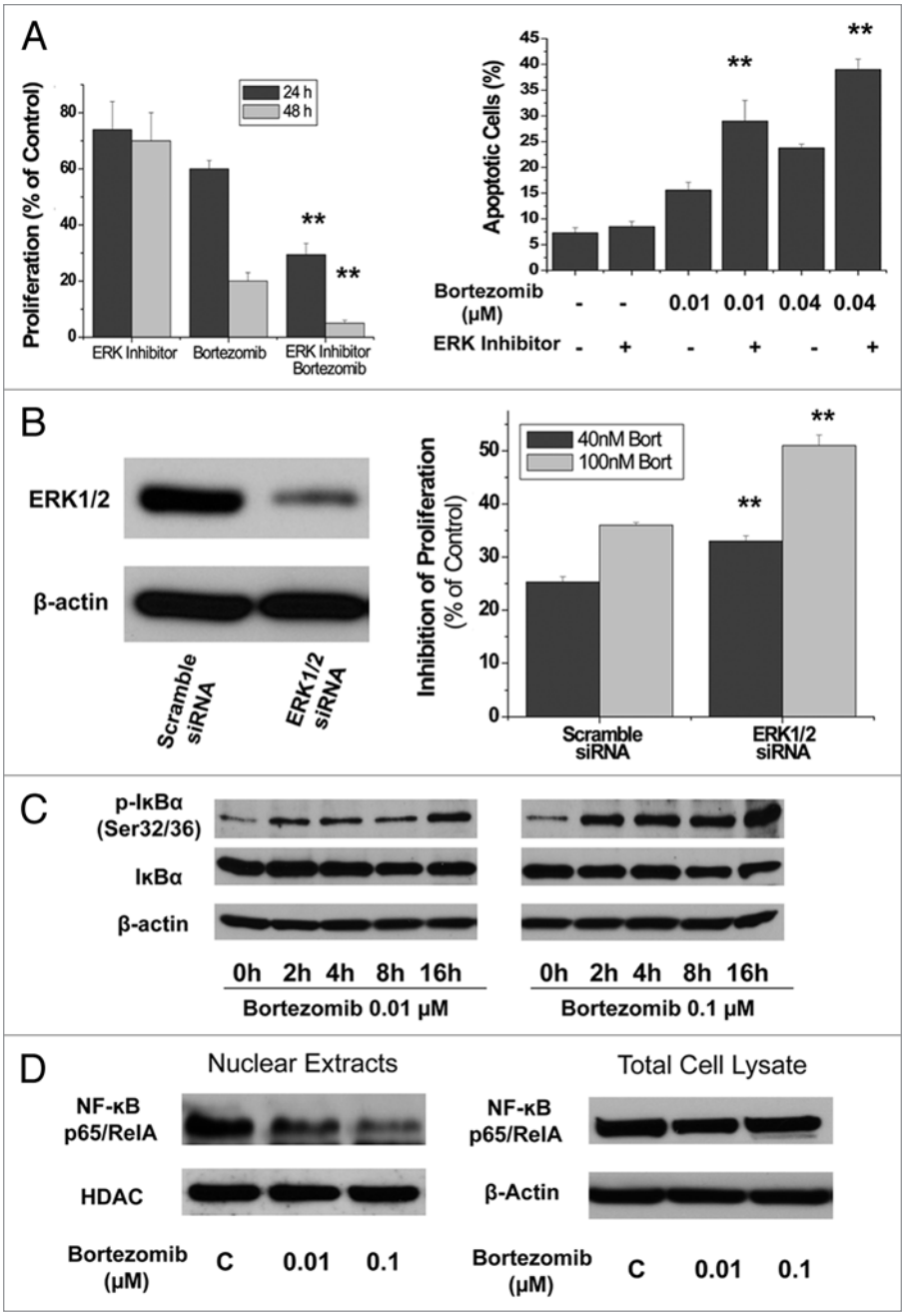


**Figure 5.** Bortezomib induced the accumulation of phosphorylated IκBα and inhibited the translocation of NFκB to the nucleus, and ERK inhibitor and ERK siRNA synergized the effects of bortezomib. (A) ERK inhibitor increased the effects of bortezomib on medulloblastoma cells. Left part, Daoy cells were treated with 10 nM bortezomib and 25 μM ERK inhibitor respectively or combination for 24 and 48 h. Then, cell proliferation was determined by MTS assay. Right part, addition of ERK inhibitor increased the induction of apoptosis after 24 h treatment with 10 or 40 nM bortezomib in Daoy cells. (B) Transfection of ERK1/2 siRNA partially decreased ERK1/2 expression in Daoy cells (left) and effects of bortezomib (40 and 100 nM) on proliferation of these cells (right) after 24 h treatment. (C) Expression of phosphorylated IκBα was determined by immunoblottings after bortezomib treatment (0.01 and 0.1 μM) with different time points as indicated. (D) Expression of NFκB (p65/RelA) in nuclear extracts and total cell lysate was examined by immunoblottings after 24 h bortezomib treatment. **p < 0.01.

