Proteasome Inhibitors Trigger NOXA-Mediated Apoptosis in Melanoma and Myeloma Cells

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Abstract

Patients with metastatic melanoma or multiple myeloma have a dismal prognosis because these aggressive malignancies resist conventional treatment. A promising new oncologic approach uses molecularly targeted therapeutics that overcomes apoptotic resistance and, at the same time, achieves tumor selectivity. The unexpected selectivity of proteasome inhibition for inducing apoptosis in cancer cells, but not in normal cells, prompted us to define the mechanism of action for this class of drugs, including Food and Drug Administration–approved bortezomib. In this report, five melanoma cell lines and a myeloma cell line are treated with three different proteasome inhibitors (MG-132, lactacystin, and bortezomib), and the mechanism underlying the apoptotic pathway is defined. Following exposure to proteasome inhibitors, effective killing of human melanoma and myeloma cells, but not of normal proliferating melanocytes, was shown to involve p53-independent induction of the BH3-only protein NOXA. Induction of NOXA at the protein level was preceded by enhanced transcription of NOXA mRNA. Engagement of mitochondrial-based apoptotic pathway involved release of cytochrome c, second mitochondria-derived activator of caspases, and apoptosis-inducing factor, accompanied by a proteolytic cascade with processing of caspases 9, 3, and 8 and poly(ADP-ribose) polymerase. Blocking NOXA induction using an antisense (but not control) oligonucleotide reduced the apoptotic response by 30% to 50%, indicating a NOXA-dependent component in the overall killing of melanoma cells. These results provide a novel mechanism for overcoming the apoptotic resistance of tumor cells, and validate agents triggering NOXA induction as potential selective cancer therapeutics for life-threatening malignancies such as melanoma and multiple myeloma. (Cancer Res 2005; 65(14): 6282-93)

Introduction

Malignancies such as melanoma and multiple myeloma are characterized by aberrant cellular responses to signals governing proliferation and apoptosis. Targeting molecular pathways that regulate diverse cellular responses has emerged as a promising therapeutic strategy (1). A novel biologically based treatment approach uses agents targeting the proteasome, a universal and broadly active cellular complex responsible for regulating protein degradation and maintenance of normal cell function (1, 2). As substrates for the proteasome include regulatory proteins involved in cell cycle progression, apoptosis, and angiogenesis, targeting the proteasome represents an attractive therapeutic approach for cancer treatment (3). Proteasome inhibitors display encouraging results in several malignancies, including multiple myeloma and a variety of solid tumors (1, 4). The unexpected selectivity of proteasome inhibitors for cancer cells versus normal cells has challenged investigators to delineate the molecular mechanism responsible for induction of apoptosis in neoplastic cells (5). As described herein, we discovered that proteasome inhibitors selectively induce the proapoptotic BH3-only protein NOXA in melanoma and myeloma cells, but not in normal melanocytes, providing new insight into the molecular basis for differential apoptotic responses of neoplastic versus normal cells.

Although the ultimate result of inhibiting proteasome activity in tumor cells is frequently apoptosis, and bortezomib was Food and Drug Administration (FDA) approved for the treatment of patients with refractory multiple myeloma that failed prior chemotherapy (6), the precise sequence of events responsible for killing malignant cells has yet to be definitively established. Currently, antilymoma activity of proteasome inhibition is partially defined as being p53 independent and involving caspase activation (7–9). To further define the molecular mechanism responsible for apoptosis, we characterized the apoptotic response in melanoma, a highly lethal tumor that is notoriously poorly responsive to treatment, and in multiple myeloma, a malignancy for which proteasome inhibitors have shown clinical efficacy. While this study was under way, a preclinical report showed the ability of a proteasome inhibitor (bortezomib) to kill melanoma cells in vivo, and postulated this was due to inhibition of the nuclear factor κB (NF-κB) survival pathway (10).

Not only is melanoma incidence on the rise (11), but mortality rates are also increasing (12). The dismal prognosis for metastatic melanoma patients reflects the resistance of tumors to conventional therapy (13). Despite improvement in the understanding of melanoma pathogenesis (14), new therapeutic strategies are still needed for metastatic melanoma patients (15). Examples of molecular mechanisms mediating drug resistance include activation of Ras signaling with enhanced survival levels of Bcl-2 (16–19); increased survivin levels (20); activation of Akt/protein kinase B and NF-κB-mediated signaling (21); loss of death receptors (22); and inactivation of effector caspases regulated by apoptotic protease activating factor-1 (23) or X-linked inhibitor of apoptosis protein (24). Many apoptotic-related abnormalities are also present in myeloma cells (8, 25). We investigated whether proteasome targeting overcomes these molecular abnormalities preventing the
engagement of an effective apoptotic program in melanoma and myeloma cells.

In this report, we show that three different proteasome inhibitors induce NOXA (at the mRNA and protein level) in a p53-independent fashion for five different melanoma cell lines and a myeloma cell line. The mechanism for induction of NOXA involved enhanced transcription, rather than protein stabilization, as blocking transcription of NOXA mRNA using an antisense oligonucleotide specific for NOXA significantly reduced killing of melanoma cells, thereby highlighting NOXA dependency for this apoptotic reaction. By defining a key role for BH3-only family proteins (i.e., NOXA) triggering a caspase cascade culminating in apoptosis in melanoma and myeloma cells, but not in normal melanocytes, the ability of these aggressive malignant cells to escape apoptosis has been overcome. These findings open the door for new therapeutic strategies targeting a NOXA-mediated apoptotic killing of cancer cells present in patients with melanoma and myeloma.

Materials and Methods

Cell lines. Normal human melanocytes were obtained from neonatal foreskins as previously described (26). Human melanoma cell lines were established from metastatic melanoma lesions obtained from patients before receiving dendritic cell-based vaccine for immunotherapy as part of a phase I FDA-approved clinical trial (physician-initiated investigational new drug by Dr. Nickoloff). Removal of metastatic lesions was done after obtaining written informed consent and approval of the Loyola Institutional Review Board. Primary early-passage melanoma cells (RJ002 and MG012) were cultured in RPMI containing 10% FCS. Late-passage melanoma cell lines (C8161, MUM2B, and SK-Mel-28), as well as a multiple myeloma cell line [RPMI8226, obtained from American Type Culture Collection (ATCC), Rockville, MD], were also used in this study as previously described (27, 28). Proliferation assays were conducted in the presence or absence of 10% FCS by manual counting of melanoma cells in triplicate wells on days 0, 1, 2, and 3.

