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Signaling mechanisms of bortezomib in TRAF3-deficient mouse B lymphoma and human multiple myeloma cells

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Abstract

Bortezomib, a clinical drug for multiple myeloma (MM) and mantle cell lymphoma, exhibits complex mechanisms of action, which vary depending on the cancer type and the critical genetic alterations of each cancer. Here we investigated the signaling mechanisms of bortezomib in mouse B lymphoma and human MM cells deficient in a new tumor suppressor gene, TRAF3. We found that bortezomib consistently induced up-regulation of the cell cycle inhibitor p21(WAF1) and the pro-apoptotic protein Noxa as well as cleavage of the anti-apoptotic protein Mcl-1. Interestingly, bortezomib induced the activation of NF- κ B1 and the accumulation of the oncoprotein c-Myc, but inhibited the activation of NF- κ B2. Furthermore, we demonstrated that oridonin (an inhibitor of NF- κ B1 and NF- κ B2) or AD 198 (a drug targeting c-Myc) drastically potentiated the anti-cancer effects of bortezomib in TRAF3-deficient malignant B cells. Taken together, our findings increase the understanding of the mechanisms of action of bortezomib, which would aid the design of novel bortezomib-based combination therapies. Our results also provide a rationale for clinical evaluation of the combinations of bortezomib and oridonin (or other inhibitors of NF- κ B1/2) or AD 198 (or other drugs targeting c-Myc) in the treatment of lymphoma and MM, especially in patients containing TRAF3 deletions or relevant mutations.

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Conflict of interest

The authors declare that they have no potential conflicts of interest.

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Keywords

Bortezomib; TRAF3; B lymphoma; multiple myeloma; Noxa; oridonin; AD 198

1. Introduction

Bortezomib (VELCADE, also called PS-341), the first proteasome inhibitor to enter the clinic, is an U.S. Food and Drug Administration (FDA) approved drug for the treatment of newly diagnosed multiple myeloma (MM), relapsed or refractory MM, and mantle cell lymphoma (MCL) [1–7]. Its specific 26S proteasome inhibitory function has been attributed to the reversible but strong covalent bond formed between its dipeptidyl boronic acid moiety and the threonine proteases of the 20S subunit [1–7]. Bortezomib thus effectively inhibits the ubiquitin-proteasome pathway (UPP), which is essential in regulating the homeostasis of proteins controlling cell cycle progression, survival and apoptosis [1–6]. Evidence accumulated in the literature reveals that bortezomib exhibits complex mechanisms of action in cancer cells. These include inhibition of canonical NF- κ B activation, suppression of cyclins and c-Myc, up-regulation of inhibitors of cell cycle progression (such as p21, p27, and p53), down-regulation or cleavage of anti-apoptotic proteins of the Bcl-2 family (such as Bcl-2, Bcl-xL, Mcl-1, and A1/bfl), and induction of pro-apoptotic proteins of the Bcl-2 family (such as Bim, Bid, Noxa, and Puma) [1–6]. Notably, bortezomib may target different oncogenic pathways in different cellular contexts, depending on the cancer cell type and the critical genetic alterations of each specific cancer [1–6].

Biallelic deletions or inactivating mutations of TRAF3, a novel tumor suppressor gene of B lymphocytes, were recently identified as a critical genetic alteration in human non-Hodgkin lymphoma (NHL), including splenic marginal zone lymphoma (MZL), B cell chronic lymphocytic leukemia (B-CLL), MCL, as well as MM and Waldenström's macroglobulinemia [8–12]. Indeed, specific deletion of TRAF3 from B lymphocytes causes prolonged survival of mature B cells, which eventually leads to B lymphoma development in mice [13,14]. Detailed mechanistic investigation elucidated that TRAF3 inactivation results in constitutive activation of the non-canonical NF- κ B (NF- κ B2) pathway, which is pivotal for the survival of B cells [10,13,15–17]. Interestingly, human MM patients with TRAF3 deletions or mutations are resistant to the chemotherapy agent dexamethasone but are sensitive to bortezomib [10]. However, the mechanisms of action of bortezomib in TRAF3-deficient NHL or MM remain unclear.

In the present study, we aimed to investigate the signaling mechanisms of bortezomib using TRAF3^{-/-} mouse B lymphoma cell lines and human MM cell lines with TRAF3 deletions or mutations as model systems. Specifically, the objectives of this study are: (1) to determine the effects of bortezomib on canonical and non-canonical NF- κ B pathways in TRAF3-deficient mouse B lymphoma and human MM cells; (2) to identify which cell cycle regulators are targeted by bortezomib in TRAF3-deficient malignant B cells; (3) to delineate pro- or anti- apoptotic proteins of the Bcl-2 family that are specifically changed by bortezomib in the context of TRAF3 inactivation. Information gained from these experiments will be helpful in rational design of combination therapy involving bortezomib

and other drugs with distinct mechanisms of action for the treatment of NHL and MM patients containing TRAF3 deletions or mutations.

