Combination bortezomib and rituximab treatment affects multiple survival and death pathways to promote apoptosis in mantle cell lymphoma

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Mantle cell lymphoma (MCL) is a distinct histologic subtype of B cell non-Hodgkins lymphoma (NHL) associated with an aggressive clinical course. Inhibition of the ubiquitin-proteasome pathway modulates survival and proliferation signals in MCL and has shown clinical benefit in this disease. This has provided rationale for exploring combination regimens with B-cell selective immunotherapies such as rituximab. In this study, we examined the effects of combined treatment with bortezomib and rituximab on patient-derived MCL cell lines (Jeko, Mino, SP53) and tumor samples from patients with MCL where we validate reversible proteasome inhibition concurrent with cell cycle arrest and additive induction of apoptosis. When MCL cells were exposed to single agent bortezomib or combination bortezomib/rituximab, caspase dependent and independent apoptosis was observed. Single agent bortezomib or rituximab treatment of Mino and Jeko cell lines and patient samples resulted in decreased levels of nuclear NFκB complex(es) capable of binding p65 consensus oligonucleotides, and this decrease was enhanced by the combination. Constitutive activation of the Akt pathway was also diminished with bortezomib alone or in combination with rituximab. On the basis of in vitro data demonstrating additive apoptosis and enhanced NFκB and phosphorylated Akt depletion in MCL with combination bortezomib plus rituximab, a phase II trial of bortezomib-rituximab in patients with relapsed/refractory MCL is underway.

Introduction

Mantle cell lymphoma (MCL) is B cell neoplasm that is classified as a morphologically distinct subtype of non-Hodgkin’s lymphoma (NHL) and is associated with a poor prognosis.1 MCL is characterized by the presence of a balanced t(11:14)(q13;q32) translocation juxtaposing the bcl-1 (cyclin D1) locus distal to the immunoglobulin heavy chain promoter located at 14q32,2 resulting in constitutive overexpression of cyclin D1 and consequent disordered cell cycle progression. While MCL comprises only 3–8% of NHL cases diagnosed in a given year, its natural history is associated with a disproportionate number of deaths with a mean survival of only three years.3-5 To date, several therapeutic options have been pursued to improve outcome for MCL patients including combination chemotherapy, high-dose chemotherapy followed by stem cell transplant, and monoclonal antibody therapy, all of which have been met with minimal success.6-8 Therefore, given the absence of curative therapy for MCL, it is essential to explore new treatment options.

Bortezomib (PS-341, Velcade™) is a therapeutic agent that targets and reversibly inhibits the 26S proteasome.9-11 The 26S proteasome is present in the cytoplasm and nucleus and represents the key non-lysosomal-mediated complex regulating proteolytic degradation of multiple cellular proteins that contribute to cellular growth and survival. The ubiquitin-proteasomal system plays a central role in regulating multiple cellular processes including cell cycle progression, apoptosis and cellular differentiation.12,13 Disorders of ubiquitin-mediated proteasomal degradation have been described in multiple malignancies and are vital to maintenance of the transformed phenotype.14-20 Important targets of the proteasome include nuclear factor κB (NFκB),21 p53, cyclin-dependent kinase (cdk) inhibitors (p21, p27) and c-myc.22-25 Given this wide variety of effects on cellular pathways that are often dysregulated in neoplastic cells, it is not surprising that bortezomib has demonstrated impressive anti-tumor activity as a single agent in MCL. To date, exploration of combination treatment strategies with bortezomib in MCL have not yet occurred.26,27

Rituximab (Rituxan™) is a CD20-specific chimeric monoclonal antibody that has activity in MCL as a single agent or in combination with chemotherapy.8,28-31 Cross-linking of surface CD20 with rituximab interferes with multiple intracellular signal transduction pathways, and sensitizes NHL B cell lines to chemotherapeutic agents in part by selectively down-modulating Bcl-2 and Bcl-xl.12,33 In a non-MCL lymphoma cell line, rituximab was shown to inhibit constitutive NFκB signaling pathways by decreasing the
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plus GAH IgG resulted in enhanced apoptosis over either agent alone. This effect was noted as early as 24 hours following treatment (Fig. 2A and B). We then repeated this experiment to investigate the induction of apoptosis with only a four hour incubation of bortezomib, using Jeko, Mino and SP53 cells (Fig. 2C) and primary MCL tumor cells (90% CD19+/CD5+) (Fig. 2D). As shown in these figures, incubation of the three cell lines with bortezomib and cross-linked rituximab resulted in significant induction of apoptosis determined by Annexin V/topro 3 and flow cytometry: SP53 cell line was the most sensitive to combination therapy, while the blastoid variant derived Jeko cell line was the least sensitive. Incubation with bortezomib plus rituximab resulted in only 13% of the SP53 cells alive at 24 hours, compared to 43% of the Mino cells and 55% of the Jeko cells, normalized to trastuzumab control (p = 0.069, p = 0.003 and p = 0.02 vs. bortezomib alone, respectively; and p = 0.006, p = 0.002 and p = 0.005 vs. rituximab alone, respectively). The combination treatment also induced an average 62.8% of apoptosis in primary MCL cells after 24 hours.