Proteasome inhibitors and antibodies. The proteasome inhibitors were obtained from either Calbiochem (MG-132; carboxbenzoxyl-I-leucyl-I-leucyl-f-Leu; Sigma Chemical Co, St. Louis, MO), Bortezomib, manufactured by Millennium Pharmaceuticals (PS-341; hydrazylcarbonyl-Phe-Leu-boronate; Millenium Pharmaceuticals, Cambridge, MA), was obtained from the pharmacy. Antibodies used were as follows: p21, Bcl-2-antagonist of cell death (Bad), Bcl-2, Bcl-Xs, Mcl-1, Bcl-2 homologous antagonist/killer (Bak), p53, apoptosis-inducing factor, poly(ADP)-ribose polymerase, and caspase 3 from Sigma Chemical Co (Santa Cruz, CA); NOXA from Calbiochem; Bcl-2-interacting death factor (BID), Bcl-2-interacting mediator of cell death (Bim), p53 up-regulated modulator of apoptosis (PUMA), and cleaved caspase 3 from Cell Signaling (Beverly, MA); caspase 8 and Bax from Upstate Biotechnology (Charlottesville, VA); and β-actin from ICN (Irvine, CA). Antibody against second mitochondria-derived activator of caspases (SMAC) was obtained from IMGENEX (San Diego, CA), and antibody against endonuclease G was obtained from ProSci, Inc (Poway, CA).

In vivo melanoma growth response to bortezomib. A xenograft animal model system was used in which C8161 melanoma cells (10⁶) were injected s.c. into nude (nu/nu) female mice (6-7 weeks old; Harlan, Indianapolis, IN). After 1 week, mice were assigned to each of the following tumor bearing groups (5 mice/group) and injected with either (a) PBS as control, (b) bortezomib—1.25 mg/kg, or (c) bortezomib—2.5 mg/kg. Treatment began on day 8 when tumors were palpable and perpendicular injected four times with either PBS (control) or bortezomib at 1.25 or 2.5 mg/kg. On day 20, mice were euthanized and tumors dissected from surrounding tissue and weighed. The mice were housed at the University of Illinois Chicago Institutional Animal Care and Use Committee approved the experimental protocol.

Bortezomib-Induced NOXA Kills Melanoma and Myeloma Cells

Retroviruses. The dominant-negative Fas-associating protein with death domain (FADD DN) cDNAs was provided by Dr. Vishva Dixit (Genentech, Inc., South San Francisco, CA), and was subcloned into the BamHI and NotI sites of LZRS retroviral expression vector as previously described (29). A Bcl-Xs retroviral construct was also used as previously described (30).

Apoptosis. Cell viability was assessed using Apo Tag Annexin V-FITC staining kits (Biosource, Camarillo, CA) according to the instructions of the manufacturer. The relative percentage of cells undergoing apoptosis was quantified by flow cytometric analysis using FACS Calibur (Beckton Dickinson, Palo Alto, CA) as described (26). The pan-caspase inhibitor carbobenzoxy-valine-aspartate-fluoromethylketone (z-VAD-fmk) was purchased from Calbiochem. Leucine zipper-Apo2L/giant/necrosis factor-like apoptosis-inducing ligand (i.e., LZ-TRAIL) was obtained from Genentech, and used as previously described (31).

Western blot analysis. Whole cell extracts were prepared as previously described (32). Briefly, cells were lysed by scraping monolayers and washed with PBS. Cell pellets were resuspended in CHAPS buffer containing a protease inhibitor cocktail. Extracts were vigorously shaken at 4°C for 15 minutes following centrifugation. Supernatants were collected and protein concentration determined using Bio-Rad reagent. Thirty- to fifty-microgram protein samples were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membrane by electroblootting. Membranes were probed with various primary antibodies overnight at 4°C, followed by detection using ECL reagents (Amersham Pharmacia Biotech, Piscataway, NJ) according to the instructions of the manufacturer.

Subcellular fractionation. To determine release of cytochrome c, SMAC/DIABLO, apoptosis-inducing factor, and endonuclease G from the mitochondria, an enriched mitochondria pellet and mitochondria-free cytosol were prepared with the Apo Alert cell fractionation kit (Clontech Laboratories, Inc., Palo Alto, CA) according to the instructions of the manufacturer. The mitochondria-free cytosolic fraction was used for Western blot analysis.

NOXA mRNA analysis. Total RNA was prepared using Trizol reagent (Invitrogen Corp., Carlsbad, CA). One microgram of total RNA was reverse transcribed using TaqMan (Roche, Branchburg, NJ). Quantitative real-time PCR was done with iQ SYBR Green Supermix (Bio-Rad Laboratories, NJ) according to the instructions of the manufacturer. The primer sequences used for NOXA were forward: 5′-AGATGCGTGGGAAGAGG-3′ and reverse: 5′-AGTCCCCCTAGT-CAAGT-3′ as previously described (33). An initial step was programmed for 5 minutes at 95°C, followed by 40 cycles at 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute. Fluorescence was automatically monitored at every cycle and at the post-temperature ramp. All expression levels were normalized to GAPDH.

The human NOXA and GAPDH cDNAs were obtained from ATCC (Manassas, VA). The coding sequence was amplified by PCR and used as a probe (labeled with [α-32P]dCTP, Amersham Biosciences) for Northern blot hybridization. Northern blot analysis was done with Northern Max System (Ambion, Austin, TX) following the protocol of the manufacturer. Twenty micrograms of total RNA were loaded for each lane, and the relative amounts of 28S and 18S RNA served as loading control.

