

Bortezomib induces apoptosis via Bim and Bik up-regulation and synergizes with cisplatin in the killing of head and neck squamous cell carcinoma cells

Changyou Li,¹ Rongxiu Li,¹ Jennifer R. Grandis,^{2,3} and Daniel E. Johnson^{1,2}

Departments of ¹Medicine, ²Pharmacology, and ³Otolaryngology, University of Pittsburgh and University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania

Abstract

Head and neck squamous cell carcinomas (HNSCC) are characterized by resistance to chemotherapy and overexpression of antiapoptotic Bcl-2 family members, including Bcl-X_L and Bcl-2. Molecular targeting of Bcl-X_L and/or Bcl-2 in HNSCC cells has been shown to promote apoptosis signaling and to sensitize cells to chemotherapy drugs, including cisplatin, which is commonly used in the treatment of HNSCC. We report that induction of HNSCC apoptosis by the proteasome inhibitor bortezomib is accompanied by up-regulation of the proapoptotic proteins Bik and Bim, natural cellular inhibitors of Bcl-X_L and Bcl-2. Additionally, bortezomib treatment of HNSCC cells caused up-regulation of antiapoptotic Mcl-1L. Inhibition of Bik or Bim up-regulation using small interfering RNA markedly attenuated bortezomib-induced cell death. By contrast, small interfering RNA-mediated inhibition of Mcl-1L expression resulted in enhanced killing by bortezomib. Further investigation showed that the combination of bortezomib and cisplatin led to synergistic killing of HNSCC cells, with calculated combination indexes well below 1.0. Taken together, these results delineate a novel mechanism of HNSCC killing by bortezomib that involves up-regulation of Bik and Bim. Moreover, our findings suggest that the combination of bortezomib plus cisplatin, or bortezomib plus an inhibitor of Mcl-1L, may have therapeutic value in the treatment of HNSCC. [Mol Cancer Ther 2008;7(6):1647–55]

Introduction

Squamous cell carcinomas of the mouth, larynx, and pharynx account for greater than 90% of all head and neck

cancers. Collectively, head and neck squamous cell carcinomas (HNSCC) represent a common human malignancy, with ~500,000 new cases per year worldwide (1). Standard treatment options for HNSCC include surgery, radiation, and chemotherapy. However, roughly half of all HNSCC patients will suffer recurrence, and the 5-year survival rate for this disease has not substantially improved over the last few decades (2, 3).

Recurring or advanced-stage HNSCC are frequently characterized by chemotherapy and radiation resistance due to failure of the tumor cells to undergo apoptosis. The overexpression of antiapoptotic members of the Bcl-2 protein family, including Bcl-X_L and Bcl-2, appears to play an important role in conferring apoptosis resistance in HNSCC (4, 5). Bcl-X_L and Bcl-2 are known to inhibit apoptosis by binding and neutralizing the activities of proapoptotic members of the Bcl-2 protein family, including the multidomain proteins Bax and Bak and the BH3 domain-only proteins Bim, Bik, Noxa, and Puma (6, 7). Overexpression of Bcl-X_L is observed in a majority of HNSCC tumors and correlates with chemotherapy resistance (4). Overexpression of Bcl-2 is also common in HNSCC, albeit to a somewhat lesser degree (8). Antisense-mediated down-regulation of Bcl-X_L and Bcl-2 in HNSCC cell lines has been shown to promote apoptosis and sensitize these cells to cisplatin and etoposide (5). Similarly, molecular targeting of Bcl-X_L and Bcl-2 with the small-molecule inhibitor (-)-gossypol (9–11) or short peptides derived from the BH3 domains of proapoptotic proteins (12) promotes apoptosis and chemosensitivity in HNSCC cells.

An alternative strategy for neutralizing the effects of Bcl-X_L and Bcl-2 overexpression in diseases such as HNSCC would involve shifting the cellular balance of proapoptotic versus antiapoptotic proteins in favor of the proapoptotic proteins. Accumulating evidence indicates that the proteasome inhibitor bortezomib (PS-341/Velcade) represents a valuable tool for perturbing this balance. Bortezomib is a potent and specific inhibitor of the chymotrypsin-like protease activity of the 26S proteasome subunit (13) and is approved for use in the treatment of refractory multiple myeloma (14, 15). In preclinical models, bortezomib has shown apoptosis-inducing and antitumor activities against both hematopoietic and solid tumor malignancies. Up-regulation of proapoptotic Noxa is frequently observed in bortezomib-treated cells and has been shown to mediate apoptosis induction by this agent in cell lines representing multiple myeloma (16, 17), non-small cell lung cancer (18), mantle cell lymphoma (19, 20), and melanoma (17). Moreover, the ability of bortezomib to sensitize non-small cell lung cancer, myeloid leukemia, and renal carcinoma