We next sought to determine whether the intrinsic or extrinsic apoptotic pathway was activated by this combination therapy by examining expression of initiating caspases involved in the intrinsic (caspase 9) and extrinsic (caspase 8) pathways, as well as downstream effector caspases. Continual incubation of Jeko and Mino cells in bortezomib resulted in cleavage of caspases 8, 9 and 3 (Fig. 1A). When Mino and Jeko cells were exposed to bortezomib for only four hours, proteasome activity was reduced by approximately 50% (Fig. 1B). This data suggests that strategies to extend bortezomib exposure may provide additional benefit in MCL.

Results

Proteasome inhibition with bortezomib. First, to confirm proteasomal inhibition, bortezomib (0, 4, 10, 20 and 100 nM) was added directly to untreated Jeko and Mino cell lysates. As expected, increasing bortezomib concentration resulted in a dose-dependent increase in proteasome inhibition which reached a maximum of 95% for both cell types at 100 nM of bortezomib. IC50 values were between 4 and 10 nM bortezomib for both cell lines evaluated in this fashion (data not shown). Based on this proteasome inhibition data and pharmacokinetic/pharmacodynamic data in treated patients, used 10 nM bortezomib in all further experiments.

We then investigated the exposure time required for proteasome inhibition in MCL cells. With continuous incubation in the presence of 10 nM bortezomib, both Mino and Jeko cells exhibited significant proteasome inhibition (>80%) at 12 and 24 hours (Fig. 1A). When Mino and Jeko cells were exposed to bortezomib for only four hours, proteasome activity was reduced by approximately 50% (Fig. 1B). This data suggests that strategies to extend bortezomib exposure may provide additional benefit in MCL.

Induction of apoptosis in MCL cells treated with bortezomib and rituximab. When Jeko and Mino cells were incubated continuously in presence of bortezomib, the addition of 1 μg/mL rituximab...
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of z-VAD-fmk did not (Jeko) or only modestly (Mino) improved cell survival in cells treated with rituximab only, suggesting that rituximab-mediated apoptosis is largely caspase-independent.43 To evaluate the effects of bortezomib and rituximab treatment on known caspase substrates, immunoblots were probed with antibodies specific for poly ADP-ribose polymerase (PARP, Fig. 4) and the pro-survival Bcl-2 family member Mcl-1 (Fig. 5). By 24 hours, virtually all detectable PARP from lysates derived from MCL cell lines treated with bortezomib alone or in combination with rituximab was in cleaved form. In contrast, MCL cell lines treated with rituximab alone demonstrated only modest PARP cleavage.

Figure 3A. Bortezomib induces caspase cleavage. (A) Jeko and Mino cell lines were cultured in the presence of 10 nM bortezomib (continuous exposure) and/or rituximab (1 μg/ml) plus cross-linker, harvested and lysed at 0 and 24 hours. 20 μg protein per lane was separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed with antibodies to caspase 8, 9, 3 and actin. Arrows show molecular weights of caspase fragments and actin.
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Figure 3B and C. Jeko (B) and Mino (C) cells were incubated with either DMSO or with 100 μM z-VAD-fmk for one hour at 37°C, then cultured in the presence of 10 nM bortezomib (continuous exposure) and/or rituximab (1 μg/ml) plus cross-linker. Cells were harvested at 24 hours and analyzed by flow cytometry with Annexin V/topro 3 and active caspase 3. Data shown are from three independent experiments.
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We then investigated the effects of bortezomib and rituximab on Mcl-1, as this protein is also a known proteasome substrate. As expected, total Mcl-1 protein levels were increased in both Jeko (Fig. 5A) and Mino (Fig. 5B) cells treated with bortezomib at 12 and 24 hours. Importantly, a cleaved form of Mcl-1 also increased at these timepoints. Cleaved Mcl-1 protein has been hypothesized to have a pro-apoptotic function, and the appearance of this fragment may indicate dual pro- and anti-apoptotic mechanisms underway. The addition of rituximab, alone or in combination with bortezomib, did not enhance Mcl-1 cleavage.