Antisense oligonucleotide treatment. The antisense oligonucleotides included a NOXA-targeted sequence (5′-IS156688: TCAGCTAGTCTGGTGCTT-GGAGTGG-3′) and a universal scrambled control oligonucleotide (5′-IS129695: TCTACCTGGCCTACCTAG-3′) as previously described (26). Melanoma lines were seeded at 2 × 10³ cells into six-well plates 1 day before transfection. Opti-MEM was preincubated for 30 minutes at room temperature using a ratio of 3 μL/mL Lipofectamine per 100 nmol/L to produce a final oligonucleotide concentration of 50 nmol/L. Cells were washed with PBS and transfection mix (1 mL) was added. After 4 hours of incubation, RPMI 1640 (1 mL) containing 20% fetal bovine serum and protease inhibitor was added.

p53 small interfering RNA treatment. Smart pools of p53 small interfering RNA (siRNA) duplexes and scrambled control duplexes were purchased from Upstate Biotechnology. RJ002L melanoma cells were plated in six-well plates at a density of 2 × 10³ cells per well and transfection was accomplished using Oligofectamine in Opti-MEM medium following the
protocol of the manufacturer (Invitrogen). After 48 hours, transfected cells were treated with proteasome inhibitors for the indicated period of time.

**Statistical analysis.** All data presented are expressed as the mean and SE, which were derived from at least three independent experiments. Statistical analysis was assessed by Student’s t test. Results were considered significant at $P < 0.05$, and asterisks in figures denote statistically significant differences.

**Results**

Proteasome inhibitors induce apoptosis in a panel of human melanoma cell lines but not in normal melanocytes *in vitro*. To begin exploring the ability of proteasome inhibitors to induce apoptosis in melanoma cells, an initial kinetic analysis was done using bortezomib (1.0 μmol/L) and RJ002L melanoma cells. During the initial 6 hours of exposure, minimal changes in viability of the culture were observed, but prominent apoptosis was detected at 18- and 24-hour time points (Fig. 1A). Next, we did a more thorough analysis involving both normal melanocytes and a panel of malignant cell lines. Defining a dose-response effect for proteasome inhibitors such as bortezomib using normal proliferating melanocytes or early-passage melanoma cells has not been well documented. To systematically examine the apoptotic response induced by blocking proteasome function, three different proteasome inhibitors were evaluated. Proteasome inhibitors included MG-132, lactacystin, and bortezomib. Bortezomib triggered an apoptotic response in less than 10% of all proliferating melanocytes (MC009 and MC011) over a concentration range of 0.01 to 10 μmol/L after 24 hours of continuous exposure (Fig. 1B, left). In contrast, bortezomib triggered a dose-dependent increase in apoptosis of all proliferating melanoma cell lines tested ranging from 30% to 70% dead cells at a 10 μmol/L concentration of the proteasome inhibitor after 24 hours of continuous exposure (Fig. 1B, right). For all melanoma cell lines examined, exposure to bortezomib at concentrations of 0.1 μmol/L or greater triggered significant apoptotic responses ($P < 0.05$). The apoptotic response of proliferating normal melanocytes (MC005, MC006, and MC008) to 24 hours of exposure to either MG-132 or lactacystin (10 μmol/L) induced less than 15% killing of the normal cell population. However, treatment of melanoma cell lines with either MG-132 or lactacystin (10 μmol/L) induced a 5- to 10-fold increase in the apoptotic response of all cell lines treated (Fig. 1C). A representative phase-contrast microscopic appearance of proliferating RJ002L melanoma cells before and 24 hours after exposure to each proteasome inhibitor is shown in Fig. 1D, triggering the rounding of tumor cells with membrane blebbing and detachment characteristic of cells undergoing apoptosis.

**Antitumor activity of bortezomib using melanoma xenograft model *in vivo*.** To confirm and extend these *in vitro* findings, proapoptotic effects for one proteasome inhibitor (i.e., bortezomib) was investigated by injecting melanoma cell line C8161 s.c. into nude mice. Compared with PBS-injected tumors that continued to grow, regression of melanoma tumors occurred using either 1.25 or 2.5 mg/kg of bortezomib (Supplementary Fig. 1A and B). There was a significant ($P < 0.01$) reduction in tumor weight comparing PBS-injected tumor versus tumor injected with 1.25 mg/kg bortezomib, and no further reduction was apparent at 2.5 mg/kg dose. Light microscopic examination of tumors revealed numerous apoptotic melanoma cells in the bortezomib-treated, but not PBS-treated, samples (Supplementary Fig. 1C, arrows).

Interaction of proteasome inhibitors with death receptor and mitochondrial-based apoptotic pathways. Two well-characterized apoptotic pathways involve either engagement of cell surface death receptors with activation of an intracellular cascade of death-inducing proteases such as caspases or a more direct disruption of the mitochondrial membrane potential (34, 35). A method to distinguish between so-called extrinsic versus intrinsic pathways is to employ dominant-negative receptors directed against a key adaptor protein linking death receptor complex to initiator caspases (36). Thus, two different melanoma cell lines were infected using a retrovirus containing a FADD DN construct and then exposed to either LZ-TRAIL or bortezomib, or to both agents (37). In a melanoma cell line (C8161), Western blotting confirmed prominent overexpression of FADD DN protein when comparing empty linker with FADD DN–infected cells (Supplementary Fig. 2, *inset*). Whereas either LZ-TRAIL or bortezomib alone induced 25% to 40% apoptosis, respectively, after 24 hours in control-infected C8161 melanoma cells (linker), over 80% of these melanoma cells were killed by combining these agents (Supplementary Fig. 2, left). When identical treatments were used on melanoma cells overexpressing FADD DN, the apoptotic response due to either LZ-TRAIL alone or in combination with bortezomib was almost completely blocked ($P < 0.05$), but no significant inhibition of bortezomib–induced apoptosis was observed (Supplementary Fig. 2, right). Identical results were observed in the melanoma cell line RJ002L, indicating bortezomib does not trigger apoptosis using the extrinsic (death receptor) pathway (Fig. 2A). Taken together, these results point to a mitochondrial-based apoptotic pathway by which proteasome inhibitors kill melanoma cells.