2. Materials and Methods

2.1. Cell lines, antibodies, and reagents

Human MM cell lines 8226 (contains bi-allelic TRAF3 deletions), KMS11 (contains bi-allelic TRAF3 deletions), and LP1 (contains inactivating TRAF3 frameshift mutations) were kindly provided by Dr. Leif Bergsagel (Mayo Clinic, Scottsdale, AZ), and were cultured as previously described [18]. TRAF3^{-/-} mouse B lymphoma cell lines 27-9.5.3, 105-8.1B6, and 115-6.1.2 were generated and cultured as described previously [14,18]. Bortezomib was generously supplied by Millennium Pharmaceuticals (Cambridge, MA). Rabbit polyclonal antibody against Noxa was purchased from AXXORA (Farmingdale, NY). Other antibodies and reagents used in this study were described previously [14,18–20].

2.2. MTT assay, annexin V staining, cell cycle analyses, and caspase cleavage assays

MTT assay, annexin V staining and cell cycle analyses were performed as previously described [14,18–20]. For caspase cleavage assays, total protein lysates were prepared from cells at different time points after treatment with bortezomib, and cleavage of caspases 3, 8, and 9 was subsequently examined by immunoblot analysis.

2.3. Protein extraction and immunoblot analysis

Total protein lysates, cytosolic and nuclear extracts were prepared as previously described [13,14]. Immunoblot analysis was performed using various antibodies as described [14,18–20]. Images of immunoblots were acquired using a low-light imaging system (LAS-4000 mini, FUJIFILM Medical Systems USA, Inc., Stamford, CT). Densitometric analyses of bands on immunoblots were performed using the Multi Gauge software (Science Lab, FUJIFILM Medical Systems USA, Inc.).

2.4. Generation of the lentiviral SBP-Noxa expression vector

Total cellular RNA was prepared from the human MM cell line 8226 cells, and the corresponding cDNA was used as the template to clone the Noxa coding sequence using the primers BamHI-hNoxa-F (5'- CGG GAT CCT ATG CCT GGG AAG AAG GCG CG -3') and XbaI-hNoxa-R (5'- GCT CTA GAT CAG GTT CCT GAG CAG AAG A -3'). To facilitate immunoprecipitation studies, we engineered an N-terminal streptavidin-binding peptide (SBP) tag [21] in frame with the Noxa coding sequence. The coding sequence of SBP-Noxa was subsequently cloned into the lentiviral expression vector pUB-eGFP-Thy1.1 [22] (generously provided by Dr. Zhibin Chen, the University of Miami, Miami, FL) by replacing eGFP with SBP-Noxa. The generated pUB-SBP-Noxa lentiviral vector was verified by DNA sequencing.

2.5. Lentiviral packaging and transduction of human MM cells

Lentiviruses of the pUB-SBP-Noxa expression vector and an empty pUB-Thy1.1 vector were packaged, titered, and used to transduce human MM cells as previously described [18–

20]. At 24 hours post transduction, the transduction efficiency of cells was analyzed by Thy1.1 immunofluorescence staining and flow cytometry. Transduced cells were subsequently analyzed for apoptosis and cell cycle distribution using annexin V staining and propidium iodide (PI) staining as previously described [14,18–20].

2.6. Co-immunoprecipitation assays

Human MM cell line 8226 cells (2×10^7 cells/condition) transduced with pUB-SBP-Noxa or an empty vector pUB-Thy1.1 were lysed and sonicated in the CHAPS lysis buffer [20] (1% CHAPS, 20 mM Tris, pH 7.4, 150 mM NaCl, 50 mM β -glycerophosphate, and 5% glycerol with freshly added 1 mM DTT and EDTA-free Mini-complete protease inhibitor cocktail). The insoluble pellets were removed by centrifugation at 10,000g for 20 minutes at 4°C. The CHAPS lysates were subsequently immunoprecipitated with streptavidin-sepharose beads (Pierce, Rockford, IL). Immunoprecipitates were washed 5 times with the Wash Buffer (0.5% CHAPS, 20 mM Tris, pH 7.4, 150 mM NaCl, 50 mM β -glycerophosphate, and 2.5% glycerol with freshly added 1 mM DTT and EDTA-free Mini-complete protease inhibitor cocktail). Immunoprecipitated proteins were resuspended in 2× SDS sample buffer, boiled for 10 minutes, and then separated on SDS-PAGE for immunoblot analyses.

2.7. Statistics

Statistical significance was assessed by Student *t* test. *P* values less than 0.05 are considered significant.