NFκB inhibition with bortezomib and rituximab in MCL cell lines and patient samples. Prior studies have demonstrated that constitutive activation of NFκB is a contributing factor to the pathogenesis of MCL. To investigate the effect of bortezomib and rituximab on NFκB activity in MCL cells, we measured p65 (relA) activity in nuclear extracts isolated from treated Jeko and Mino cells (Fig. 6). We found that by 12 hours, single agent bortezomib, rituximab or combination treatment reduced levels of p65 NFκB complexes capable of binding a p65 consensus oligonucleotide. Combination treatment with bortezomib and rituximab led to a significant reduction in relative NFκB activity compared to control and each treatment alone (p = 0.00009 and p = 0.00015 vs. control; p = 0.00035 and p = 0.000006 vs. bortezomib alone; p = 0.000015 and p = 0.000025 vs. rituximab alone, respectively) (Fig. 6A and B). We then investigated this effect using MCL patient samples. As shown in Figure 6C and D, combination treatment of two patient samples (patients 1 and 3) induced a significant decrease in NFκB activity compared to the control (p = 0.015 and p = 0.033, respectively), but not compared to each treatment alone. To verify these results, we isolated nuclear and cytoplasmic protein fractions from Jeko and Mino MCL cells treated for 6, 12 and 24 hours with or without bortezomib and rituximab. Fractions were analyzed by immunoblot for p65, α-tubulin (cytoplasmic control) and Brg-1 (nuclear control). As shown in Figure 6E and F, nuclear p65 levels were reduced in cells treated with either bortezomib or rituximab alone as early as 12 hours after exposure to the respective drug. Combined exposure to bortezomib plus rituximab resulted in near elimination of nuclear NFκB/p65 in both Jeko (Fig. 6E) and Mino (Fig. 6F) cells at 12 hours.

Combination bortezomib and rituximab results in decreased Akt activation. Recent work demonstrates that the PI3K/Akt signaling pathway is constitutively activated in MCL, suggesting that this pathway contributes to the pathogenesis of MCL.46 Furthermore, prior work has shown that rituximab treatment interferes with the Akt pathway.47 To determine if combination therapy with bortezomib and rituximab affect the Akt pathway, we performed a series of immunoblots with whole-cell lysates collected from Jeko and Mino cells treated with bortezomib, rituximab with cross-linking, or combination treatment. Immunoblots were probed with monoclonal antibodies specific for Akt (total), phosphorylated Akt (active) or actin. As shown in Figure 7, levels of phosphorylated Akt were diminished in single agent bortezomib and rituximab treated Jeko and Mino cells at 24 hr. Furthermore, combination treatment moderately enhances this effect.

Discussion

MCL is an aggressive B-cell lymphoma, largely resistant to traditional chemotherapy and characterized by nondurable remissions. Therefore, new therapeutic options are crucial to improve patient outcome. Because of the data demonstrating activity of both bortezomib and rituximab as single agents in patients with relapsed/ refractory MCL and the potential for targeting NFκB from two different mechanisms, we examined the biological effects of these two compounds in combination, using MCL cell lines and patient samples.
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Bortezomib is a potent and reversible inhibitor of the proteasome which has therapeutic efficacy in multiple myeloma, MCL, other B-cell NHL, and several solid tumors. However, the precise mechanism by which proteasome inhibition induces cell death is not clear. Inhibiting the proteasome can lead to the accumulation of cellular cdk inhibitors p21 and p27, which play a critical role in regulating cell cycle and apoptosis. In addition to the effects on cell cycle control, proteasome inhibition can affect the levels of Bcl-2 family members, leading to the induction of apoptosis. Furthermore, bortezomib inhibits the nuclear translocation of the p50/p65 (NFκB) heterodimer by preventing proteasomal degradation of IkBα. NFκB is a transcription factor that regulates multiple anti-apoptotic genes such as Bcl-2 homologs A1/Bfl-1, Bcl-xL, IEX-1 and XIAP, and its constitutive activation has been shown in MCL and other malignancies including pancreatic cancer, colon cancer, breast cancer, T cell leukemia, Hodgkin’s lymphoma, diffuse large B cell lymphoma and chronic lymphocytic leukemia. NFκB may also antagonize p53 function and induce cell growth through binding to the promoter of cyclin D1. Abnormal regulation of the NFκB pathway has been associated with chemotherapy resistance in both solid tumors and hematologic malignancies.