To further probe into a mitochondrial-based pathway, RJ002L melanoma cells were infected with a retroviral construct to overexpress Bcl-xL. Bcl-xL blocks apoptosis by influencing mitochondrial potential via interaction with proapoptotic proteins Bax and Bak (35). After confirming overexpression of Bcl-xL (Fig. 2A), melanoma cells were exposed to bortezomib. Overexpression of Bcl-xL provided significant ($P < 0.05$) protection against bortezomib–induced apoptosis, further reinforcing the importance of mitochondrial-based pathway. As previously reported in non-melanoma cells, Bcl-xL overexpression reduced LZ-TRAIL–mediated apoptosis in RJ002L cells (38).

**Activation of mitochondrial-based apoptotic pathway in melanoma cells.** When the intrinsic or mitochondrial-based cell death pathway is engaged, there is release of proapoptotic factors such as cytochrome c and SMAC/DIABLO (39) from mitochondria with subsequent activation of caspase 9 and other caspases such as caspases 3 and 8 (40–42). Release of both cytochrome c and SMAC/DIABLO from mitochondria into cytoplasm was detected in RJ002L cells following bortezomib exposure (Fig. 2B). Cytosolic levels of cytochrome c began to increase at 3 to 6 hours, with more prominent levels detected at 18 to 24 hours posttreatment. SMAC/DIABLO levels were readily detectable in cytoplasmic fraction at 18- to 24-hour time points, as were prominent levels of apoptosis-inducing factor. Release of apoptosis-inducing factor from mitochondria triggers a caspase-independent apoptotic response (43). Using C8161 melanoma cells, cytoplasmic levels of cytochrome c and apoptosis-inducing factor were enhanced following bortezomib treatment, and accompanied by increased endonuclease G (44) beginning at 3 hours posttreatment (Fig. 2C).

**NOXA: A critical p53-independent determinant of specificity for proteasome inhibitor–mediated killing of melanoma cells.** Given aforementioned results highlighting a mitochondrial-based apoptotic pathway following exposure to proteasome inhibitors,
a search was conducted to identify potentially important proteins mediating the killing of melanoma cells. Many regulators of cellular life or death switches belong to the Bcl-2 family (45). These proteins include opposing factions of antiapoptotic and proapoptotic members. Beginning with proapoptotic proteins belonging to BH3-only family (46), four different melanoma cell lines were examined before and 18 hours after exposure to bortezomib (Fig. 3A).

Among the five different BH3-only family members examined, only NOXA was consistently induced in all four melanoma cells by bortezomib. Other BH3-only proteins examined in these melanoma cell lines revealed constitutive levels of Bad, Bid, PUMA, and Bim. After bortezomib exposure, Bad, Bid, and PUMA levels decreased, with no changes in Bim levels in all melanoma cell lines (Fig. 3A). These results indicate that among the two categories of BH3-only proteins, the only ‘sensitizing’ molecule was NOXA in melanoma cells following treatment with bortezomib (47).

Moving to an examination of multiple-BH related family members (Fig. 3B) revealed constitutive levels of prosurvival proteins Bcl-xL and Mcl-1L in all cell lines with two different melanoma cell lines (C8161 and MUM2B) constitutively expressing Bcl-2. All four melanoma cells constitutively expressed proapoptotic proteins Mcl-1S, Bax, and Bak. Exposure for 18 hours to bortezomib had differential effects on levels of the multiple-BH family members, with some protein levels being reduced (i.e., Bcl-xL in RJ002L and SK-Mel-28 cells; Bax in RJ002L, C8161, and MUM2B), whereas Mcl-1S and Bak levels were enhanced (minimal changes were identified in the other proteins).

To compare and contrast melanocytic responses to melanoma cell responses, two different melanocyte cultures (MC010 and MC012) were examined and immunoblots prepared to detect BH3-only and multiple-BH related family members before and after bortezomib exposure. Figure 3A reveals constitutive expression of Bad and Bim with slight reductions in Bim levels following treatment, accompanied by variable levels of Bid. No NOXA or PUMA levels were detected before or after bortezomib exposure. Figure 3B reveals constitutive levels of Bcl-2 and Bax with barely detectable levels of Bak accompanied by variable levels of Bcl-xL and Mcl-1L. The Mcl-1S levels increased following bortezomib exposure, with variable responses for the other Bcl-2 family members. Thus, overall, the melanoma cells responded very differently than the melanocyte cultures as regards bortezomib-induced proteins belonging to the BH3-only family as...
after addition of bortezomib, by 80% (data not shown). Thus, apoptotic response, and the induction of NOXA protein, 24 hours
with cycloheximide (1 μg/mL). Induction of NOXA mRNA was identified at 2, 4, 6, and 8 hours. In the next set of
studies (Fig. 1 inset), the apoptotic susceptibility to either bortezomib (1 μmol/L) or LZ-TRAIL (100 ng/mL) was determined. Whereas FADD DN overexpression reduced the LZ-TRAIL–mediated apoptotic response (*, P < 0.05) but not the bortezomib-induced apoptosis, the Bcl-xL overexpression reduced both bortezomib– as well as LZ-TRAIL–induced apoptosis. These results indicate an important role for mitochondrial-based (intrinsic) rather than death receptor (extrinsic)–mediated pathway for bortezomib in melanoma cell lines. B and C, proliferating RJ002L (B) and C8161 (C) melanoma cells were subjected to a mitochondrial isolation procedure before and after various time intervals following exposure to bortezomib (1 μmol/L). Protein extracts were prepared from the mitochondrial-free cytosolic fractions and relative levels of either cytochrome c, apoptosis-inducing factor (AIF), SMAC, or endonuclease G (endo G) were determined by Western blot analysis. The results indicate that bortezomib triggered release of several proapoptotic proteins that contribute to both caspase-dependent and caspase-independent cell death pathways.

Next, the relationship between proliferation and NOXA induction was investigated. Inducing a relatively quiescent state in C8161 melanoma cells was accomplished by serum withdrawal.

Well as the multiple-BH related family members with only NOXA up-regulated by proteasome inhibitors in melanoma cells.