3. Results

3.1. Potent tumoricidal activity of bortezomib on TRAF3^{-/-} mouse B lymphoma and human MM cell lines

It has been previously shown that human MM patients with TRAF3 deletions or mutations are sensitive to bortezomib [10]. This prompted us to test the tumoricidal activity of bortezomib on TRAF3^{-/-} mouse B lymphoma cell lines and human MM cell lines with TRAF3 deletions or mutations. The results of our MTT assays demonstrated that bortezomib exhibited potent anti-proliferative/survival-inhibitory effects on all the examined TRAF3^{-/-} mouse B lymphoma and human MM cell lines in a dose-dependent manner (Fig. 1A). To understand the mechanism of bortezomib, we first performed cell cycle analysis using propidium iodide (PI) staining followed by flow cytometry. We found that bortezomib induced TRAF3^{-/-} mouse B lymphoma and human MM cells to undergo apoptosis, as demonstrated by the drastic increase of the sub-G1 population with DNA content < 2n (Fig. 1B). Bortezomib also inhibited the proliferation of TRAF3^{-/-} tumor B cells, as shown by the marked decrease of the population at the S/G2/M phase (2n < DNA content < 4n) (Fig. 1B). We verified bortezomib-induced apoptosis using annexin V staining, which showed phosphatidylserine exposure (Supplementary Fig. 1A). We further demonstrated that bortezomib triggered mitochondrial membrane permeabilization as analyzed by MitoProbe JC-1 staining (Supplementary Fig. 1B). We next determined whether bortezomib induced the activation of key caspases involved in apoptosis. We found that bortezomib induced rapid activation of caspases 9, 8, and 3, as evidenced by the cleavage of these caspases after

treatment with bortezomib in TRAF3^{-/-} mouse B lymphoma and human MM cells (Fig. 2A). Collectively, our data demonstrate that bortezomib induces caspase-mediated apoptosis via the mitochondrial apoptotic pathway in TRAF3^{-/-} malignant B cells.

3.2. Contrasting roles of bortezomib on canonical versus non-canonical NF-κB pathways

From the beginning of its clinical use, the principal mechanism of bortezomib was believed to be the inhibition of the NF-κB pathway, which promotes cell survival by activating anti-apoptotic genes [2–5]. However, contradictory effects of bortezomib on the canonical NF-κB pathway have been reported in NHL and MM [4,23,24]. Ma *et al.* showed that bortezomib substantially inhibits the canonical NF-κB pathway in chemosensitive and chemoresistant MM cell lines [23]. Inhibition of the canonical NF-κB pathway has also been demonstrated in MCL and primary effusion lymphomas (PEL) [3]. In contrast, Hideshima *et al.* reported that bortezomib induces canonical NF-κB activation in MM cell lines and primary tumor from MM patients [24]. In addition, Markovina *et al.* found that constitutive NF-κB activity in primary tumor cells from MM patients is refractory to inhibition by bortezomib [25]. We recently demonstrated that TRAF3^{-/-} mouse B lymphomas exhibit constitutive activation of both canonical and non-canonical NF-κB pathways [14]. It is known that both NF-κB pathways require proteasome-dependent activity, including IκBα degradation of the canonical pathway and processing of p100 NF-κB2 of the non-canonical pathway [4,10,24,26]. In this context, we examined and compared the cytosolic and nuclear levels of proteins of both canonical and non-canonical NF-κB pathways in TRAF3^{-/-} malignant B cells after treatment with bortezomib. Consistent with the study by Hideshima *et al.* [24], our results demonstrated that bortezomib induced canonical NF-κB activation in TRAF3^{-/-} mouse B lymphoma and human MM cell lines (Fig. 2B, and Supplementary Fig. 2 and 3). This effect of bortezomib is evidenced by induction of the phosphorylation and degradation of the inhibitory protein IκBα, induction of the phosphorylation of RelA, and increased nuclear levels of the processed NF-κB1 (p50), RelA and c-Rel (Fig. 2B, and Supplementary Fig. 2 and 3). In contrast, we found that bortezomib moderately inhibited non-canonical NF-κB activation in most TRAF3^{-/-} cell lines examined in this study (Fig. 2B, and Supplementary Fig. 2 and 3). Thus, the anti-proliferative/survival-inhibitory effects of bortezomib are difficult to be attributed to its contrasting roles on the two NF-κB pathways in TRAF3^{-/-} malignant B cells.