Rituximab, a CD20-specific chimeric monoclonal antibody, exerts considerable cytotoxicity against MCL cells when it is cross-linked, and interferes with multiple apoptosis regulatory proteins such as Bcl-xl (downregulation) and Apaf-1 (upregulation). In Burkitt lymphoma cell lines (Daudi and Ramos), rituximab has been shown to inhibit constitutive NFκB signaling pathways by reducing the phosphorylation of the components of the NFκB pathway and by inhibition of IKK activity.

In our in vitro experiments we showed that bortezomib can induce apoptosis at concentrations as low as 10 nM, a concentration successfully reached in patients. We utilized four-hour drug exposures, as this reflects the transient exposure time of drug in vivo as reported in phase I studies. Thus, our experiments were designed to use doses of drug and incubation times that are realistically achieved in patients. A recent report communicated by Wang et al. evaluated a three-drug regimen combining bortezomib, rituximab and cyclophosphamide (BRC) in MCL cell lines and MCL patient samples. Our study evaluated the two drug bortezomib and rituximab regimen.
a combination that was not explored by Wang et al. While our results evaluating the activity of single agent bortezomib or rituximab are consistent with the data reported by Wang et al., our data with combination bortezomib and rituximab demonstrate equivalent potency when compared to the BRC regimen suggesting that the addition of cyclophosphamide does not add any significant antitumor activity to this regimen. In particular, our results detailed in Figure 2A and B show apoptosis of MCL cell lines Jeko and Mino treated with bortezomib and rituximab at identical concentrations used by Wang et al. (without cyclophosphamide), for a 24 hour incubation period. The resulting apoptosis seen at 24 hours was 87.6% in Jeko cells and 87.5% in Mino cells treated with bortezomib and rituximab versus 80% and 55% in Jeko and Mino cells treated with the three drug BRC drug regimen described by Wang et al. Furthermore, we repeated the same experiment with a four hour exposure intended to mimic the rapid clearance of bortezomib in vivo and even with this short exposure time we were able to appreciate a significant induction of apoptosis in Jeko, Mino, SP53 and primary cells from MCL patient samples treated with bortezomib and rituximab without the addition of cyclophosphamide (Fig. 2C and D).

Our data demonstrate that combination bortezomib plus rituximab treatment produced greater apoptotic activity than either agent alone in MCL cell lines as well as MCL patient samples. We observed that the tested cell lines accurately reflected the sensitivity of the three MCL patient samples tested. While this number of samples is low and represents a limitation of these studies, the observation is interesting and may provide an indication of the direct activity of this regimen when delivered to patients in the clinic. It is interesting to note that while the combination induces apoptosis in all three MCL cell lines tested, there is a range in sensitivity, with the SP53 being most sensitive and Jeko the most resistant. One explanation for this variation in sensitivity may lie in the p53 mutational/deletion status of each cell line. Jeko cells are p53-null, Mino cells possess a mutated p53 allele, and SP53 cells carry two wild-type p53 alleles. These results are in agreement with those published previously in which bortezomib was shown to have cytotoxic effect through p53-dependent and -independent mechanisms. Possible resistance through this pathway should therefore be investigated in future studies. In addition, differences in the extent of proteasome inhibition may contribute to the enhanced effect of bortezomib in these cell lines. For example, we showed that in Jeko cells, proteasome inhibition occurs for a shorter period of time compared to Mino cells. The mechanism of reduced proteasome inhibition is not understood and may explain the relative resistance of Jeko cells to the combination treatment.

Caspase activation is a common pathway leading to programmed cell death induced by antineoplastic agents. Our data demonstrate that caspase activation is critical for bortezomib-induced apoptosis, as demonstrated in other studies. Furthermore, the addition of rituximab significantly increased cell death without further caspase activation, showing that both caspase dependent and independent pathways are mobilized with this combination therapy. The broad spectrum caspase inhibitor z-VAD-fmk partially blocked bortezomib-induced cell death in MCL cell lines at 24 hours. Pérez-Galan et al. showed that combination bortezomib-induced apoptosis is a consequence of the alteration of mitochondrial membrane potential, reactive oxygen species (ROS) generation, Bax/Bak conformational changes and caspase activation. They further showed that ROS generation was upstream of caspase activation, and that induction of bortezomib-induced apoptosis could be completely rescued by ROS scavengers but only partially by z-VAD-fmk, in agreement with our data presented here. In contrast, Wang et al. showed that bortezomib-induced apoptosis could be nearly completely rescued in presence of z-VAD-fmk in MCL cell lines. The reasons for these differences remain to be identified.