Expanding the in vitro studies to in vivo studies, s.c. tumors produced in nude mice were tested to detect NOXA using whole cell protein extracts. Tumors of C8161 melanoma cells injected with PBS did not contain detectable NOXA, but injection of bortezomib (2.5 mg/kg) did induce NOXA in these treated tumors (Fig. 3C). These results support a role for NOXA in the apoptotic response of melanoma cells to bortezomib in vivo.

Regulation of NOXA induction in melanoma cells by proteasome inhibitors included an assessment of the potential roles for new transcription and translation. Using quantitative, real-time PCR analysis, exposure of RJ002L and C8161 melanoma cells to bortezomib (1 μmol/L) triggered a 6- to 7-fold increase in NOXA mRNA levels 6 hours after treatment (data not shown). To confirm and extend these findings, Northern blot analysis was initially done using RNA extracted from C8161 melanoma cells before and after (2, 4, 6, and 8 hours) bortezomib (1 μmol/L) exposure (Fig. 3D). Compared with barely detectible constitutive NOXA mRNA levels, a 2- to 3-fold increase in levels of NOXA mRNA was identified at 2, 4, 6, and 8 hours. In the next set of experiments, four different melanoma cell lines were examined by Northern blots before and 6 hours after bortezomib (1 μmol/L) exposure (Fig. 3E). Once again, low constitutive mRNA levels of NOXA were observed, but addition of bortezomib triggered a severalfold induction of NOXA mRNA levels in all of these melanoma cell lines (Fig. 3E).

Both the delayed apoptotic response derived from the kinetic studies (Fig. 1A) and the Northern blot analysis (Fig. 3D and E) suggested a requirement for new protein synthesis for NOXA (rather than protein stabilization). Preincubation of melanoma cells with cycloheximide (1 μg/mL, 1 hour) reduced the subsequent apoptotic response, and the induction of NOXA protein, 24 hours after addition of bortezomib, by 80% (data not shown). Thus, proteasome inhibitors induce NOXA at the mRNA and protein level.

To further explore a role for NOXA in the apoptotic response, relative levels of NOXA were examined in three different melanocyte cultures (MC005, MC006, and MC008) before and 18 hours after exposure to either MG-132 or lactacystin (Fig. 4A). Also, because previous reports indicated NOXA induction was p53 dependent (48–50), relative levels of p53 were also examined. There was no induction of NOXA in any of the normal melanocytes, despite accumulation of ubiquitinated p53 consistent with inhibition of the proteasome activity. By contrast, all three melanoma cell lines (RJ002L, MUM2B, and C8161) treated with either MG-132 or lactacystin (18 hours) induced high NOXA levels (Fig. 4B). Interestingly, whereas two cell lines expressed p53 (RJ002L and C8161), including ubiquitinated forms, after exposure to the proteasome inhibitor, one melanoma cell line (MUM2B) failed to accumulate detectable p53 consistent with a homozygous inactivating mutation (R196 stop) as previously described (26). To determine if NOXA induction by bortezomib was dependent on p53 levels, RJ002L cells were pretreated with either scrambled siRNA or p53 siRNA. Figure 4C reveals the p53 siRNA reduced p53 levels, as well as bortezomib-induced MDM2 and GADD45 (two genes known to be regulated by p53), with only minimal reduction in NOXA levels. These results indicate that NOXA induction by bortezomib is relatively insensitive to decreases in p53 levels in RJ002L melanoma cells.

To more fully exclude a role for p53 in the induction of NOXA, a time-course experiment using SAOS2 cells, an osteogenic sarcoma cell line known to be p53-null, was also exposed to bortezomib (1 μmol/L). Induction of NOXA occurred beginning at 3 to 6 hours with more prominent levels detected at 18 to 24 hours (Fig. 4D). These data indicate proteasome inhibitors are unable to trigger NOXA induction in normal melanocytes, and that p53 is not absolutely required for NOXA induction.

Figure 2. Interaction of proteasome inhibitors with death receptor and mitochondrial-based apoptotic pathways in melanoma cells. A, proliferating RJ002L melanoma cells were infected with either empty retrovirus (i.e., linker) or with a FADD DN–containing retrovirus or a Bcl-xL–containing retrovirus. After confirming overexpression by Western blot analysis (insets), the apoptotic susceptibility to either bortezomib (1 μmol/L) or LZ-TRAIL (100 ng/mL) was determined. Whereas FADD DN overexpression reduced the LZ-TRAIL–mediated apoptotic response (*, P < 0.05) but not the bortezomib-induced apoptosis, the Bcl-xL overexpression reduced both bortezomib– as well as LZ-TRAIL–induced apoptosis. These results indicate an important role for mitochondrial-based (intrinsic) rather than death receptor (extrinsic)–mediated pathway for bortezomib in melanoma cell lines. B and C, proliferating RJ002L (B) and C8161 (C) melanoma cells were subjected to a mitochondrial isolation procedure before and after various time intervals following exposure to bortezomib (1 μmol/L). Protein extracts were prepared from the mitochondrial-free cytosolic fractions and relative levels of either cytochrome c, apoptosis-inducing factor (AIF), SMAC, or endonuclease G (endo G) were determined by Western blot analysis. The results indicate that bortezomib triggered release of several proapoptotic proteins that contribute to both caspase-dependent and caspase-independent cell death pathways.

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Proliferation assay revealed minimal increase in cell number for C8161 melanoma cells after 2 and 3 days in serum-free medium, compared with significantly increased cell number (P < 0.05) in the presence of 10% FCS (Supplementary Fig. 3A). Phase-contrast microscopy showed that the withdrawal of FCS arrested cell growth (data not shown), which was confirmed by induction of the cyclin-dependent kinase inhibitor, p21 (Supplementary Fig. 3B).