and $p27^{Kip1}$ after bortezomib treatment was markedly increased. AKT downregulates the expression and function of $p21^{Cip1}$ and $p27^{Kip1}$ at both transcriptional and post-transcriptional levels.[28] Since bortezomib inhibits AKT activity in medulloblastoma cells, downregulation of AKT function also contributes to the increased expression of $p21^{Cip1}$ and $p27^{Kip1}$ in these cells. Subsequently, $p21^{Cip1}$ and $p27^{Kip1}$ induced by bortezomib inhibits cell proliferation in medulloblastoma cells. In addition, expression of pro-apoptotic proteins, Bad and Bak, was increased by bortezomib, resuting in activation of cytochrome *c*/caspases cascade and induction of apoptosis in medulloblastoma cells. Caspase inhibitors completely blocked the effects of bortrzomib on the cytotoxicity of medulloblastoma cells.

The p44 ERK1 and p42 ERK2 MAPKs have attracted intense research interest because of their critical involvement in the regulation of cell proliferation and survival. In particular, aberrant regulation of this pathway contributes to the development and metastasis of tumors.[29] Bortezomib increases expression ERK1/2 in medulloblastoma cells. To address the possibility that elevated ERK1/2 attenuates antitumor effects of bortezomib in these tumor cells, we combined bortezomib with ERK inhibitor or ERK siRNA, resulting in increased anticancer effects of bortezomib. This synergic effect of bortezomib with ERK inhibitors or ERK siRNA may potentially be beneficial for medulloblastoma treatment.

Bortezomib is the first proteasome inhibitor to have shown anticancer activity in both solid and hematological malignancies.[12] In this report, we demonstrate that bortezomib exhibits antitumor effects in two established cell lines, a primary culture and a mouse xenograft model of human medulloblastoma. Treatment of medulloblastoma is complicated by the blood-brain barrier, which acts as a physiologic barrier for delivery of drugs to the central nervous system. Bortezomib is difficult to pass through this blood-brain barrier.[40] Therefore, local delivery of bortezomib to the cerebrospinal fluid is necessary, such as convection-enhanced delivery.[41] Local delivery may result in more effective antitumor activity with reduced systemic toxicity. In summary, bortezomib is a potentially promising drug for the treatment of pediatric medulloblastomas.