3.3. Bortezomib induced accumulation of p21 and c-Myc

Among the proteins degraded by the ubiquitin-proteasome pathway are cyclins (A, B, D and E), cyclin-dependent kinase inhibitors such as p21 and p27, the tumor suppressor p53 and the oncoprotein c-Myc [2,3,6,27]. Decreased degradation and increased accumulation of these proteins may disrupt cell cycle progression [2,3,6,27]. To understand its mechanisms of action, we investigated the effects of bortezomib on the protein levels of these cell cycle regulators. We found that treatment with bortezomib led to a time-dependent increase in total cellular levels of the cyclin-dependent kinase inhibitor p21(WAF1) in all examined TRAF3^{-/-} mouse B lymphoma and human MM cell lines (Fig. 3A). Interestingly, accumulation of c-Myc was induced by bortezomib in all examined cell lines except 105-8 (Fig. 3A). Although total cellular levels of cyclin D2 were decreased and those of cyclin B1 were increased by bortezomib in all TRAF3^{-/-} mouse B lymphoma cell lines, these

observations were not mirrored in human MM cell lines (Fig. 3A). In contrast, bortezomib did not consistently affect the protein levels of other cell cycle regulators in different cell lines examined in our study, including p27, cyclin D1, cyclin A, and p53 (Fig. 3A). In light of the consistent accumulation of p21 in all examined cell lines, we further determined the subcellular distribution of p21 in TRAF3^{-/-} malignant B cells after treatment with bortezomib. Our results showed that bortezomib increased p21 protein levels in both the cytosol and nucleus in a dose-dependent and time-dependent manner (Fig. 3B, and Supplementary Fig. 4). Our findings thus suggest that bortezomib-induced accumulation of the cell cycle inhibitor p21 is associated with its anti-proliferative effects in TRAF3^{-/-} malignant B cells.

3.4. Bortezomib induced up-regulation of Noxa and cleavage of Mcl-1

Pro- and anti-apoptotic proteins of the Bcl-2 family are also known substrates of the proteasome. Increasing evidence indicates that proteasome inhibition-dependent regulation of the Bcl-2 family is required for the anti-tumor activity of bortezomib [1–4]. In particular, stabilization of BH3-only proteins Bik, Noxa and Bim, appears to be essential for effecting Bax/Bak-dependent apoptosis triggered by bortezomib [1–4]. However, the effects of bortezomib on the Bcl-2 family proteins are variable and appear to be context-dependent [1–4]. We thus examined the regulation of the Bcl-2 family proteins by bortezomib in TRAF3^{-/-} malignant B cells. We found that bortezomib induced striking up-regulation of the proapoptotic protein Noxa in a time-dependent manner in all examined TRAF3^{-/-} mouse B lymphoma and human MM cell lines (Fig. 4A). Interestingly, bortezomib also induced the phosphorylation and cleavage of the anti-apoptotic protein Mcl-1 in a time-dependent manner in all examined cell lines (Fig. 4A and Supplementary Fig. 6A). In contrast, although increases of Bax, Bak and Bim as well as decreases in Bcl-2, Bcl-xL and A1/Bfl have been previously observed in NHL or MM after treatment with bortezomib [1–4], we did not detect any effects of bortezomib on these proteins in all examined TRAF3^{-/-} cell lines (Fig. 4A and Supplementary Fig. 5). Bid cleavage was induced by bortezomib in TRAF3^{-/-} mouse B lymphoma cell lines, but this effect was not recapitulated in human MM cell lines (Supplementary Fig. 5). Similarly, Bik was decreased by bortezomib in TRAF3^{-/-} mouse B lymphoma cell lines, but slightly increased by bortezomib in human MM cell lines (Fig. 4A). Therefore, the most consistent effects of bortezomib on the Bcl-2 family are the induction of Noxa up-regulation and Mcl-1 cleavage in TRAF3^{-/-} malignant B cells. Given the previous evidence that Noxa induction and Mcl-1 cleavage are crucial for the cytotoxic effects of bortezomib on MM [1–4], we further verified that bortezomib induced Noxa up-regulation and Mcl-1 cleavage in a dose-dependent manner in TRAF3^{-/-} malignant B cells (Fig. 4B and Supplementary Fig. 6B). Taken together, our results highlight the importance of Noxa and Mcl-1 in bortezomib-mediated tumoricidal activities in TRAF3^{-/-} malignant B cells.

3.5. Overexpression of Noxa induced apoptosis and cleavage of Mcl-1 in human MM cells

To further investigate the functional consequences of Noxa up-regulation in TRAF3^{-/-} malignant B cells, we employed genetic means to overexpress Noxa in human MM cells by generating a lentiviral expression vector, pUB-SBP-Noxa. Cells transduced with an empty lentiviral expression vector (pUB-Thy1.1) were used as control in these experiments. We