The growth-arrested cells appeared viable which was confirmed by apoptosis assays revealing less than 10% dead cells at 1 or 2 days in serum-free medium (Supplementary Fig. 3B). However, addition of either MG-132 or bortezomib still triggered prominent NOXA induction after serum withdrawal (Supplementary Fig. 3B), accompanied by markedly enhanced apoptosis to levels comparable with the presence of serum (Supplementary Fig. 3C). Thus, not

Figure 3. Proteasome inhibitors differentially induce BH3-only and multiple-BH related family members in melanoma cell lines with prominent induction of NOXA compared with normal melanocytes. A, proliferating RJ002L, C8161, MUM2B, and SK-Mel-28 melanoma cells and melanocytic cultures MC010 and MC012 were examined either before (−) or after (+) 18 hours of exposure to bortezomib (1 μmol/L) and a series of Western blots done to detect relative levels of the induced BH3-only proteins. Only NOXA was consistently and strongly induced in all four melanoma cell lines compared with the absence of NOXA in normal melanocytes (actin serving as loading control). B, using the same protocol as in (A), protein extracts were also analyzed for multiple-BH related proteins before and after bortezomib exposure. Note the lack of consistent induction of Bcl-2 or Bcl-xL compared with the induction of Mcl-1S and Bak after bortezomib exposure. Both Bax and Bak were constitutively expressed in all melanoma cell lines, with similar constitutive levels for Bax and lower levels for Bak in normal melanocytes. Mcl-1S was increased in bortezomib-treated melanocytes (actin serving as loading control). C, immunoblot of protein extracted from PBS-injected versus bortezomib (2.5 mg/kg)–injected tumors reveals NOXA induction following bortezomib injection (actin serving as loading control). D, Northern blot analysis of NOXA mRNA using RNA extracted from C8161 melanoma cells before and at 2, 4, 6, and 8 hours after treatment with bortezomib (1 μmol/L). Induction of NOXA mRNA was quantified using laser-scanning densitometry. Loading control included detection of the housekeeping gene GAPDH. E, Northern blot analysis of NOXA mRNA using RNA extracted from four different melanoma cell lines before and 6 hours after treatment with bortezomib (1 μmol/L). Induction of NOXA mRNA was quantified using laser-scanning densitometry. Loading control included detection of the housekeeping gene GAPDH.
only can proteasome inhibitors selectively induce NOXA and kill melanoma cells and not kill melanocytes independent of p53 but also melanoma cells are susceptible to killing even when maintained in a relatively quiescent state. The withdrawal of growth factors in the melanoma cells maintained in a serum-free environment indicates that proteasome inhibitors can induce NOXA and apoptosis in nonproliferating cells in an equivalent fashion as rapidly proliferating melanoma cells (Supplementary Fig. 3B). It is not possible to directly equate lack of growth in serum-free media to tumor dormancy as other microenvironmental factors may also contribute to tumor dormancy beyond growth factors, and only a clinical trial or additional animal model studies can determine if proteasome inhibitors could affect dormant tumor cells in vivo.

Proteasome inhibitor–mediated killing of melanoma cells is partially caspase dependent, and kinetics of NOXA induction correlates with activation of apoptotic machinery. To confirm a role for caspases in the apoptotic response triggered by proteasome inhibitors, three different melanoma cells (RJ002L, C816L, and MUM2B) were preincubated (2 hours) with a pan-caspase inhibitor (z-VAD-fmk) and then exposed to bortezomib (Fig. 5A–C). Bortezomib triggered significant apoptosis in all three melanoma cells ($P < 0.05$). The pan-caspase inhibitor significantly reduced the apoptotic response to bortezomib in all three cell lines ($P < 0.05$) although ~15% to 20% of the melanoma cells remained resistant to bortezomib-induced killing. These results indicate an important role for caspase activation in the apoptotic response of melanoma cells to proteasome inhibitors. However, the incomplete protection by the pan-caspase inhibitor z-VAD-fmk indicates that there are other non-caspase-dependent apoptotic pathways involved in the proteasome inhibitor–mediated apoptotic response in human melanoma cells. One such non-caspase-dependent pathway involves apoptosis-inducing factor release from mitochondria as depicted by the cytoplasmic accumulation of apoptosis-inducing factor following bortezomib treatment in RJ002L and C816L melanoma cells (Fig. 2).

To characterize the apoptotic machinery mediating proteasome inhibitor killing of melanoma cells, Western blot analysis was done...
on proliferating cells before and after exposure to MG-132, lactacystin, and bortezomib. A representative set of results using RJ002L melanoma cells exposed to either bortezomib, lactacystin, or MG-132 (10 μmol/L) for various time intervals is portrayed in Fig. 6A. Kinetic analysis revealed the induction of NOXA became detectible 6 hours following exposure to these proteasome inhibitors. Note the near simultaneous appearance of activation (cleavage) for caspases 9, 3, and 8 as well as poly(ADP)-ribose polymerase beginning to appear between 3 and 6 hours. When the C8161 melanoma cells, which display a lower apoptotic response, but did not reduce the apoptosis to baseline levels, the ability of the NOXA antisense oligonucleotide to block NOXA induction was accompanied by significant reduction in the apoptotic response for all three proteasome inhibitors in all three melanoma cell lines (Fig. 7A-C). Because the inhibition of apoptosis using the NOXA antisense oligonucleotide was incomplete, other components of the apoptotic machinery are likely to be involved. Nonetheless, these data indicate an important role for NOXA induction in mediating the killing of melanoma cells achieved by the use of proteasome inhibitors.

To show a link between reduction in NOXA levels and the apoptotic response involving caspase activation, several additional experiments were done as illustrated in Fig. 7D. Using RJ002L melanoma cells (left), a kinetic analysis was done in which either control antisense oligonucleotide or NOXA antisense oligonucleotide–pretreated cells were exposed to bortezomib (1 μmol/L; 3, 6, 18, and 24 hours). Not only were NOXA levels reduced in NOXA antisense oligonucleotide–pretreated cells but also activated (e.g., cleaved) caspases 9 and 3 levels were delayed and reduced. Using the same batch of protein lysates as shown in Fig. 7B in which MUM2B cells were pretreated with either control antisense oligonucleotide or NOXA antisense oligonucleotide, immunoblotting to detect activated (cleaved) caspas 9 and 3 was done. As can be seen in Fig. 7D (middle), the relatively lower apoptotic response to lactacystin was accompanied by lower levels of activated caspases 9 and 3 (compared with MG-132 and bortezomib); and the levels of activated caspases 9 and 3 were also lower for all three proteasome inhibitors when the NOXA antisense oligonucleotide–treated melanoma cells were compared with control antisense oligonucleotide–treated melanoma cells. Using protein lysates derived from C8161 cells before and after bortezomib exposure in Fig. 7C, immunoblot results reveal NOXA antisense oligonucleotide–pretreated cells contained reduced activated (cleaved) caspas 9 and 3. In all experiments, actin levels indicate equivalent protein loading. Taken together, these results lend further support to the role for NOXA in regulating the levels of activated caspases 9 and 3 in several melanoma cells exposed to proteasome inhibitors.