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Requests for reprints: Daniel E. Johnson, Division of Hematology/Oncology, University of Pittsburgh, Hillman Cancer Center, Room 2.18c, 5117 Centre Avenue, Pittsburgh, PA 15213-1863. Phone: 412-623-3245; Fax: 412-623-7768. E-mail: johnsond@pitt.edu
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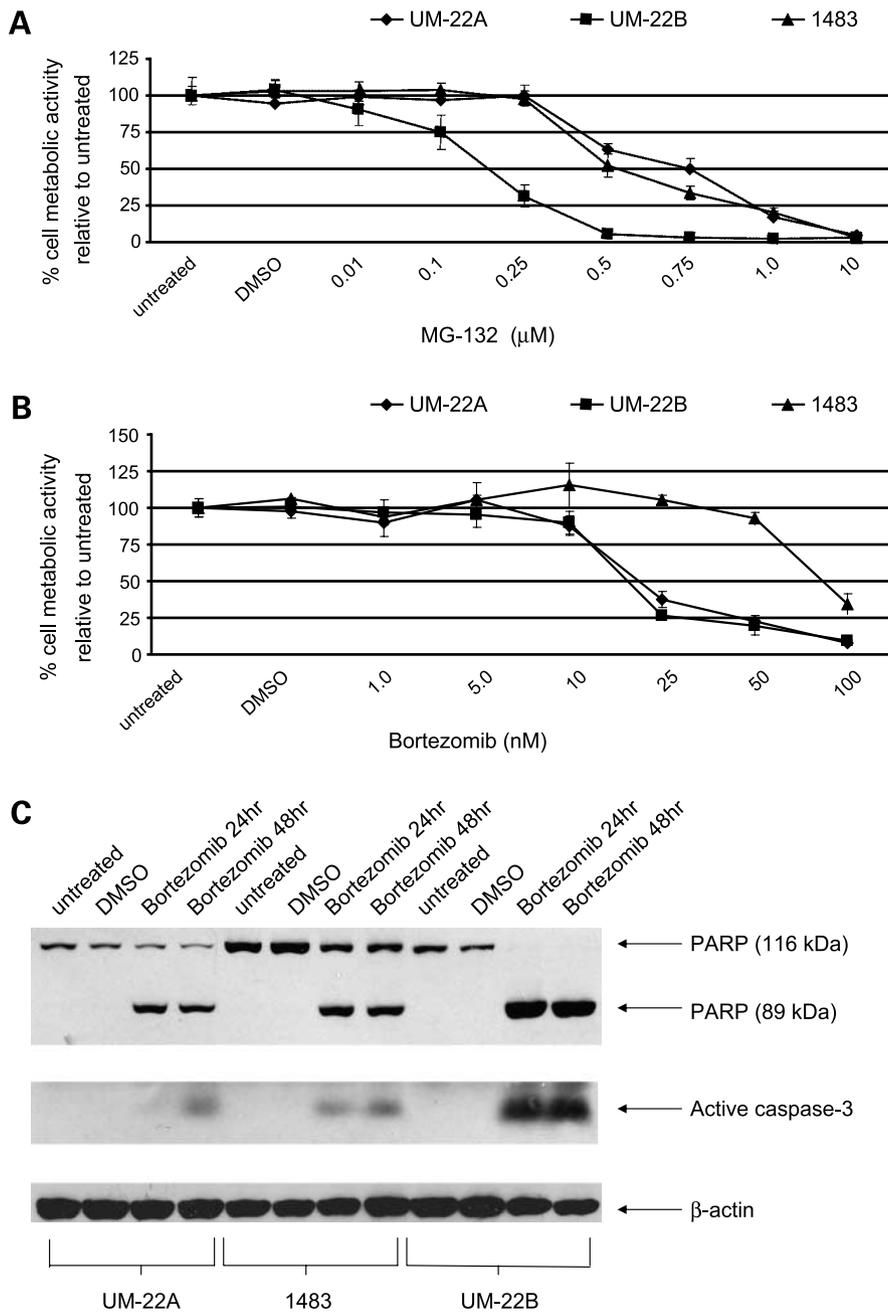


Figure 1. Induction of HNSCC cell death by proteasome inhibitors. UM-22A, UM-22B, and 1483 cells were left untreated or were treated for 48 h with 0.1% DMSO or varying concentrations of MG-132 (A) or bortezomib (B). Following treatment, MTS assays were done. Points, average of triplicate wells; bars, SD. The experiments were done twice, with similar results each time. C, UM-22A, UM-22B, and 1483 cells were left untreated or were treated for 24 or 48 h with concentrations of bortezomib corresponding to the IC₅₀ of each cell line (20 nmol/L for UM-22A and UM-22B and 80 nmol/L for 1483). As a control, cells were treated with 0.1% DMSO for 24 h. Whole-cell lysates (40 μg/lane) were subjected to immunoblotting with anti-PARP or anti-caspase-3. The processed, active forms of caspase-3 are depicted. Blots were stripped and reprobbed with anti-β-actin to show equal protein loading.

cells to tumor necrosis factor-related apoptosis-inducing ligand is associated with up-regulation of the DR5 death receptor and down-regulation of c-FLIP (21, 22), whereas tumor necrosis factor-related apoptosis-inducing ligand sensitization of prostate and colon cancer cells has been reported to involve Bik and Bim (23–25).

Bortezomib treatment of HNSCC cells leads to induction of apoptosis *in vitro* and antitumor effects *in vivo* (26–28). Several mechanisms have been proposed to explain the effects of bortezomib on HNSCC cells. Treatment with bortezomib results in inhibition of nuclear factor-κB

(26, 28), an important antiapoptotic factor that is constitutively activated and contributes to the survival of HNSCC cells (29–31). Bortezomib also promotes generation of reactive oxygen species in HNSCC, and inhibition of reactive oxygen species production attenuated bortezomib-induced cell death (27). In addition, HNSCC cells up-regulate Noxa following bortezomib treatment, and inhibition of Noxa up-regulation using small interfering RNA (siRNA) was found to moderately suppress cell death (32). Treatment of HNSCC tumors with bortezomib *in vivo* results in suppression of nuclear factor-κB target genes

and important proangiogenic cytokines including interleukin-6, GRO-1, and vascular endothelial growth factor (26, 28). Inhibition of HNSCC cell migration and cell-cell adhesion by bortezomib has also been reported (33).

In this study, we investigated the effect of bortezomib on apoptosis-regulatory proteins in HNSCC cells. We found that bortezomib induced expression of the proapoptotic proteins Bim and Bik and the antiapoptotic protein Mcl-1L. Inhibition of Bim or Bik up-regulation using siRNA strategies significantly attenuated bortezomib-induced apoptosis. By contrast, inhibition of Mcl-1L up-regulation served to enhance killing by bortezomib. In further experiments, we determined that bortezomib synergizes with cisplatin in the killing of HNSCC cells. Together, these findings delineate a new mechanism of bortezomib-induced apoptosis in HNSCC and support dual targeting of HNSCC cells using bortezomib plus an inhibitor of Mcl-1L or bortezomib plus cisplatin.

Materials and Methods

Cells and Reagents

The human HNSCC cell lines UM-22A, UM-22B, and 1483 (34) were grown at 37°C and 5% CO₂ in DMEM