Bortezomib induced a marked accumulation of the anti-apoptotic protein Mcl-1 in both Jeko and Mino cell lines as early as 12 hours after exposure, likely due to the inhibition of ongoing proteasome-mediated Mcl-1 turnover. While results reported by Nencioni et al. suggested that the accumulation of Mcl-1 in bortezomib-induced apoptosis played a pro-survival role, our data demonstrate that this accumulation does not block apoptosis induced by bortezomib or combination treatment. The accumulation could slow down apoptosis induction, as was suggested by Pérez-Galan et al. However, we also showed the appearance of a cleaved form of Mcl-1 with as little as 12 hours of treatment with bortezomib. This cleaved form of Mcl-1 may play a pro-apoptotic role similar to that seen with cleaved Bcl-2 and Bcl-xL. The ratio of intact Mcl-1 to cleaved Mcl-1 may be a critical element that alters the apoptotic threshold; this possibility remains to be fully investigated.

To further clarify the multiple mechanisms through which combination bortezomib and rituximab induce cell death, we also examined the expression of Akt and p-Akt in Jeko and Mino cell lines treated with bortezomib and rituximab. PI3K/Akt is a major signaling pathway that controls multiple cellular processes such as cell growth, proliferation and survival. The constitutive activation of Akt in MCL induces phosphorylation of multiple targets including MDM2, which leads to inhibition of p53 activity; Bad, which inactivates its proapoptotic function; and p27, which leads to its proteasomal degradation. We observed a near complete down-modulation of p-Akt without loss of total Akt in cells treated with bortezomib plus rituximab after 24 hours. Our data on p-Akt further confirm that the combination of bortezomib and rituximab interferes with multiple pro-survival pathways important in the pathogenesis and survival of MCL.

In summary, we have demonstrated that the combination of bortezomib and rituximab induce enhanced apoptosis both in MCL
cell lines and in patient samples. The molecular signature of MCL is complex and involves multiple signals that favor cell survival and growth.45 Our data supports the rationale for using bortezomib in combination with rituximab in MCL therapy, as this strategy interferes with both NFκB and Akt signaling, which are both critical in the pathogenesis of MCL. These findings further confirm the efficacy of bortezomib as a therapeutic agent in MCL, and the enhanced cytotoxic effect seen in combination with rituximab established the basis for the design of a phase II trial delivering this combination therapy to patients with relapsed/refractory MCL that is currently underway.

Materials and Methods

Cell lines and patient samples. Human MCL cell lines (SP53, Mino and Jeko) were established from patient biopsy samples as previously described.35-37 All cell lines were cultured in RPMI 1640 medium (Invitrogen, Rockville, MD), supplemented with 100 U/ml penicillin, 2 mM L-glutamine, 100 μg/ml streptomycin, and 10% fetal bovine serum (Invitrogen, Rockville, MD) and were maintained at 37°C in a humidified atmosphere of 5% CO2. Cell counts and viability were initially determined by trypan blue dye exclusion and then confirmed by topro 3 uptake. The Jeko cell line was derived from a patient with blastoid variant MCL and Mino cells were derived from classical MCL. Jeko is p53 null and Mino has a mutated but functional p53.35-37 SP53 cells express wild-type p53 and were derived from classical MCL. Characteristics for each sample were known. Patient 1 and 2 were derived from peripheral blood of patients affected by the blastoid variant of MCL. Patient 3 was derived from a lymph node biopsy that was at least 90% involved by MCL (CD19+/CD5+ by flow cytometry). All samples were confirmed for the characteristic t(11;14) translocation and immunophenotype (CD19/CD5 positive). Fresh MCL cells were obtained from The Ohio State University’s Tissue Procurement and Banking core facility. Written, informed consent was obtained from all patients on an IRB-approved tissue procurement protocol.

Reagents. Bortezomib (Millennium Pharmaceuticals, Inc., Cambridge, MA), trastuzumab (Herceptin) (Genentech Inc., San Francisco, CA) and rituximab (Genentech) were obtained commercially. Cells were incubated with or without 4-hour or continuous exposures to bortezomib (10–100 nM). Cells were treated for 48 hours were washed with phosphate buffered saline (PBS) and plated in standard growth medium. Trastuzumab or rituximab (1 μg/ml each), together with 20 μg/ml of goat anti-human (GAH) IgG (Jackson ImmunoResearch), were added to cross-link surface CD20.