verified that the transduction efficiency of lentiviruses was >90% in human MM 8226 cells (Fig. 5A). We found that overexpression of SBP-Noxa drastically induced apoptosis in human MM cells within 48 hours post transduction, as shown by >50% of annexin V+ cells (Fig. 5B and 5C). Our results of cell cycle analyses also demonstrated that overexpression of SBP-Noxa led to a robust increase in the apoptotic cell population (DNA content < 2n) and a remarkable decrease in the proliferating cell population (S/G2/M phase: 2n < DNA content 4n) of transduced cells (Fig. 5D and 5E). Western blot data revealed that overexpression of SBP-Noxa resulted in the cleavage of caspase 3 and Mcl-1 at 36 hours post transduction (Fig. 5F and 5G). Furthermore, we found that SBP-Noxa co-immunoprecipitated specifically with both full-length and cleaved Mcl-1, but not with other examined proteins of the Bcl2 family, including Bcl-xL, Bcl2, Bax and Bim. Therefore, overexpression of Noxa is sufficient to induce apoptosis as well as cleavage of caspase 3 and Mcl-1 in human MM cells. These results indicate that up-regulation of Noxa contributes to the tumoricidal activities of bortezomib in TRAF3^{-/-} malignant B cells.

3.6. Combined effects of bortezomib and oridonin or AD 198

Detailed elucidation of its mechanisms of action will not only provide better understanding of the molecular determinants of sensitivity of malignant B cells to bortezomib, but also suggest new, rational combination therapies with potential to improve outcome in patients. Our evidence that bortezomib exhibited contrasting roles on canonical and non-canonical NF- κ B pathways prompted us to test the hypothesis that a universal NF- κ B inhibitor may enhance the anti-tumor activity of bortezomib on TRAF3^{-/-} malignant B cells. We recently found that oridonin, an active diterpenoid isolated from a traditional Chinese herbal medicine, potently inhibits both canonical and non-canonical NF- κ B pathways in TRAF3^{-/-} mouse B lymphoma cells [14]. We thus compared the cytotoxic effects of bortezomib and oridonin, alone or in combination, on TRAF3^{-/-} mouse B lymphoma and human MM cell lines. Our results demonstrated that bortezomib and oridonin exhibited additive or synergistic tumoricidal activities on all examined cell lines except 8226 (Fig. 6A). Furthermore, our finding that bortezomib induced the accumulation of c-Myc in most examined cell lines suggests that simultaneous targeting of the proteasome and c-Myc may be more effective in the treatment of TRAF3-deficient B cell neoplasms. We thus examined the effects of bortezomib in combination with AD 198, a new drug that we recently found to specifically target c-Myc in malignant B cells [18]. We found that AD 198 potently enhanced the cytotoxic activities of bortezomib in all examined TRAF3^{-/-} cell lines (Fig. 6B). Taken together, our results provide a rationale for further clinical evaluation of the combinations of bortezomib and oridonin or AD 198 in the treatment of NHL and MM patients containing TRAF3 deletions or relevant mutations.

4. Discussion

During the past decade, the clinical success of the proteasome inhibitor bortezomib as a front-line and second-line regimen has dramatically improved the outcome of patients with MM and MCL [6,7,28]. However, it should be noted that bortezomib also has side-effects, including peripheral neuropathy, diarrhea, vomiting, fatigue, dizziness, anorexia, anemia, and thrombocytopenia [2,3,5,6,27,29]. Moreover, some patients do not benefit from

bortezomib treatment, about 60% of responding patients eventually acquire resistance, and relapse is common in patients that initially responded to bortezomib [5,28,29]. In this context, although bortezomib has demonstrated efficacy as monotherapy, combination therapies are expected to maximize benefit while minimizing toxicity [4,7,28,29]. Understanding of signaling mechanisms of bortezomib provides a basis for rational design of novel combination therapies that target non-overlapping pathways. Given the significant heterogeneity in the outcomes despite similar clinical stage classifications as seen in MM patients, it is now recognized to be crucial to consider genetic risk factors for optimum therapeutic recommendation for individual groups of patients [6]. In the present study, we thus investigated detailed signaling mechanisms of bortezomib in mouse B lymphoma and human MM cells deficient in a new tumor suppressor gene, TRAF3.