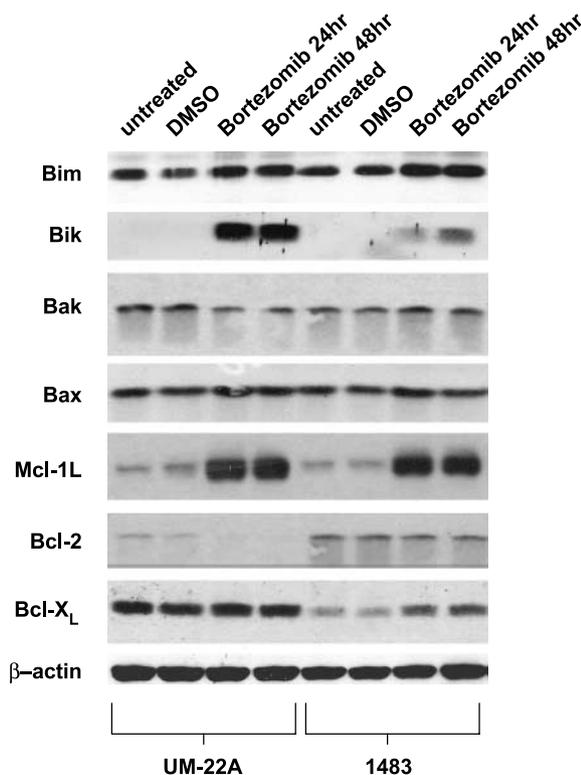


Figure 2. Bortezomib regulates the expression of proapoptotic and antiapoptotic Bcl-2 family members in HNSCC cells. UM-22A and 1483 cells were left untreated or were treated for 24 or 48 h with bortezomib (UM-22A: 20 nmol/L; 1483: 80 nmol/L). Control cells were treated for 24 h with 0.1% DMSO. Whole-cell lysates (40 µg/lane) were subjected to immunoblotting for the indicated proteins. Probing with anti-β-actin was used to show equal protein loading. Similar results were obtained in three independent experiments.

(Mediatech) containing 10% heat-inactivated fetal bovine serum and antibiotics. UM-22A cells were derived from a primary HNSCC tumor and UM-22B cells were derived from a cervical lymph node metastasis from the same patient. Both of these cell lines were kindly provided by Dr. Thomas Carey (University of Michigan). 1483 cells were derived from a primary HNSCC tumor and were provided by Dr. Gary Clayman (M. D. Anderson Cancer Center). Bortezomib was purchased from the University of Pittsburgh Cancer Institute Pharmacy, and a stock solution (10 mmol/L) was prepared in DMSO and stored at -80°C. MG-132 was obtained from Calbiochem, and a stock solution (10 mmol/L) was similarly prepared in DMSO and stored at -80°C. Antibodies against Bik, Bax, Mcl-1, and Bcl-X_L were purchased from Santa Cruz Biotechnology. Antibodies against poly(ADP-ribose) polymerase (PARP) and Bak were obtained from Cell Signaling Technology. Anti-Bcl-2 antibody was from DAKO, whereas anti-Bim antibody was from Calbiochem. Anti-caspase-3 was obtained from Assay Designs. Secondary horseradish peroxidase-conjugated antibodies were from Promega.

Immunoblotting

Cells were harvested by scraping of the monolayers followed by centrifugation at 4°C for 5 min and washing once in cold PBS. The cell pellets were resuspended in cell lysis buffer [50 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 0.1% SDS, 1% NP-40] containing 1.5 mmol/L phenylmethylsulfonyl fluoride, 3 µg/mL leupeptin, and 20 µg/mL aprotinin. After 1 h on ice, the lysates were centrifuged at 13,000 rpm for 10 min at 4°C. Protein concentrations in the clarified lysates were determined using Bio-Rad Protein Assay dye concentrate (Bio-Rad Laboratories). Proteins (40 µg/lane) were electrophoresed on 10% or 12.5% SDS-PAGE gels, transferred to nitrocellulose filters, and blocked for 1 h at room temperature in 5% nonfat dry milk in 10 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 0.1% Tween 20 (TBST). The blocked filters were briefly rinsed in TBST buffer, incubated with primary antibody at 4°C overnight, and then washed again in TBST. The filters were then incubated with secondary antibody for 1 h at room temperature followed by washing in TBST. Blots were developed using enhanced chemiluminescence reagent according to the guidelines of the manufacturer (Perkin-Elmer Life Science).

Cell Viability Assays

To determine cellular sensitivities to proteasome inhibitors, UM-22A cells (10,000 per well), UM-22B cells (5,000 per well), and 1483 cells (8,000 per well) were plated in triplicate in 96-well plates 1 day before treatment. Varying concentrations of MG-132 and bortezomib were then added to cells followed by incubation for 48 h at 37°C. The concentration of DMSO in the wells never exceeded 0.1%, and treatment with 0.1% DMSO was used as a control in each experiment. Following treatment, 20 µL MTS reagent (5 mg/mL) was added to each well and incubation was continued for 1 h at 37°C. Absorbance was measured at 570 nm using a VERSA MAX microplate reader (Molecular Devices).