Induction of NOXA by proteasome inhibitors in a myeloma cell line and downstream effector caspase cascade. To compare and contrast the response of melanoma cell lines to a myeloma cell line, RPMI8226 myeloma cells were examined before and after exposure to proteasome inhibitors. All three proteasome inhibitors

**Figure 5.** Relative functional role for caspase activation in mediating apoptotic response induced by proteasome inhibitors. A. RJ002L, C8161, and MUM2B were pretreated with either PBS or the pan-caspase inhibitor Z-VAD-fmk, and then exposed to bortezomib (1 μmol/L). Bortezomib exposure significantly induced apoptosis in all three melanoma cell lines (*, P < 0.05). The pan-caspase inhibitor led to a significant reduction compared with absence of inhibitor (*, P < 0.05) in the bortezomib-induced apoptotic response, indicating a non-caspase-dependent pathway.
also triggered significant apoptotic response after 18 hours in the myeloma cell line, and a concentration-dependent representative result using bortezomib is displayed in Fig. 8A. The time course for induction of NOXA in the myeloma cell line following treatment with bortezomib (1 μmol/L) revealed induction of NOXA at 6 hours with decreases in Bim levels, accompanied by cleavage (activation) of caspases 9, 3, and 8 as well as poly(ADP)-ribose polymerase first becoming apparent at 6 hours of treatment (Fig. 8B). The 18-hour response of a myeloma cell line to proteasome inhibitors revealed a concentration-dependent induction of NOXA by bortezomib (0.01-10 μmol/L) and by MG-132 (10 μmol/L), accompanied by activation of caspases 9, 3, and 8 and poly(ADP)-ribose polymerase cleavage (Fig. 8C). The results indicate that proteasome inhibitors not only induce NOXA in malignant melanoma cell lines but also trigger NOXA induction in a multiple myeloma cell line, indicating that NOXA induction is not limited to melanoma cells but also occurs in cells from the disease for which bortezomib was originally approved by the FDA. Thus, these results suggest that this mechanism of apoptosis may be broadly applicable in multiple malignant cell types.

**Discussion**

Despite the growing interest and successful clinical use of targeted therapies for various malignancies, the precise mechanistic pathway responsible for triggering apoptosis in human tumor cells has lagged behind drug development (51, 52). In this report we show that several structurally distinct proteasome inhibitors trigger significant apoptosis in a panel of malignant melanoma cell lines and a myeloma cell line. This apoptotic response did not involve the death receptor (extrinsic) pathway, but did involve the intrinsic or mitochondrial-based pathway. Moreover, the apoptosis in all of the melanoma cell lines tested was consistently accompanied by induction of the proapoptotic BH3-only family member NOXA, and we have shown that this NOXA induction did not require wild-type p53. Induction of NOXA in melanoma cells occurred at both the mRNA and protein level. Importantly, we showed that not only were proliferating melanocytes much less sensitive to proteasome inhibitor–induced apoptosis but also that these inhibitors did not trigger NOXA induction in these untransformed primary cells. Antisense oligonucleotide–based experiments indicate that NOXA is required for apoptosis of melanoma cells treated with proteasome inhibitors. It should be noted that additional apoptotic pathways are likely to be involved beyond NOXA as only partial protection was provided using the NOXA antisense oligonucleotide. Further studies using a broad caspase inhibitor (z-VAD-fmk) revealed both a caspase-dependent as well as a caspase-independent apoptotic pathway in the melanoma cells.

Defining the mechanism of apoptotic action for proteasome inhibitors has been previously elusive, and the current results clearly highlight a key role for NOXA in triggering apoptosis in melanoma and myeloma cells. The data nicely complement earlier studies indicating that the breakdown of apoptosis resistance in melanoma and myeloma cells can be accomplished using proteasome inhibitors irrespective of the p53 status of the tumor cells (7, 9). Such a p53-independent mechanism for NOXA induction confirms and extends our earlier study whereby a tripeptide aldehyde compound with γ secretase inhibitory activity was shown to induce apoptosis through a NOXA-dependent, but p53-independent, mechanism (26). Indeed, most recent evidence revealed that this γ secretase inhibitor not only could interfere with Notch signaling pathways but also possessed proteasome inhibitory activity as well.\(^5\)

We postulate that proteasome inhibitors induce apoptosis through the rapid and prominent accumulation of NOXA, which can then negate the multiple Bcl-2 pro survival family members.

\(^5\) J-Z. Qin, B. Nickoloff, and L. Miele, unpublished observations.
and facilitate mitochondrial cytochrome c, SMAC/DIABLO, and apoptosis-inducing factor release with subsequent apoptosome activation with ultimate DNA degradation and apoptosis. As Bcl-2 family members can significantly control mitochondrial integrity, thereby contributing to the apoptosis resistance to conventional chemotherapeutic agents, it is remarkable that proteasome inhibitors can overcome the relatively high levels of survival factors present within melanoma cells (53). Our data suggest that even extremely low levels of apoptotic protease activating factor-1 are sufficient for apoptosis induced by proteasome inhibitors, as some of the melanoma cell lines we tested have barely detectable apoptotic protease activating factor-1 expression (23) as previously

![Figure 7](https://www.aacrjournals.org/doi/figure/10.1158/0008-5472.CAN-04-2666-19)