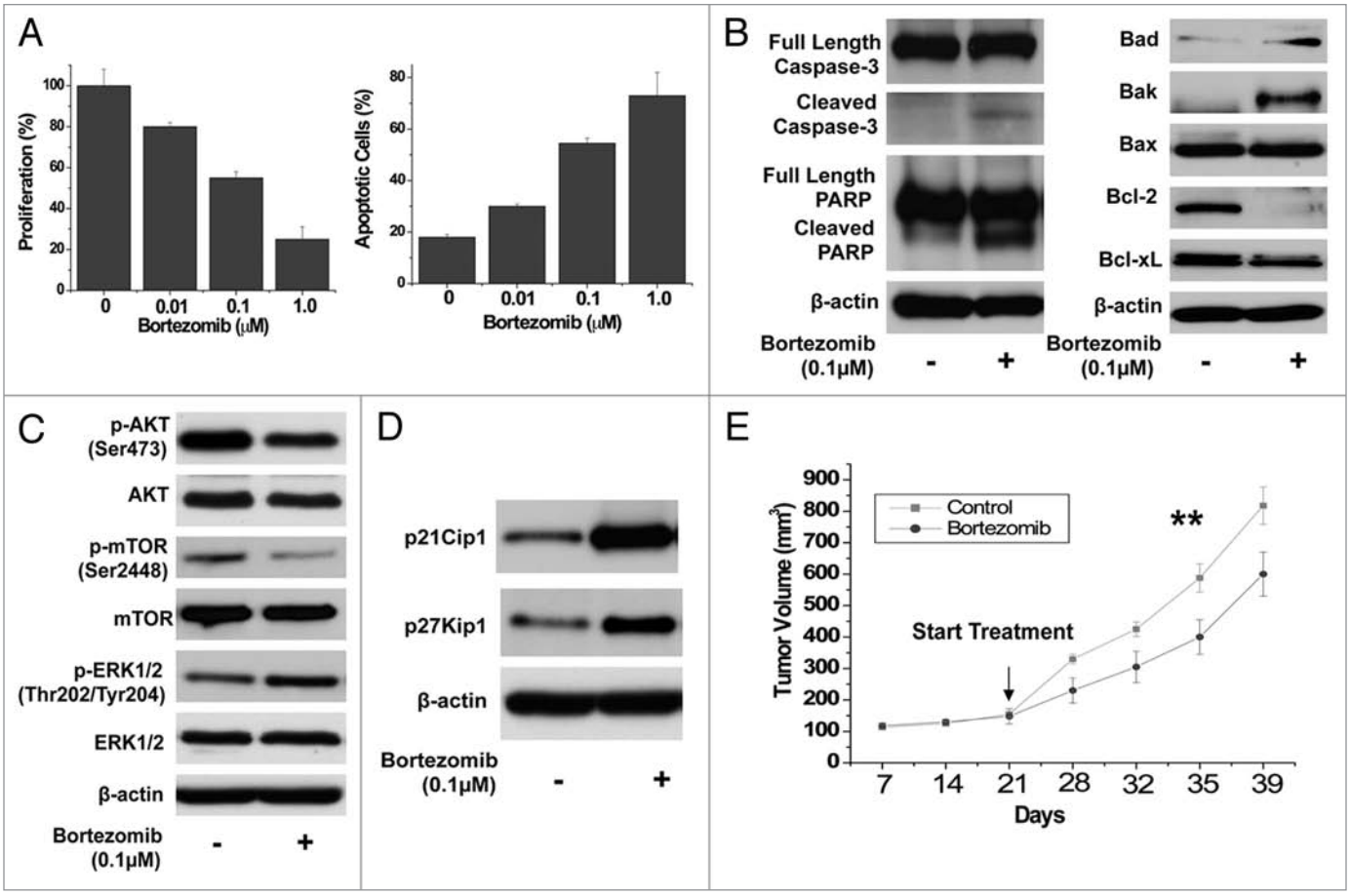


**Figure 6.** Bortezomib inhibited cell growth and survival in a primary culture of human medulloblastomas, and suppressed growth of a human medulloblastoma tumor xenograft in nude mice. (A) Bortezomib inhibited cell proliferation and induced apoptosis in VC312 cells, a primary culture of human medulloblastomas, after 48 h treatment. (B) Bortezomib increased cleavage of caspase-3 and PARP in VC312 cells after 24 h treatment. Bortezomib also increased expression of Bad and Bak, and decreased expression of Bcl-2 and Bcl-$x_L$ in these cells after 24 h treatment. (C) Effects of bortezomib on expression of phosphorylated and total AKT, mTOR and ERK in VC312 cells after 24 h treatment. (D) Bortezomib increased expression of $p21^{Cip1}$ and $p27^{Kip1}$ in VC312 cells. (E) Bortezomib inhibited the growth of Daoy medulloblastoma tumor xenograft in nude mice. Daoy tumor cells ($5 \times 10^6$ per animal) were implanted s.c. in nude mice as described in the Materials and Methods. Treatment was initiated on day 21. Bortezomib (0.5 mg/kg) was administered by intraperitoneal injection two times a week. Tumor volume ($mm^3$) exhibited mean ± SD (n = 11). **$p < 0.01$.

## Materials and Methods

**Reagents and antibodies.** Bortezomib (VELCADE) was obtained from Millennium Pharmaceutics, Inc. ERK inhibitor and triciribine was obtained from EMD Chemicals Inc. RAD001 was purchased from Selleck Chemicals. Z-VAD-FMK, an inhibitor of caspases, was purchased from Sigma. All primary antibodies were obtained from Cell Signaling. Horseradish peroxidase-labeled anti-mouse and anti-rabbit secondary antibodies were from GE Healthcare.