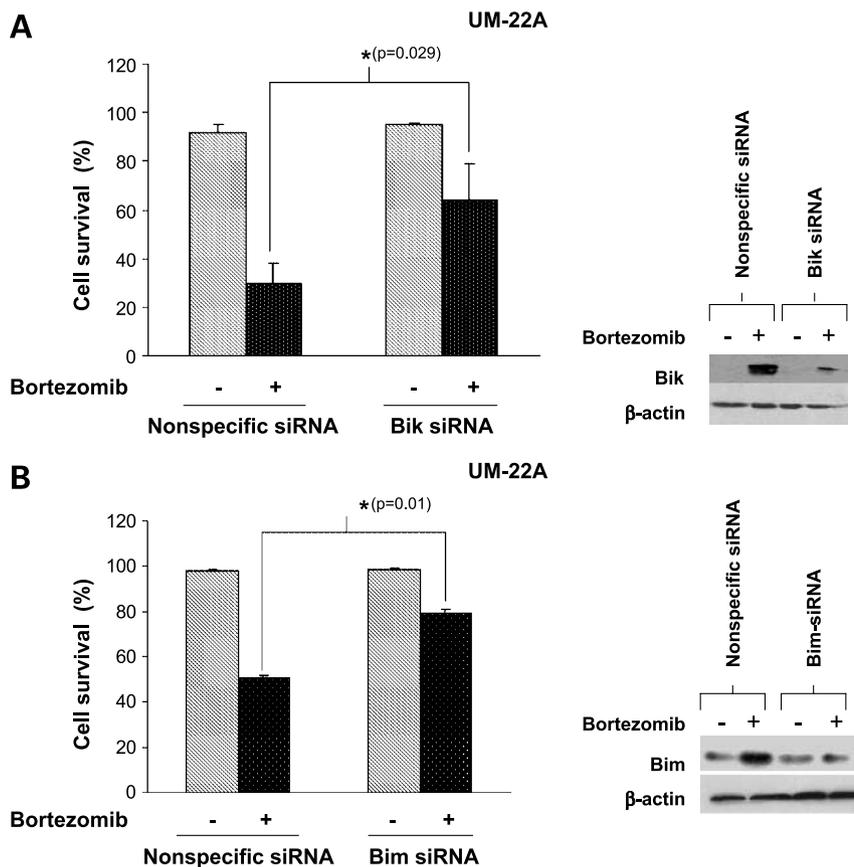


Figure 3. Bik and Bim mediate bortezomib-induced HNSCC cell death. **A**, UM-22A cells were transfected with Bik siRNA or a nonspecific siRNA as described in Materials and Methods. After 24 h, the transfected cells were either left untreated or were treated for 48 h with 20 nmol/L bortezomib. Cell viabilities were determined by trypan blue exclusion assays, and Bik expression levels were determined by immunoblotting. *Columns*, average of means obtained in three independent experiments; *bars*, SE. ***, $P = 0.029$, compared with bortezomib-treated cells transfected with nonspecific siRNA versus Bik siRNA. **B**, UM-22A cells were transfected with Bim siRNA or a nonspecific siRNA, then treated, and analyzed as in **A**. *Columns*, average of means from three independent experiments; *bars*, SE. ***, $P = 0.01$, compared with bortezomib-treated cells transfected with nonspecific siRNA versus Bim siRNA.

For experiments involving siRNA down-regulation of apoptosis-regulatory proteins, cell viabilities were assessed by trypan blue exclusion assays. A minimum of 300 cells were counted per data point and the average of triplicate experiments was plotted. Error bars represent the SE.

siRNA Transfection

Cells (6.0×10^5 per well) were seeded into 6-cm dishes 1 day before transfection. Immediately before transfection, the culture medium was removed and replaced with DMEM containing 10% FBS but lacking antibiotics. Duplex siRNA oligonucleotides (100 nmol/L) were then transfected into cells using LipofectAMINE 2000 (Invitrogen) diluted in Opti-MEM (Invitrogen) according to the manufacturer's protocol. After 12 h, the medium was removed and fresh DMEM containing 10% FBS and antibiotics was added. Following incubation for an additional 12 h, bortezomib was added and the cells treated for an additional 48 h. Down-regulation of protein expression was then analyzed by immunoblotting and cell viability was assessed by trypan blue exclusion assays. siRNA for Bim (targeting sequence: 5'-CCUUCUGAUGUAAGUUCUGTT-3'), Bik (targeting sequence: 5'-CUAUUACUCCAAGACCUCU-3'), and Mcl-1 (targeting sequence: 5'-CCUGAAAUCUAAAUCACUTT-3') were synthesized at Ambion. Nonspecific siRNA was also purchased from Ambion.

Analysis of Synergy

UM-22A cells (10,000 per well) and 1483 cells (8,000 per well) were plated in triplicate in 96-well plates 1 day in advance of treatment. The cells were then treated with cisplatin alone, bortezomib alone, or the combination of cisplatin plus bortezomib for 24 h at 37°C followed by performance of MTS assays. Synergy was determined using the method of Chou and Talalay (35). Combination indexes (CI) were calculated using CalcuSyn V2 software (Biosoft). A CI = 1 indicates an additive effect, a CI < 1 indicates synergy, and a CI > 1 indicates antagonism.

Statistics

Statistical analysis of the data was done using SigmaStat software (Jandel Scientific Software). Comparison between groups was made using one-way ANOVA followed by the Student's-Newman-Keuls' test. $P < 0.05$ was considered significant.

Results

Bortezomib Killing of HNSCC Cells Is Accompanied by Changes in the Expression Levels of Bcl-2 Family Members

In initial studies, we evaluated the effect of proteasome inhibition on three HNSCC cell lines: UM-22A, UM-22B, and 1483 (34, 36–38). UM-22A and 1483 cells were

originally derived from primary HNSCC tumors from different patients. UM-22B cells were derived from a cervical lymph node metastasis from the same patient that yielded UM-22A cells. In Fig. 1A, cells were treated with varying doses of the proteasome inhibitor MG-132, and 48 h later, MTS assays were done to obtain a measure of cell viability. In this assay, UM-22B cells exhibited an IC_{50} of $\sim 0.2 \mu\text{mol/L}$ for MG-132, whereas UM-22A and 1483 exhibited IC_{50} values of 0.5 and $0.75 \mu\text{mol/L}$, respectively. Controls cells that were left untreated or were treated with 0.1% DMSO, the drug diluent, did not show any loss of viability over the 48-h timeframe. All three HNSCC cell lines showed even greater sensitivity to the proteasome inhibitor bortezomib (Fig. 1B). In this case, UM-22A and UM-22B cells showed essentially identical sensitivities, with IC_{50} values of $\sim 20 \text{ nmol/L}$. 1483 cells were moderately less sensitive to bortezomib, with an IC_{50} of $\sim 80 \text{ nmol/L}$. The killing of UM-22A, UM-22B, and 1483 cells by bortezomib was accompanied by molecular features of apoptosis, including processing of procaspase-3 to active subunits and cleavage of PARP protein (39), indicating an apoptotic form of cell death (Fig. 1C).

We next evaluated the effect of bortezomib treatment on the expression levels of both proapoptotic and antiapoptotic members of the Bcl-2 protein family. As shown in Fig. 2, bortezomib treatment did not substantially alter the expression of the multidomain proapoptotic proteins Bak and Bax. By contrast, bortezomib treatment for 24 or 48 h resulted in moderate up-regulation of the BH3 domain-only protein Bim and dramatic induction of the BH3 domain-only protein Bik. In addition, bortezomib caused marked up-regulation of Mcl-1L, an antiapoptotic protein whose expression levels in other cell types have been shown to be regulated by the proteasome (40). The expression levels of antiapoptotic Bcl-2 and Bcl-X_L were not appreciably changed following bortezomib treatment, although a modest up-regulation of Bcl-X_L was observed in 1483 cells. Collectively, these data indicated that bortezomib treatment of HNSCC cells induced the expression of both proapoptotic proteins, which might serve to mediate the killing effects of this compound, and an

antiapoptotic protein, which might act to dampen the induction of apoptosis by bortezomib.