Proteasome inhibition assay. A spectrofluorometric assay was used to measure proteasome activity based on a previously described method.38 Briefly, bortezomib-treated or untreated cells were lysed by repeated freeze-thaw and 5 mM EDTA (pH 8.0) for 1 hour on ice and then centrifuged at 6,600 xg for 10 minutes at 4°C. The protein content of the resultant lysate was determined using a Coomassie protein assay (Pierce Biotechnology, Rockford, IL). Five micrograms of total protein was added to wells of a microtiter plate, with substrate buffer containing the peptide Suc-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (AMC), and the mixtures were incubated at 37°C for 25 minutes. Liberated AMC dye was measured using an excitation wavelength of 380 and an emission wavelength of 460 nm. Percent inhibition was calculated by comparing the fluorescence of treated samples to untreated controls at each indicated time point. A total of 8 lysates from Jeko and Mino cell lines were processed for 4 hours versus continual bortezomib incubation, and five replicates were performed for each sample.

Apoptosis assessments. Following treatments for 24 or 48 hr, 5 x 10⁵ cells were evaluated for apoptosis by dual staining with Annexin V conjugated to fluorescein isothiocyanate (FITC; BD Pharmingen, San Diego, CA) and topro 3 iodide (Sigma, St. Louis, MO) as described.39 Samples were analyzed on a FACS Calibur flow cytometer (Beckton Dickinson, Mountain View, CA). Cells that were both annexin V-negative and topro 3-negative were considered viable. Flow cytometry data were collected with CellQuest (Becton-Dickinson) and analyzed using WinMDI software (Scripps Research Institute, San Diego, CA).

Caspase inhibition studies. Cells were incubated with either vehicle control (DMSO) or with 100 μM of the broad-spectrum caspase inhibitor z-VAD-fmk (Enzyme Product Systems, Livermore, CA), 1 hour prior to continuous exposure to bortezomib (10 nM). Following bortezomib treatment, rituximab, trastuzumab (1 μg/ml each) and GAH IgG were added as indicated in each figure. Cells were harvested after 24 or 48 hours and analyzed for viability and caspase activation. Intracellular staining was performed using Cytofix/Cytoperm reagent and a FITC-labeled monoclonal antibody specific for active caspase 3 (both from BD Pharmingen) as previously described.39 Nonspecific binding of monoclonal antibodies was blocked by pre-incubating samples with unconjugated mouse immunoglobulin G (Sigma, St. Louis, MO).

Immunoblot analysis. For whole cell extracts, cells were lysed in RIPA buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% SDS and 1% sodium deoxycholate] containing protease and phosphatase inhibitors (all from Sigma) and cell lysates were clarified by centrifugation. Nuclear and cytosolic extracts were prepared using the NE-PER kit (Pierce) according to the manufacturer’s recommendations. Proteins were analyzed by immunoblot using standard procedures. Antibodies to the following proteins were used: caspase 3, caspase 8, caspase 9, PARP, tAkt, phospho-AKT and p65/NFκB (Cell Signaling Technology, Boston, MA); Mcl-1, Brg-1, actin and α-Tubulin (Santa Cruz Biotechnology, Santa Cruz, CA). Appropriate horseradish peroxidase-conjugated secondary antibodies were obtained from Cell Signaling Technology. Immunoreactivity was visualized with Super Signal West Pico Chemiluminescent kit (Pierce Biotechnology, Rockford, IL) and exposed to Kodak X-OMAT MR film (Kodak, Rochester, NY). Actin (whole-cell extract), Brg-1 (nuclear extract) or tubulin (cytosolic extract) were used as loading controls.

NFκB analyses. After 12 hr treatments with bortezomib and rituximab alone or in combination, nuclear extracts were prepared and examined using the NFκB Trans-Am kit according to the manufacturer’s instructions (Active Motif, Carlsbad, CA). Tumor necrosis factor α-stimulated HeLa cell extracts were used as a positive control. To determine specificity, wild-type and mutant NFκB consensus oligonucleotides were added to HeLa extracts. Relative NFκB activity was measured as the absorbance in the treated samples minus the absorbance in the negative control.

Statistical analyses. All assays were performed in triplicate, and data are expressed as mean values ± standard deviation. Statistical analyses and p value determinations were done by two-tailed paired
t-test with a confidence interval of 95% for determination of the significance of differences observed between experimental groups. p < 0.05 was considered to be significant.