We identified the most striking and consistent signaling events of bortezomib in TRAF3-deficient malignant B cells, including induction of the cell cycle inhibitor p21(WAF1) and the pro-apoptotic protein Noxa as well as cleavage of the anti-apoptotic protein Mcl-1. Our findings corroborate several previous studies of p21, Noxa, and Mcl-1 in MM cells [30–32]. Both p21 and Noxa are previously known as p53 target genes. Interestingly however, the 6 cell lines have different functional status of p53. Human MM cell line KMS11 contains wild type p53, 8226 contains a temperature-sensitive p53 mutation, and LP1 has a p53 functional mutation [33–37]. Our sequencing data revealed that mouse B lymphoma cell line 105-8 contains wild type p53, 27-9 contains the p53 point mutation V170M, and 115-6 has one allele of truncated p53 (p53 1-233aa). This suggests that both p53-dependent and p53-independent mechanisms may be involved in the up-regulation of Noxa and p21 induced by bortezomib in TRAF3-deficient malignant B cells. Indeed, multiple p53-independent mechanisms of Noxa and p21 induction have been described in the literature, including those dependent on Myc, RelA, p73, or E2F1 [38–42]. Regardless of the detailed mechanisms, the functional consequences of Noxa and p21 up-regulation are clear. Induction of p21 is known to inhibit proliferation and induce cell cycle arrest predominantly at the G1/S checkpoint [2,3,6,27]. Noxa promotes apoptosis by directly interacting with Mcl-1, thereby releasing the apoptotic protein Bak to trigger mitochondrial depolarization [5,31,38,43]. Furthermore, the cleaved Mcl-1 fragment (28 kDa) has strong pro-apoptotic activity by enhancing Mcl-1 turnover and impairing its ability to interact with BH3-only pro-apoptotic proteins [31,32]. Indeed, silencing of p21 reverses the anti-proliferative effects of bortezomib [44–46], knockdown of Noxa protects against apoptosis induced by bortezomib, and overexpression of cleaved Mcl-1 fragment sensitize MM cells to bortezomib-induced apoptosis [31,38,43]. Here we further demonstrated that overexpression of Noxa is sufficient to induce apoptosis and inhibit proliferation in human MM cells by inducing cleavage of Mcl-1 and caspase 3. Collectively, up-regulation of p21 and Noxa together with cleavage of Mcl-1 appears to be key mediators of the cytotoxic effects of bortezomib in TRAF3^{-/-} malignant B cells. Therefore, to improve efficacy or prevent relapse, we recommend the combination of bortezomib with agents that are independent of p21, Noxa and Mcl-1.

In addition to the consistent effects of bortezomib on p21, Noxa and Mcl-1, we observed differential effects of bortezomib on cyclin D2, cyclin B1, Bid and Bik in mouse B lymphoma versus human MM cell lines. This likely reflects the difference in differentiation

stage between mouse B lymphoma cell lines (derived from peripheral mature B cells) [14] and human MM cell lines (derived from plasma cells) [28]. Hence, down-regulation of cyclin D2 and Bik, cleavage of Bid, and up-regulation of cyclin B1 by bortezomib observed in mouse B lymphoma cell lines may represent distinct regulatory mechanisms of mature B cells that are not present in plasma cells or MM cells. Our observations suggest that future studies are required to examine these regulatory mechanisms in human MCL, which are also derived from mature B cells [2]. Furthermore, we detected reproducible variations in the responses of p27, cyclin D1, cyclin A, and p53 to bortezomib among the 6 TRAF3-deficient cell lines examined in this study. We reason that such variations may be caused by different combinations of genetic mutations in the cell lines, considering that all cancers contain numerous somatic mutations [47,48] and that each cancer cell may have 10^3 – 10^4 mutations [47–50]. In this regard, one example is the different mutational status of the p53 gene in the 6 cell lines of our study as described above. Thus, all the 6 cell lines share the genetic deficiency in TRAF3, but each cell line has a unique combination of genetic mutations, which mimic the case of heterogeneity observed in different human patients. Therefore, the complexity and variations of bortezomib signaling mechanisms observed in different TRAF3-deficient cell lines point to the value and strength of personalized (or precision) medicine, in which therapeutic regimens are tailored and modified according to the genetic mutation profile of each patient [51,52].

Although NF- κ B inhibition was initially believed to be responsible for the anti-cancer activity of bortezomib [3,5,6,27,28], we detected contrasting roles of bortezomib on NF- κ B1 and NF- κ B2 pathways in TRAF3^{-/-} malignant B cells. We demonstrated that bortezomib induced canonical NF- κ B activation while moderately inhibiting non-canonical NF- κ B activation in TRAF3^{-/-} malignant B cells. Our results reinforce recent evidence that argues against a critical role for NF- κ B inhibition in the mechanisms of action of bortezomib in MM [24,25,28]. Notably, NF- κ B1 and NF- κ B2 control the expression of a variety of target genes crucial for cell survival (e.g., Bcl-xL and Mcl-1), proliferation (e.g., cyclin D1 and c-Myc), adhesion (e.g., CD54 and CD106), and cytokine signaling (e.g., IL-6 and BAFF secretion) [3,5,6,27,28]. Frequent genetic abnormalities of the NF- κ B2 pathway in MM further highlight the importance of NF- κ B2 signaling in the pathogenesis of MM [10,11]. We recently demonstrated that knockdown of NF- κ B2 induced apoptosis in TRAF3^{-/-} mouse B lymphoma cells [14]. Based on the above evidence, we tested the combined effects of bortezomib and an inhibitor of both NF- κ B1 and NF- κ B2, oridonin [14]. We found that co-treatment with bortezomib and oridonin remarkably potentiated the anti-cancer effects of bortezomib in all examined TRAF3^{-/-} cell lines except 8226 (which might have acquired additional mutations and NF- κ B-independent oncogenic pathways). Similarly, Fabre *et al.* recently reported that another inhibitor of NF- κ B1 and NF- κ B2, PBS-1086, markedly enhanced the cytotoxicity of bortezomib on MM cells [53]. Therefore, combination of bortezomib and oridonin (or other inhibitors of both NF- κ B1 and NF- κ B2) represent a rational therapeutic regimen for the treatment of NHL and MM, especially in patients that are refractory to bortezomib-mediated inhibition of NF- κ B pathways.