Bik and Bim Mediate, Whereas Mcl-1L Suppresses, Bortezomib-Induced HNSCC Apoptosis

To determine whether Bik or Bim may be acting to mediate bortezomib-induced apoptosis in HNSCC cells, a siRNA strategy was used to prevent the up-regulation of these proteins following bortezomib treatment. UM-22A cells were transfected with either Bik (Fig. 3A) or Bim (Fig. 3B) siRNA duplex oligonucleotides (100 nmol/L) or a nonspecific siRNA as control. After 24 h, bortezomib (20 nmol/L) was added to the cells, and incubation continued for an additional 48 h. The cells were then harvested for analysis of protein expression levels by immunoblotting and assessment of cell viabilities by trypan blue exclusion assays. As expected, cells transfected with the nonspecific siRNA exhibited dramatic up-regulation of Bik and moderate up-regulation of Bim following bortezomib treatment. By contrast, bortezomib-induced up-regulation of Bik or Bim was markedly attenuated in cells transfected with Bik or Bim siRNA, respectively. Attenuation of Bik up-regulation resulted in a significant inhibition of bortezomib-induced cell death ($P = 0.029$). Similarly, prevention of Bim up-regulation also resulted in reduced killing by bortezomib ($P = 0.01$). These results showed that Bik and Bim play important roles in mediating bortezomib-induced killing of HNSCC cells.

Whereas the up-regulation of Bik and Bim was found to be important for the activity of bortezomib against HNSCC cells, the simultaneous up-regulation of antiapoptotic proteins such as Mcl-1L may serve to blunt the potency of this compound. To determine the consequence of bortezomib-induced Mcl-1L up-regulation, transfection of Mcl-1L siRNA was used to prevent up-regulation of the protein (Fig. 4). In contrast to what was seen using Bik or Bim siRNA, prevention of Mcl-1L up-regulation resulted in significant enhancement of bortezomib-induced cell death. ($P = 0.004$). This indicated that the ability of bortezomib to kill HNSCC cells is partially restrained due to bortezomib-induced up-regulation of antiapoptotic Mcl-1L.

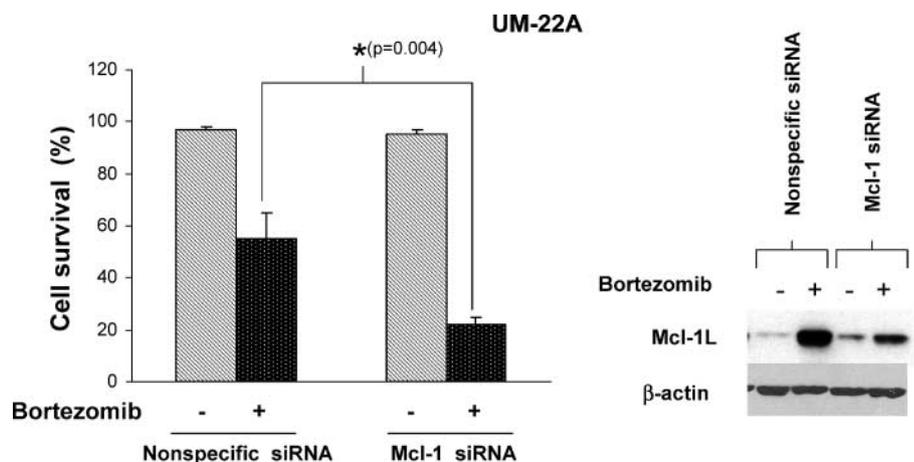
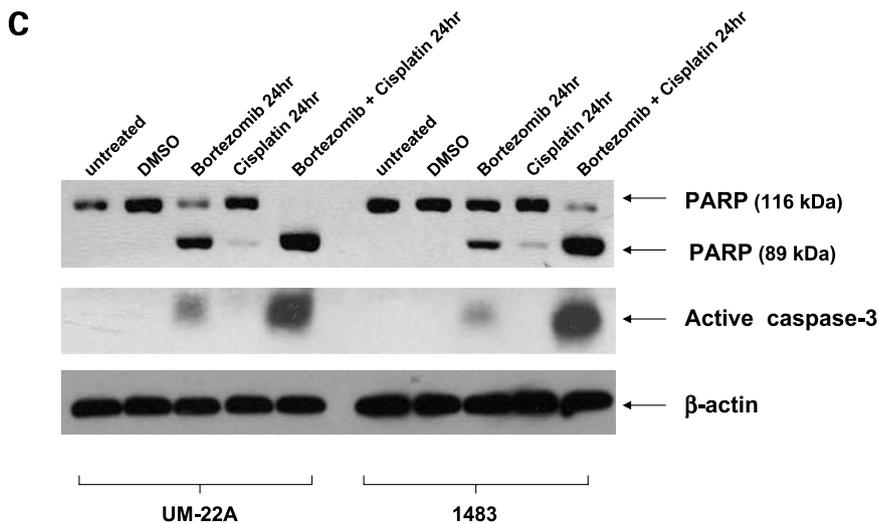
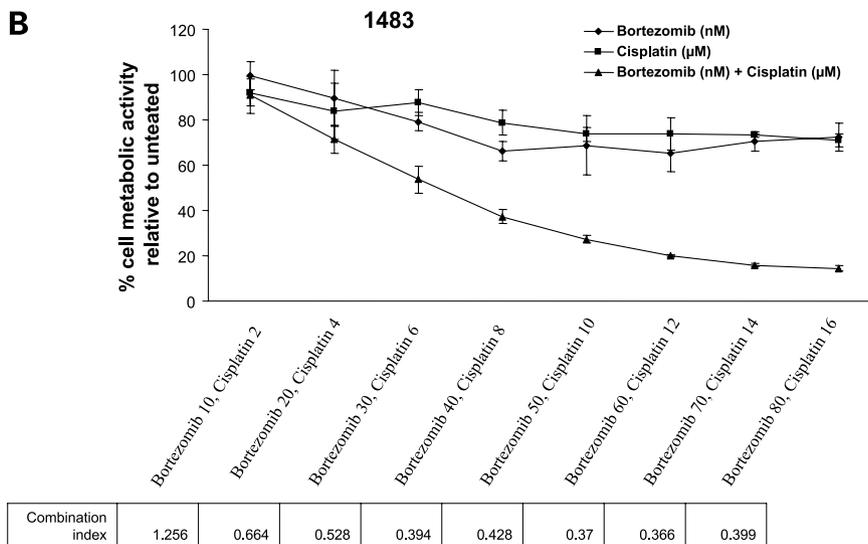
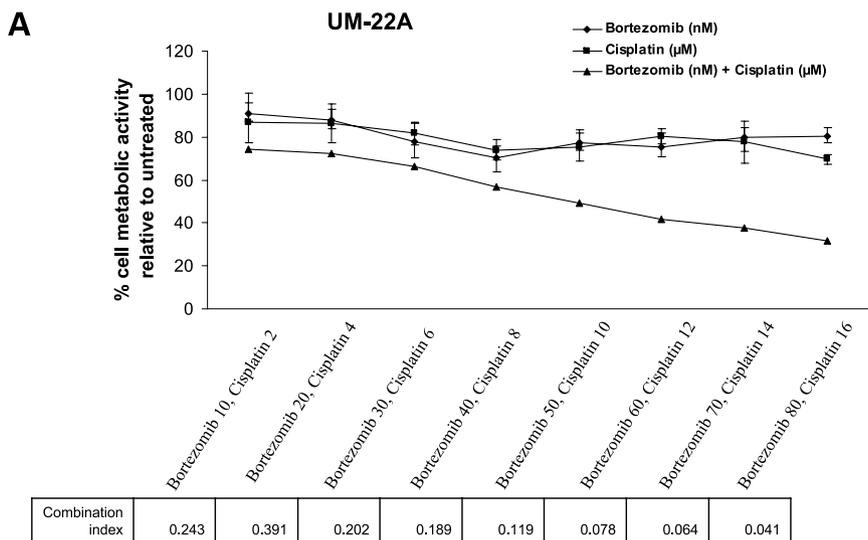