Figure 7. Knockdown of NOXA with antisense oligonucleotide reduced induction of apoptosis and caspase processing in melanoma cell lines exposed to proteasome inhibitors. A to C, proliferating melanoma cells lines RJ002L, MUM2B, and C8161 were exposed to either a control antisense oligonucleotide (antisense oligonucleotide) or to a NOXA-targeted antisense oligonucleotide, and then exposed to either MG-132, lactacystin, or bortezomib (1 μmol/L, 24 hours). After confirming reduction in the proteasome inhibitor-mediated induction of NOXA by the NOXA antisense oligonucleotide (inset), the apoptotic response was determined. For each melanoma cell line, the NOXA antisense oligonucleotide reduced the apoptotic response by ~30% to 50% depending on the proteasome used and the type of melanoma cell line, and all of these reductions were significant (*, P < 0.05). D, to explore links between NOXA induction and caspase processing, whole cell extracts from control antisense oligonucleotide and NOXA-targeted antisense oligonucleotide–pretreated cells were examined before and after proteasome inhibitor exposure. RJ002L melanoma cells were studied in a kinetic analysis (3, 6, 18, and 24 hours) in which reduction in NOXA by use of NOXA-targeted antisense oligonucleotide was accompanied by delay in activated caspase 9 and reduction in activated caspase 3 following bortezomib (1 μmol/L) treatment (left). For MUM2B cells, the same extracts derived from (B) were analyzed for activated (cleaved) caspases 9 and 3 using different proteasome inhibitors (MG-132, lactacystin, and bortezomib), and the relative levels of activated caspases correlated to the extent of apoptosis (middle). Also, the NOXA-directed antisense oligonucleotide reduced the levels of activated caspases 9 and 3 in MUM2B cells (middle). For C8161 cells, extracts derived from bortezomib-treated cultures as shown in (C) were analyzed and reduction in NOXA levels was associated with reduced activated caspase 9 and 3 levels compared with control antisense oligonucleotide–treated cells (right). Actin levels confirmed equal loading.
described for C8161 cells (26). In addition, a non-caspase-dependent pathway can be inferred to be engaged given the incomplete protection afforded by the pan-caspase inhibitor results. Likely participants in this non-caspase-dependent mechanism include apoptosis-inducing factor and endonuclease G, which can directly translocate to the nucleus to induce chromatin condensation and/or DNA fragmentation as previously reported for staurosporine-induced apoptosis of melanoma cell lines (54).

Although our current results are in agreement with an earlier report focused on myeloma cells and bortezomib indicating a role for caspase 3 (8), we disagree with their conclusion that caspase 9 is not activated in myeloma cells following bortezomib exposure. Not only do we consistently detect activated caspase 9 in both melanoma cells (Fig. 6A) and myeloma cells (Fig. 8) exposed to proteasome inhibitors, but the NOXA antisense oligonucleotide results also point to a key role for a mitochondrial-based apoptotic machinery involved in the demise of these malignant cells. Thus, the death pathway includes release of cytochrome c and activation of caspase 9, which can function in a proximal fashion to subsequent activation of caspase 3, as well as caspase 8 and Bid.

Although metastatic melanoma cells are notoriously difficult to kill using conventional chemotherapeutic agents, exposure of relatively early passage melanoma cell lines to proteasome inhibitors triggered rapid and substantial apoptosis in vitro and in vivo. The induction of cell death was at least partially specific to transformed cells because these proteasome inhibitors showed minimal cytotoxicity in normal proliferating melanocytes. There is a growing interest in the role of BH3-only proteins such as NOXA from both a basic biological perspective (55) as well as a therapeutic perspective (56). The ability to induce NOXA in a p53-independent fashion will greatly expand the potential of therapeutic applications to include tumor cells that harbor p53 mutations (26, 57).

In contrast to previous investigators who identified an Achilles’ heel of some types of cancer cells by antagonizing inhibitors of caspases such as X-linked inhibitor of apoptosis protein (58, 59), we have identified a different pathway in which a potent proapoptotic protein (e.g., NOXA) could be selectively induced in tumor cells using proteasome inhibitors. Because melanoma and myeloma cells do not seem to maintain constitutive levels of a proteolytically processed profile of caspases, the current therapeutic strategy can be applied in many clinical settings. In other words, rather than reducing essential survival factors, we have targeted induction of proapoptotic molecules that can overcome several survival factors such as Bcl 2, Mcl 1, and survivin.

The rapidly rising incidence of melanoma, coupled with the resistance of metastatic lesions to conventional chemotherapy, makes this deadly form of skin cancer a large public health problem (11, 12). Using relatively early as well as late-passage cell lines derived from metastatic melanomas, we describe the rapid and efficient killing by proteasome inhibitors. These data suggest the breaking of apoptosis resistance in metastatic melanoma cells and myeloma cells can be achieved by the use of proteasome inhibitors, and for the first time a precise mechanistic link to a distinct BH3-only family member (i.e., NOXA) has been established. The discovery of this novel mechanistic pathway should pave the way for future clinical trials using proteasome inhibitors, either singly

Figure 8. Proteasome inhibitors trigger apoptosis and NOXA induction in RPMI8226 myeloma cells accompanied by activation of caspases 9, 3, and 8 and poly(ADP)-ribose polymerase. A, proliferating multiple myeloma cells (RPMI8226) were exposed to bortezomib (24 hours) at the indicated concentration and the apoptotic response determined, with doses as low as 0.01 μmol/L triggering significant apoptosis (*, P < 0.05). B, kinetic analysis using Western blots from myeloma cells reveals that bortezomib (1 μmol/L) triggered rapid induction of NOXA (first detectable at 6 hours) accompanied by diminished Bim levels with activation of caspases 9, 3, and 8 and poly(ADP)-ribose polymerase. C, myeloma cells were exposed to increasing concentrations of bortezomib (0.01-10 μmol/L) or MG-132 (10 μmol/L) for 24 hours and then whole cell extracts prepared for Western blot analysis. Note that the induction of NOXA was detected at the lowest bortezomib concentration (0.01 μmol/L), accompanied by cleavage (activation) of caspases 9, 3, and 8 and poly(ADP)-ribose polymerase.
or in combination with other agents, to produce synergistic effects in notoriously difficult and clinically aggressive malignancies such as malignant melanoma and multiple myeloma. Finally, our ability to detect NOXA in vivo using bortezomib-treated melanoma cells undergoing apoptosis suggests that NOXA may also be used as a biomarker for responsiveness in clinical trials in which in vivo inhibitors are used alone or in combination with other agents.

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