Interestingly, bortezomib induced the accumulation of c-Myc, an oncoprotein of B cells [54], in most examined TRAF3^{-/-} cell lines. We thus also examined the combined effects of

bortezomib and a drug specifically targeting c-Myc, AD 198 [18]. We found that this combination killed TRAF3^{-/-} malignant B cells to a much greater extent than either drug alone. Given the ubiquitously elevated expression of c-Myc in B cell malignancies [55], our findings suggest that combination of bortezomib and AD 198 (or other drugs targeting c-Myc) is another promising therapy for NHL and MM.

In summary, our study provides a better understanding of the mechanistic basis for the anti-cancer effects of bortezomib in NHL and MM, which would aid the design of novel bortezomib-based combination regimens. Additionally, we found that combinations of bortezomib with oridonin or AD 198 allow the use of lower doses of bortezomib to reduce side-effects while enhancing anti-cancer efficacy in TRAF3^{-/-} malignant B cells through complementary mechanisms of action. Our findings thus provide the preclinical framework for clinical evaluation of the combinations of bortezomib and oridonin or AD 198, which would expand the therapeutic armamentarium for NHL and MM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Bortezomib induced Noxa and p21, and caused Mcl1 cleavage in TRAF3^{-/-} tumor B cells.
- Bortezomib exhibited contrasting roles on NF-κB1 and NF-κB2 pathways.
- Oridonin or AD 198 drastically potentiated the anti-cancer effects of bortezomib.

Figure 1. Bortezomib induced apoptosis in TRAF3^{-/-} mouse B lymphoma and human MM cells TRAF3^{-/-} mouse B lymphoma cell lines examined include 27-9.5.3 (27-9), 105-8.1B6 (105-8), and 115-6.1.2 (115-6). Human patient-derived MM cell lines examined include 8226 (with TRAF3 bi-allelic deletions), KMS11 (with TRAF3 bi-allelic deletions), and LP1 (with TRAF3 frameshift mutations). (A) Viable cell curves analyzed by MTT assay. Cells were treated with various concentrations (1:2 serial dilutions) of bortezomib for 24 h. Total viable cell numbers were subsequently determined by MTT assay. The graphs depict the results of three independent experiments with duplicate samples in each experiment (mean $\pm$ SEM). (B) Cell cycle distribution determined by PI staining and flow cytometry. Cells were cultured in the absence or presence of bortezomib for 24 h. Concentrations of bortezomib used: 10 nM for 27-9, 5 nM for 105-8, 10 nM for 115-6, 50 nM for 8226, 20 nM for KMS11, and 50 nM for LP1. Cells were fixed, and then stained with PI. Stained cells were subsequently analyzed by FACS. Representative FACS histograms of PI staining are shown, and percentages of apoptotic cells (DNA content < 2n; sub-G1) and proliferating cells (2n <

DNA content 4n; S/G2/M) are indicated. Results are representative of three independent experiments.

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Figure 2. Bortezomib induced cleavage of caspases and NF-κB1 activation in TRAF3^{-/-} malignant B cells

Cells were cultured in the absence or presence of bortezomib for indicated time periods. Concentrations of bortezomib used: 10 nM for 27-9, 5 nM for 105-8, 10 nM for 115-6, 50 nM for 8226, 20 nM for KMS11, and 50 nM for LP1. **(A)** Cleavage of caspases. Total cellular lysates were prepared, and then immunoblotted for caspase 9, caspase 8, and caspase 3, followed by actin. Immunoblots of actin were used as loading control. **(B)** Cytosolic and nuclear levels of NF-κB subunits. Cytosolic and nuclear proteins were

extracted, and then immunoblotted for NF- κ B1 (p105 and p50), RelA, c-Rel, NF- κ B2 (p100 and p52), and RelB, followed by YY1 and actin. Immunoblots of actin and YY1 were used as loading control for cytosolic and nuclear proteins, respectively. Results (A and B) are representative of three independent experiments.