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References

1. The non-Hodgkin's Lymphoma Classification Project. A clinical evaluation of the International Lymphoma Study Group classification of non-Hodgkin's lymphoma. Blood 1997; 89:3909-18.
2. Swerdlow SH, Berger F, Isaacson PI, Muller-Hermelink HK, Nathwani BN, Piiri MA et al. World Health Organization Classification of Tumors: Pathology and Genetics of tumors of Hematopoietic and Lymphoid Tissues. IARC Press 2001; 168-70.
3. Fisher RI, Dahlberg S, Nathwani B, Banks PM, Miller TP, Gruppen TM. A clinical analysis of two indolent lymphomentities: mantle cell lymphoma and marginal zone lymphoma (including plasmablastic and lymphoepithelioid B-cell subcategories). Southwest Oncology Group Study. Blood 1995; 85:1075-82.
4. Boccadoro M, Campo E, Rödel M, Ribera JM, Conde E, Piiri MA, Vallespi T et al. Mantle cell lymphoma: presenting features, response to therapy and prognostic factors. Cancer 1998; 82:567-75.
5. Campo E, Raffeld M, Gillis AE. Constitutive nuclear factor-kappaB-RelA activation is required for proliferation and survival of reactive oxygen species. Blood 2001; 98:2771-7.
6. Ishiyama K, Takami A, Okumura H, Ozaki J, Shimadou S, Yamanaka S et al. Complete and durable remission of refractory mantle cell lymphoma with rituximab monotherapy. Int J Hematol 2005; 79:193-7.
7. Khouri IF, Romaguera J, Kantarjian H, Palmer JL, Pugh WC, Korbling M et al. HyperCVAD and high-dose methotrexate/cytarabine followed by stem cell transplantation: an active regimen for aggressive mantle cell lymphoma. J Clin Oncol 1998; 16:3803-9.
8. Lenz G, Dreyling M, Hoster E, Wormald B, Duhues N, Metzner B et al. Immunomorphometry with rituximab and cyclophosphamide, doxorubicin, vincristine and prednisone significantly improves response and time to treatment failure, but not long-term outcome in patients with previously untreated mantle cell lymphoma: results of a prospective randomized trial of the German Low Grade Lymphoma Study Group (GLSG). J Clin Oncol 2005; 23:1894-92.
9. Adams J, Kaufman M. Development of the proteasome inhibitor Velcade (Bortezomib). Cancer Invest 2004; 22:304-11.
10. Richardson PG, Mistodes C, Hideshima T, Anderson KC. Bortezomib: proteasome inhibition as an effective anticancer therapy. Ann Rev Med 2006; 57:33-47.
11. Voorhees PM, Orlowski RZ. The proteasome and proteasome inhibitors in cancer therapy. Clin Cancer Res 2004; 10:630-9.
12. Shan D, Leadbetter JA. PRESS: Apoptosis of malignant human B cells by ligation of CD20 with monoclonal antibodies. Cancer 1998; 91:1644-52.
13. Bellosillo B, Villamor N, Lopez-Guillermo A, Marcé S, Esteve J, Campo E et al. Phase II trial of the proteasome inhibitor bortezomib in patients with indolent non Hodgkin's lymphoma and previously treated mantle cell lymphoma, immunocytoma and small B-cell lymphocytic lymphoma. J Clin Oncol 2000; 18:317-24.
14. Ghielmini M, Schütt M, Cognati S, Berton F, Walterz U, Fry MF et al. Effect of single-agent rituximab given at the standard schedule or as prolonged treatment in patients with mantle cell lymphoma: a study of the Swiss Group for Clinical Cancer Research (SAKK). J Clin Oncol 2005; 23:705-11.
15. Bonavida B, Liptak D, Cortes J, Zinzani P, Gertz M, Zinzani P et al. Phase I clinical experience with the novel proteasome inhibitor bortezomib in patients with indolent non Hodgkin's lymphoma and mantle cell lymphoma. J Clin Oncol 2005; 23:676-84.
16. Wang M, Han XH, Zhang Y, Yang J, Qian JF, Shi YK et al. Bortezomib is synergistic with rituximab and cyclophosphamide in inducing apoptosis of mantle cell lymphoma cells in vitro and in vivo. Leukemia 2008; 22:178-95.
17. Ishiyama K, Takami A, Okumura H, Ozaki J, Shimadou S, Yamanaka S et al. Complete and durable remission of refractory mantle cell lymphoma with repeated rituximab monotherapy. Int J Hematol 2005; 79:193-7.
18. Nakshatri H, Bhat-Nakshatri P, Martin DA, Goulet RJ Jr, Sledge GW Jr. Constitutive activation of c-Cyc in Burkitt's lymphoma cells. Mol Cell Biol 2000; 20:2423-35.
19. Boccadoro M, Tam SW, Teddy AM, Beer-Romero P, Del Sal G, Chian V et al. Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin dependent kinase inhibitor p27. Science 1995; 269:682-5.
20. Chiarle R, Bodel LM, Skolnik J, Fruzzia G, Chilosi M, Corato A et al. Increased protease-240some degradation of cyclin-dependent kinase inhibitor p27 is associated with decreased overall survival in mantle cell lymphoma. Blood 2000; 95:619-26.
21. O'Connor OA, Wright J, Monshouwer M, Murray J, Magnone Correlli B, Stribblefield M et al. Phase II clinical experience with the novel proteasome inhibitor bortezomib in patients with indolent non Hodgkin's lymphoma and mantle cell lymphoma. J Clin Oncol 2005; 23:676-84.
22. Wang M, Han XH, Zhang Y, Yang J, Qian JF, Shi YK et al. Bortezomib is synergistic with rituximab and cyclophosphamide in inducing apoptosis of mantle cell lymphoma cells in vitro and in vivo. Leukemia 2008; 22:178-95.
23. Gregory MA, Hann SR, c-myc proteolysis by the ubiquitin-proteasome pathway: stabilization of c-Myc in Burkitt's lymphoma cells. Mol Cell Biol 2000; 20:2423-35.
24. Campo E, Hamelmann E, Kampf K, Mappara MY, Arnold W et al. Constitutive nuclear factor kappaB RelA transcription factor is constitutively activated in human pancreatic adenocarcinoma cells. Clin Cancer Res 1999; 5:119-27.
25. Adams J, Kaufman M. Development of the proteasome inhibitor Velcade (Bortezomib). Cancer Invest 2004; 22:304-11.
26. Roychowdhury S, Baiocchi RA, Vourganti S, Bhatt D, Blaser BW, Freud AG et al. Selective inhibition of c-Myc in Burkitt's lymphoma cells. Mol Cell Biol 2000; 20:2423-35.
27. Ishiyama K, Takami A, Okumura H, Ozaki J, Shimadou S, Yamanaka S et al. Complete and durable remission of refractory mantle cell lymphoma with repeated rituximab monotherapy. Int J Hematol 2005; 79:193-7.
28. Nakshatri H, Bhat-Nakshatri P, Martin DA, Goulet RJ Jr, Sledge GW Jr. Constitutive activation of c-Cyc in Burkitt's lymphoma cells. Mol Cell Biol 2000; 20:2423-35.
29. Boccadoro M, Tam SW, Teddy AM, Beer-Romero P, Del Sal G, Chian V et al. Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin dependent kinase inhibitor p27. Science 1995; 269:682-5.
30. Chiarle R, Bodel LM, Skolnik J, Fruzzia G, Chilosi M, Corato A et al. Increased protease-240some degradation of cyclin-dependent kinase inhibitor p27 is associated with decreased overall survival in mantle cell lymphoma. Blood 2000; 95:619-26.
31. O'Connor OA, Wright J, Monshouwer M, Murray J, Magnone Correlli B, Stribblefield M et al. Phase II clinical experience with the novel proteasome inhibitor bortezomib in patients with indolent non Hodgkin's lymphoma and mantle cell lymphoma. J Clin Oncol 2005; 23:676-84.
32. Wang M, Han XH, Zhang Y, Yang J, Qian JF, Shi YK et al. Bortezomib is synergistic with rituximab and cyclophosphamide in inducing apoptosis of mantle cell lymphoma cells in vitro and in vivo. Leukemia 2008; 22:178-95.
Bortezomib and rituximab induce apoptosis in mantle cell lymphoma