Figure 4. Inhibition of Mcl-1L up-regulation enhances bortezomib-induced death of HNSCC cells. UM-22A cells were transfected with Mcl-1 siRNA or nonspecific siRNA followed by 48 h of treatment with 20 nmol/L bortezomib. Trypan blue exclusion assays were used to determine cell viabilities, and immunoblotting was used to assess siRNA-mediated inhibition of Mcl-1L up-regulation. Columns, average of means from three independent experiments; bars, SE. *, $P = 0.004$, compared with bortezomib-treated cells transfected with nonspecific siRNA versus Mcl-1 siRNA.



Bortezomib Synergizes with Cisplatin in the Killing of HNSCC Cells

An overall strategy in developing anticancer drug regimens is to identify synergistic drug combinations that will allow lowering of drug doses to prevent undesirable side effects in patients. In the case of HNSCC, cisplatin is commonly used as a frontline chemotherapeutic agent. To determine whether bortezomib might synergize with cisplatin in the killing of HNSCC cells, UM-22A and 1483 cells were treated for 24 h with varying doses of bortezomib plus cisplatin while maintaining a constant ratio of the two drugs (Fig. 5A and B). Synergy was then assessed by determining CI (CalcuSyn V2 software) according to the method of Chou and Talalay (35). According to this algorithm, $CI < 1.0$ is indicative of synergy. CI well below 1.0 was achieved at multiple combinations of bortezomib and cisplatin, showing synergy between these drugs in the killing of HNSCC cells. Synergy between bortezomib and cisplatin was also shown using clonogenic survival assays (Supplementary Fig. S1; data not shown).⁴

We next sought to confirm the observed synergy between bortezomib and cisplatin by using a molecular marker of apoptosis induction. For these experiments, we examined PARP cleavage and caspase-3 processing in cells treated with bortezomib alone, cisplatin alone, or a combination of bortezomib plus cisplatin known to yield a $CI < 1.0$ (as determined in Fig. 5A and B). Treatment of either UM-22A or 1483 cells for 24 h with the combination of 40 nmol/L bortezomib plus 8.0 μ mol/L cisplatin resulted in a dramatic increase in PARP cleavage and caspase-3 processing relative to that observed in cells treated with either drug alone (Fig. 5C). Thus, the combination of bortezomib plus cisplatin resulted in synergistic induction of apoptosis signaling, suggesting the potential utility of combining these agents in a therapeutic regimen for HNSCC.

Discussion

Members of the Bcl-2 protein family act as key regulators of cellular apoptosis and are important determinants of cellular sensitivity or resistance to chemotherapy drugs (6). Overexpression of Bcl-X_L and Bcl-2, antiapoptotic members of this family, is commonly observed in HNSCC, and Bcl-X_L overexpression correlates with chemoresistance in this disease (4, 8). Previously, we and others have shown

that molecular targeting of Bcl-X_L and/or Bcl-2 with BH3 peptides (12), (-)-gossypol (9–11), or antisense (5) promotes apoptosis signaling and cell death in HNSCC cells. The natural cellular antagonists of Bcl-X_L and Bcl-2 are proapoptotic members of the same protein family and include the BH3 domain-only proteins Bik, Bim, Noxa, and Puma. In this study, we found that treatment of HNSCC cells with the proteasome inhibitor bortezomib results in up-regulation of proapoptotic Bik and Bim as well as up-regulation of antiapoptotic Mcl-1L. Inhibition of Bik or Bim up-regulation using siRNA significantly inhibited bortezomib-induced cell death, showing an important role for these proteins in mediating the killing activity of bortezomib. An opposite effect was seen when siRNA was used to prevent Mcl-1L up-regulation, indicating that bortezomib-induced up-regulation of Mcl-1L serves to dampen the potency of this compound in HNSCC.

It is important to note that our experiments involving siRNA-mediated suppression of Bik or Bim up-regulation never resulted in complete inhibition of bortezomib-induced cell death. This suggests that Bik- and Bim-independent pathways also contribute to HNSCC cell killing by bortezomib. Sunwoo et al. (26) have shown that bortezomib inhibits nuclear factor- κ B, an antiapoptotic transcription factor that is aberrantly hyperactivated in a majority of HNSCC cell lines and primary specimens (29–31). Interestingly, an early clinical trial of bortezomib in combination with radiation showed effective down-regulation of nuclear factor- κ B target genes in HNSCC tumors from patients who exhibited a reduction in tumor size (28). This suggests the potential utility of bortezomib inclusion in HNSCC therapeutic regimens. Others have reported that bortezomib induces generation of reactive oxygen species in HNSCC cells and activation of the unfolded protein response pathway (27). Activation of this pathway was associated with phosphorylation of the stress kinase PERK, induction of ATF-4 transcription factor, and up-regulation of Noxa (32). Inhibition of Noxa up-regulation in these cells modestly inhibited bortezomib-induced cell death (32). Thus, up-regulation of Bik, Bim, and Noxa all may play a role in the killing of HNSCC cells by bortezomib. At the same time, it is also apparent that bortezomib induces responses that lessen the killing activity of this compound in HNSCC cells as evidenced by our observation of Mcl-1L up-regulation. This supports the use of combination approaches targeting multiple pathways as a reasonable strategy in HNSCC.