**Cell culture.** Human meduloblastoma cell lines, Daoy and D283, were from American Type Culture Collection (ATCC). Cells were maintained in MEM (Eagle) with L-glutamine supplemented with 10% fetal bovine serum (FBS), and 1% Antibiotic-Antimycotic (AA). The primary culture (VC312) of medulloblastoma was derived from a tumor of a 4-y-old male patient treated at the Virginia Commonwealth University Health System's Medical College of Virginia Hospital under an IRB approved protocol as previously described in reference 30. VC312 cells were maintained in DMEM (with L-glutamine) supplemented with 10% FBS and 1% AA, and used at low passage number (below passage 22 for all studies).

**Proliferation assay.** Cell proliferation assays were performed with CellTiter 96 Aqueous One Solution Cell proliferation Assay (Promega) which contains 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS). Each well of 96-well plates was seeded with 3,000 cells in culture medium. After overnight culture the cells were treated with different concentrations of bortezomib and controls were treated with vehicle ($H_2O$). After 24 or 48 h treatments, MTS was added to the cells according to the supplier's protocol and absorbance was measured at 490 nm using an automated ELISA plate reader.

**Apoptosis assay.** Tumor cells ($2 \times 10^5$) were seeded in 60 mm culture dishes in culture medium. The following day the cells were treated with indicated concentrations of bortezomib for 24 and 48 h period. After treatment, all cells including both floating and attached cells were collected, and the apoptotic cells were detected

by Annexin V-FITC Apoptosis Detection Kit (BD Biosciences). The cells were stained with Annexin V-FITC and propidium iodide (PI) according to the supplier's instructions. Viable and dead cells were detected by flow cytometry in the Analytical Cytometry Core at City of Hope National Medical Center.

**Immunoblotting analysis.** Twenty micrograms total proteins were resolved in 4–15% gradient TRIS-HCl gels (BIO-RAD). After gel electrophoresis, the proteins were transferred to Hybond-C membranes (Amersham). The membranes were blocked for 1 h at room temperature (RT) in 10% non-fat dry milk in PBST (1x PBS with 0.1% Tween-20), followed by an overnight incubation at 4°C with primary antibodies in PBST with 2% non-fat dry milk. The membranes were then incubated with horseradish peroxidase labeled anti-mouse or anti-rabbit secondary antibodies for 1 h at RT. Immunoreactivity was detected with SuperSignal West Pico substrate (Pierce).

**ERK1/2 siRNA transfection.** Human ERK1/2 siRNA was purchased from Cell Signaling and transfected to Daoy cells by using RNAiFect Transfection Reagent (QIAGEN). After 24 h transfection, cells were treated with bortezomib for 24 h, and then cell proliferation was determined.

**Cytosol isolation.** Cytosol of Daoy cells was isolated as described in reference 42. In brief, after 24 h treatment with bortezomib, cells were collected and resuspended in 1 ml of lysis buffer containing 20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 250 mM sucrose. After homogenization with a 26-gauge needle syringe 5 times, cells were centrifuged at 750x g for 10 min at 4°C. Then the supernatant was centrifuged at 10,000x g for 15 min at 4°C, and the resulting supernatant was collected (i.e., the cytosolic extract).

**Nuclear extract preparation.** For the detection of translocation of NFκB, nuclear protein extracts were prepared using high-salt extraction as previously described in reference 43.

**Tumor xenografts.** Mouse xenograft studies were performed in 4- to 6-week-old nude mice obtained from National Cancer Institute-Charles Rivers Laboratories. The mice were held in a pathogen-free animal facility at City of Hope Medical Center and were fed standard rodent chow and water ad libitum. All procedures followed the NIH guidelines for the care and use of laboratory animals. Tumors were generated by harvesting Daoy cells from mid-log phase cultures. Cells were pelleted and resuspended in a 50% mixture of Matrigel (BD Biosciences) in MEM (Eagle) medium to $2.5 \times 10^7$ cells/mL. This cell suspension (0.2 mL) was injected s.c. in the right flank of each mouse. Bortezomib treatment was started at 21 d after injection of Daoy cells. Animals with palpably established tumors of at least 65 $mm^3$ were designated to treatment groups. Treatment with 0.5 mg/kg bortezomib in 0.9% NaCl was administered by intraperitoneal injection two times per week. Mice in the control group were injected with the same amount of 0.9% NaCl with vehicle. Tumors were measured every 3–4 d with Vernier calipers, and tumor volumes were calculated by the formula $\pi/6$ x (larger diameter) × (small diameter)$^2$.

**Statistics.** Student's t-test was used to evaluate statistical significance of differences between two groups and $p < 0.05$ was considered statistically significant.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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