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Figure 3. Bortezomib increased the accumulation of p21 and c-Myc in TRAF3^{-/-} malignant B cells

(A) Time-dependent effects of bortezomib on the protein levels of cell cycle regulators. Cells were cultured in the absence or presence of bortezomib for indicated time periods. Concentrations of bortezomib used: 10 nM for 27-9, 5 nM for 105-8, 10 nM for 115-6, 50 nM for 8226, 20 nM for KMS11, and 50 nM for LP1. Total cellular lysates were prepared, and then immunoblotted for p21, p27, cyclin D1, cyclin D2, cyclin B1, cyclin A, p53, and c-Myc, followed by actin. Immunoblots of actin were used as loading control. (B) Dose-

dependent effects of bortezomib on p21 protein levels in the cytosol and nucleus. Cells were cultured in the absence or presence of bortezomib of indicated concentrations for 6 h. Cytosolic and nuclear proteins were extracted, and then immunoblotted for p21, followed by YY1 and actin. Immunoblots of actin and YY1 were used as loading control for cytosolic and nuclear proteins, respectively. Similar results were also obtained with other cell lines examined. Results (A and B) are representative of three independent experiments.

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Figure 4. Bortezomib induced up-regulation of Noxa and cleavage of Mcl-1 in TRAF3^{-/-} malignant B cells

(A) Time-dependent effects of bortezomib on the protein levels of cell survival regulators of the Bcl-2 family. Cells were cultured in the absence or presence of bortezomib for indicated time periods. Concentrations of bortezomib used: 10 nM for 27-9, 5 nM for 105-8, 10 nM for 115-6, 50 nM for 8226, 20 nM for KMS11, and 50 nM for LP1. Total cellular lysates were prepared, and then immunoblotted for Noxa, phosphorylated Mcl-1, Mcl-1, Bcl-xL, Bcl2, Bik, Bax, and Bak, followed by actin. Immunoblots of actin were used as loading

control. **(B)** Dose-dependent effects of bortezomib on the protein levels of Noxa and Mcl-1. Cells were cultured in the absence or presence of bortezomib of indicated concentrations for 6 h. Total cellular lysates were prepared, and then immunoblotted for Noxa, phosphorylated Mcl-1, and Mcl-1, followed by actin. Similar results were also obtained with other cell lines examined. Results (A and B) are representative of three independent experiments.

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Figure 5. Overexpression of SBP-Noxa induced apoptosis and Mcl-1 cleavage in human MM cells

Human MM 8226 cells were transduced with a lentiviral expression vector of Noxa (pUB-SBP-Noxa) or an empty lentiviral expression vector (pUB-Thy1.1). (A) Transduction efficiency analyzed by Thy1.1 staining and flow cytometry. Cells were analyzed at 24 hours post transduction. Gated population (Thy1.1+) indicates cells that were successfully transduced with the lentiviral expression vector. (B) Representative FACS profiles of transduced cells analyzed by annexin V staining. Cells were stained at 48 hours post transduction. Apoptotic cells were identified as annexin V+ PI-, dead cells were annexin V+

PI+, and live cells were annexin V– PI–. **(C)** Summary graph of the results of annexin V staining. The graph depicts the results of 3 independent experiments (mean $\pm$ SEM). **(D)** Cell cycle distribution analyzed by PI staining. Cells were stained at 48 hours post transduction. Gated populations in the FACS histograms indicate apoptotic cells (DNA content $< 2n$) or proliferating cells ($2n < \text{DNA content} \leq 4n$). **(E)** Summary graph of the results of cell cycle analyses. The graph depicts the results of 3 independent experiments (mean $\pm$ SEM). **(F)** Noxa overexpression induced cleavage of caspase 3. Total cellular proteins were prepared at 36 hours post transduction, and then immunoblotted for Caspase 3, followed by SBP-Noxa and actin. **(G)** Co-immunoprecipitation of Mcl-1 with SBP-Noxa. Total cellular lysates were prepared at 36 hours post transduction using 1% CHAPS lysis buffer. Immunoprecipitation of SBP-Noxa was performed using streptavidin-sepharose beads as described in Methods. Input lysates (shown in the left panel) and proteins pulled-down by streptavidin-sepharose beads (SA IP; shown in the right panel) were immunoblotted for Mcl-1, Bcl-xL, Bcl2, Bax, and Bim, followed by SBP-Noxa and actin. Results shown are representative of three independent experiments.

Figure 6. Oridonin or AD 198 potently augmented the cytotoxic activity of bortezomib on TRAF3^{-/-} malignant B cells

Cells were treated with indicated concentrations of bortezomib (BTZ), oridonin (Ori) or AD 198 (AD), alone or in combination, for 24 h. Total viable cell numbers were subsequently determined by MTT assay. (A) Combined effects of bortezomib and oridonin. (B) Combined effects of bortezomib and AD 198. The graphs depict the results of three independent experiments with duplicate samples in each experiment (mean $\pm$ SEM). *, significantly different from single agent treatment (*t* test, *p* < 0.05); **, very significantly

different from single agent treatment (t test, $p < 0.01$); ***, highly significantly different from single agent treatment (t test, $p < 0.001$).

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