48. Wang CY, Guttridge DC, Mayo MW, Baldwin AS Jr. NFkappaB induces expression of the Bcl-2 homologue A1/Bfl-1 to preferentially suppress chemotherapy-induced apoptosis. Mol Cell Biol 1999; 19:5923-9.

49. Hinz M, Krappmann D, Eichten A, Heder A, Scheidereit C, Straus M. NFkappaB function in growth control: regulation of cyclin D1 expression and G0/G1-to-S-phase transition. Mol Cell Biol 1999; 19:2690-8.

50. Bogner C, Ringshausen I, Schneller F, Fend F, Quintanilla-Martinez L, Hacker G, et al. Inhibition of the proteasome induces cell cycle arrest and apoptosis in mantle cell lymphoma cells. Br J Haematol 2003; 122:260-8.

51. Pérez-Galán P, Roué G, Villamor N, Villamor N, Monnerrat E, Campo E, et al. 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.

52. Furman RR, Asgary Z, Mascarenhas JO, Liou HC, Schattner EJ. Modulation of NFkappaB activity and apoptosis in chronic lymphocytic leukemia B cells. J Immunol 2000; 164: 2209-6.

53. Nencioni A, Hua F, Dillon CP, Yokoo R, Scheiermann C, Cardone MH, et al. Evidence for a protective role of Mcl-1 in proteasome inhibitor-induced apoptosis. Blood 2005; 105: 3255-62.