The ability of bortezomib to enhance apoptosis induction by other anticancer agents has been reported. Bortezomib

⁴ Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

Figure 5. Bortezomib synergizes with cisplatin in the killing of HNSCC cells. UM-22A cells (A) and 1483 cells (B) were treated for 24 h with bortezomib alone, cisplatin alone, or a constant ratio of bortezomib plus cisplatin. Following treatment, MTS assays were done. Points, average of triplicate wells; bars, SD. CI was determined as described in Materials and Methods. Similar results were obtained in three independent experiments. C, UM-22A and 1483 cells were left untreated or were treated for 24 h with 0.1% DMSO alone, 40 nmol/L bortezomib alone, 8 μ mol/L cisplatin alone, or the combination of 40 nmol/L bortezomib plus 8 μ mol/L cisplatin. Whole-cell lysates were analyzed by immunoblotting for cleavage of PARP protein and processing/activation of caspase-3. The blot was stripped and reprobbed with anti- β -actin to show equal protein loading. The experiment was done twice, with similar results each time.

enhances cell death caused by the death ligand tumor necrosis factor-related apoptosis-inducing ligand (21–23), the BH3 mimetic GX15-070 (20), and the histone deacetylase inhibitors trichostatin A, sodium butyrate, and PXD101 (41). In addition, bortezomib has been shown to enhance the activities of the epidermal growth factor receptor inhibitors PKI166 and PD153035 (33, 42). These findings may be particularly relevant for the treatment of HNSCC, because overexpression and/or hyperactivation of epidermal growth factor receptor is observed in a majority of HNSCC tumors and contributes to malignant cell growth and survival in this disease (43–45). In our studies, we observed that bortezomib potently synergizes with the chemotherapy drug cisplatin in the killing of HNSCC cells. Interestingly, Fribley et al. (32) have reported that bortezomib is able to kill HNSCC cells that are resistant to cisplatin. As cisplatin is commonly used in the treatment of HNSCC, it will be important to evaluate the combination of bortezomib plus cisplatin as a therapeutic strategy.

In summary, we have identified a novel mechanism of bortezomib-induced killing of HNSCC cells, which involves up-regulation of the proapoptotic proteins Bik and Bim. Moreover, our finding that bortezomib up-regulation of Mcl-1L lessens the killing activity of this compound supports the development of Mcl-1L targeting agents as a means of enhancing bortezomib potency against HNSCC tumors. Lastly, the potent synergy we observed between bortezomib and cisplatin suggests that the combination of these agents may prove useful for improving therapeutic efficacy in a disease where little improvement in survival rates has been seen over the past few decades.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

- Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2006. *CA Cancer J Clin* 2006;56:106–30.
- Forastiere A, Koch W, Trotti A, Sidransky D. Head and neck cancer. *N Engl J Med* 2001;345:1890–900.
- Mao L, Hong WK, Papadimitrakopoulou VA. Focus on head and neck cancer. *Cancer Cell* 2004;5:311–6.
- Trask DK, Wolf GT, Bradford CR, et al. Expression of Bcl-2 family proteins in advanced laryngeal squamous cell carcinoma: correlation with response to chemotherapy and organ preservation. *Laryngoscope* 2002; 112:638–44.
- Sharma H, Sen S, Lo Muzio L, Mariggio A, Singh N. Antisense-mediated downregulation of anti-apoptotic proteins induces apoptosis and sensitizes head and neck squamous cell carcinoma cells to chemotherapy. *Cancer Biol Ther* 2005;4:720–7.
- Danial NN, Korsmeyer SJ. Cell death: critical control points. *Cell* 2004; 116:205–19.
- Cheng EH, Wei MC, Weiler S, et al. BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. *Mol Cell* 2001;8:705–11.
- Drenning SD, Marcovitch AJ, Johnson DE, Melhem MF, Twardy DJ, Grandis JR. Bcl-2 but not Bax expression is associated with apoptosis in normal and transformed squamous epithelium. *Clin Cancer Res* 1998;4: 2913–21.
- Oliver CL, Bauer JA, Wolter KG, et al. *In vitro* effects of the BH3 mimetic, (-)-gossypol, on head and neck squamous cell carcinoma cells. *Clin Cancer Res* 2004;10:7757–63.
- Bauer JA, Trask DK, Kumar B, et al. Reversal of cisplatin resistance with a BH3 mimetic, (-)-gossypol, in head and neck cancer cells: role of wild-type p53 and Bcl-xL. *Mol Cancer Ther* 2005;4:1096–104.
- Wolter KG, Wang SJ, Henson BS, et al. (-)-gossypol inhibits growth and promotes apoptosis of human head and neck squamous cell carcinoma *in vivo*. *Neoplasia* 2006;8:163–72.
- Li R, Boehm AL, Miranda MB, Shangary S, Grandis JR, Johnson DE. Targeting antiapoptotic Bcl-2 family members with cell-permeable BH3 peptides induces apoptosis signaling and death in head and neck squamous cell carcinoma cells. *Neoplasia* 2007;9:801–11.
- Adams J, Kauffman M. Development of the proteasome inhibitor Velcade (bortezomib). *Cancer Invest* 2004;22:304–11.
- Bross PF, Kane R, Farrell AT, et al. Approval summary for bortezomib for injection in the treatment of multiple myeloma. *Clin Cancer Res* 2004; 10:3954–64.
- Richardson PG, Mitsiades C, Hideshima T, Anderson KC. Bortezomib: proteasome inhibition as an effective anticancer therapy. *Ann Rev Med* 2006;57:33–47.
- Gomez-Bougie P, Wulleme-Toumi S, Menoret E, et al. Noxa up-regulation and Mcl-1 cleavage are associated to apoptosis induction by bortezomib in multiple myeloma. *Cancer Res* 2007;67:5418–24.
- Qin JZ, Ziffra J, Stennett L, et al. Proteasome inhibitors trigger NOXA-mediated apoptosis in melanoma and myeloma cells. *Cancer Res* 2005;65: 6282–93.
- Voortman J, Checinska A, Giaccone G, Rodriguez JA, Kruyt FA. Bortezomib, but not cisplatin, induces mitochondria-dependent apoptosis accompanied by up-regulation of noxa in the non-small cell lung cancer cell line NCI-H460. *Mol Cancer Ther* 2007;6:1046–53.
- Perez-Galan P, Roue G, Villamor N, Montserrat E, Campo E, Colomer D. The proteasome inhibitor bortezomib induces apoptosis in mantle-cell lymphoma through generation of ROS and Noxa activation independent of p53 status. *Blood* 2006;107:257–64.
- Perez-Galan P, Roue G, Villamor N, Campo E, Colomer D. The BH3-mimetic GX15-070 synergizes with bortezomib in mantle cell lymphoma by enhancing Noxa-mediated activation of Bak. *Blood* 2007;109:4441–9.
- Sayers TJ, Brooks AD, Koh CY, et al. The proteasome inhibitor PS-341 sensitizes neoplastic cells to TRAIL-mediated apoptosis by reducing levels of c-FLIP. *Blood* 2003;102:303–10.
- Liu X, Yue P, Chen S, et al. The proteasome inhibitor PS-341 (bortezomib) up-regulates DR5 expression leading to induction of apoptosis and enhancement of TRAIL-induced apoptosis despite up-regulation of c-FLIP and survivin expression in human NSCLC cells. *Cancer Res* 2007;67:4981–8.
- Nikrad M, Johnson T, Puthalalath H, Coultas L, Adams J, Kraft AS. The proteasome inhibitor bortezomib sensitizes cells to killing by death receptor ligand TRAIL via BH3-only proteins Bik and Bim. *Mol Cancer Ther* 2005;4:443–9.
- Zhu H, Zhang L, Dong F, et al. Bik/NBK accumulation correlates with apoptosis-induction by bortezomib (PS-341, Velcade) and other proteasome inhibitors. *Oncogene* 2005;24:4993–9.
- Zhu H, Guo W, Zhang L, et al. Proteasome inhibitors-mediated TRAIL resensitization and Bik accumulation. *Cancer Biol Ther* 2005;4:781–6.
- Sunwoo JB, Chen Z, Dong G, et al. Novel proteasome inhibitor PS-341 inhibits activation of nuclear factor- κ B, cell survival, tumor growth, and angiogenesis in squamous cell carcinoma. *Clin Cancer Res* 2001;7: 1419–28.
- Fribley A, Zeng Q, Wang CY. Proteasome inhibitor PS-341 induces apoptosis through induction of endoplasmic reticulum stress-reactive oxygen species in head and neck squamous cell carcinoma cells. *Mol Cell Biol* 2004;24:9695–704.
- Van Waes C, Chang AA, Lebowitz PF, et al. Inhibition of nuclear factor- κ B and target genes during combined therapy with proteasome inhibitor bortezomib and reirradiation in patients with recurrent head-and-neck squamous cell carcinoma. *Int J Rad Oncol Biol Phys* 2005;63:1400–12.
- Ondrey FG, Dong G, Sunwoo J, et al. Constitutive activation of transcription factors NF- κ B, AP-1, and NF-IL6 in human head and neck squamous cell carcinoma cell lines that express pro-inflammatory and pro-angiogenic cytokines. *Mol Carcinogenesis* 1999;26:119–29.
- Duffey DC, Chen Z, Dong G, et al. Expression of a dominant-negative mutant inhibitor- κ B α of nuclear factor- κ B in human head and neck squamous cell carcinoma inhibits survival, proinflammatory cytokine expression, and tumor growth *in vivo*. *Cancer Res* 1999;59:3468–74.

31. Loercher A, Lee TL, Ricker JL, et al. Nuclear factor- κ B is an important modulator of the altered gene expression profile and malignant phenotype in squamous cell carcinoma. *Cancer Res* 2004;64:6511–23.
32. Fribley AM, Evenchik B, Zeng Q, et al. Proteasome inhibitor PS-341 induces apoptosis in cisplatin-resistant squamous cell carcinoma cells by induction of Noxa. *J Biol Chem* 2006;281:31440–7.
33. Lorch JH, Thomas TO, Schmoll HJ. Bortezomib inhibits cell-cell adhesion and cell migration and enhances epidermal growth factor receptor inhibitor-induced cell death in squamous cell cancer. *Cancer Res* 2007;67:727–34.
34. Lin CJ, Grandis JR, Carey TE, et al. Head and neck squamous cell carcinoma cell lines: established models and rationale for selection. *Head Neck* 2007;29:163–88.
35. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Reg* 1984;22:27–55.
36. Krause CJ, Carey TE, Ott RW, Hurbis C, McClatchey KD, Regezi JA. Human squamous cell carcinoma. Establishment and characterization of new permanent cell lines. *Arch Otolaryngol* 1981;107:703–10.
37. Sacks PG, Parnes SM, Gallick GE, et al. Establishment and characterization of two new squamous cell carcinoma cell lines derived from tumors of the head and neck. *Cancer Res* 1988;48:2858–66.
38. Grenman R, Carey TE, McClatchey KD, et al. *In vitro* radiation resistance among cell lines established from patients with squamous cell carcinoma of the head and neck. *Cancer* 1991;67:2741–7.
39. Kaufmann SH, Desnoyers S, Ottaviano Y, Davidson NE, Poirier GG. Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. *Cancer Res* 1993;53:3976–85.
40. Opferman JT. Unraveling MCL-1 degradation. *Cell Death Differ* 2006;13:1260–2.
41. Duan J, Friedman J, Nottingham L, Chen Z, Ara G, Van Waes C. Nuclear factor- κ B p65 small interfering RNA or proteasome inhibitor bortezomib sensitizes head and neck squamous cell carcinomas to classic histone deacetylase inhibitors and novel histone deacetylase inhibitor PXD101. *Mol Cancer Ther* 2007;6:37–50.
42. An J, Rettig MB. Epidermal growth factor receptor inhibition sensitizes renal cell carcinoma cells to the cytotoxic effects of bortezomib. *Mol Cancer Ther* 2007;6:61–9.
43. Rubin Grandis J, Melhem MF, Gooding WE, et al. Levels of TGF- α and EGFR protein in head and neck squamous cell carcinoma and patient survival. *J Natl Cancer Inst* 1998;90:824–32.
44. Kalyankrishna S, Grandis JR. Epidermal growth factor receptor biology in head and neck cancer. *J Clin Oncol* 2006;24:2666–72.
45. Cohen EE. Role of epidermal growth factor receptor pathway-targeted therapy in patients with recurrent and/or metastatic squamous cell carcinoma of the head and neck. *J Clin Oncol* 2006;24:2659–65.

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