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Treatment-induced oxidative stress and cellular antioxidant capacity determine response to bortezomib in mantle cell lymphoma

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Abstract

Purpose—Proteasome inhibition disrupts protein homeostasis and induces apoptosis. Up to 50% of patients with relapsed mantle cell lymphoma (MCL) respond to bortezomib. We used gene expression profiling to investigate the connection between proteasome inhibition, cellular response, and clinical efficacy.

Experimental Design—We assessed transcriptional changes in primary tumor cells from five patients during treatment with bortezomib in vivo, and in 10 MCL cell lines exposed to bortezomib in vitro, on Affymetrix microarrays. Key findings were confirmed by Western blotting.

Results—MCL cell lines exposed to bortezomib in vitro showed upregulation of endoplasmic reticulum and oxidative stress response pathways. Gene expression changes were strongest in bortezomib sensitive cells and these cells were also more sensitive to oxidative stress induced by H₂O₂. Purified tumor cells obtained at several timepoints during bortezomib treatment in five previously untreated patients with leukemic MCL showed strong activation of the antioxidant

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Authorship

Contributions: Marc A. Weniger, Edgar G. Rizzatti, Patricia Pérez-Galán and Adrian Wiestner designed the study, analyzed data, and wrote the paper. Qiuyan Wang and Yihong Ye provided reagents, conducted molecular and cellular assays and performed data analysis. Delong Liu and Peter J. Munson evaluated gene expression data and performed statistical analysis. Nalini Raghavachari performed gene expression profiling. Wyndham H. Wilson initiated and supervised the clinical study. Adrian Wiestner, Kieron Dunleavy, and Wyndham H. Wilson evaluated patients and collected clinical data. Adrian Wiestner coordinated and supervised laboratory studies.

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response controlled by NRF2. Unexpectedly, activation of this homeostatic program was significantly stronger in tumors with the best clinical response. Consistent with its pro-apoptotic function we found upregulation of NOXA in circulating tumor cells of responding patients. In resistant cells gene expression changes in response to bortezomib were limited and upregulation of NOXA was absent. Interestingly, at baseline, bortezomib resistant cells displayed a relatively higher expression of the NRF2 gene expression signature than sensitive cells ($P < 0.001$).

Conclusion—Bortezomib triggers an oxidative stress response in vitro and in vivo. High cellular antioxidant capacity contributes to bortezomib resistance.

Introduction

Mantle cell lymphoma (MCL) is a mature B-cell NHL that is typically disseminated at diagnosis, involves the lymphoid tissues, gastrointestinal tract, and bone marrow, and in 20-30% of patients presents with leukemic disease.¹ MCL patients typically respond to conventional first-line chemoimmunotherapy regimens but most patients relapse within a few years.² Thus MCL has a relatively short median overall survival of 5-7 years, which is amongst the shortest of all B-cell lymphomas.³ Bortezomib (Velcade™), the first proteasome inhibitor to reach the clinic, was approved by the US Food and Drug Administration as second-line treatment for MCL.⁴ Bortezomib has a response rate of 30-50% in relapsed disease and was equally effective in patients who were sensitive or refractory to prior therapy.⁵⁻⁷ Onset of response is fast, with a median time to response of only four weeks.⁸ Bortezomib is also highly active as a single agent in Multiple Myeloma (MM), Waldenström's Macroglobulinemia, and systemic AL amyloidosis.⁴

Bortezomib is a reversible inhibitor of the proteasome, the major pathway for intracellular protein degradation. Bortezomib preferentially targets the chymotrypsin-like activity of the $\beta 5$ subunit and to a lesser extent inhibits the caspase-like activity of the $\beta 1$ subunit.⁴ Proteasome inhibition results in stabilization of key proteins involved in cell cycle control, DNA replication and repair, and cell survival. Whether an effect on any specific protein or pathway is responsible for bortezomib cytotoxicity remains unresolved.⁹ Inhibition of NF- κ B signaling through stabilization of the cytoplasmic inhibitor of NF- κ B (I κ B) was initially thought to be a major mechanism of bortezomib action. However, recent studies in MCL and MM cells do not support this hypothesis,^{10,11} and in some settings bortezomib may actually increase NF- κ B activity.^{10,12} On a more general level, proteasome inhibition disrupts protein homeostasis in both cytoplasm and the endoplasmic reticulum (ER). Bortezomib thus can elicit ER stress¹³⁻¹⁵ that triggers a homeostatic response termed the unfolded protein response (UPR). The UPR orchestrates a complex transcriptional program in order to alleviate ER stress and restore protein homeostasis.^{16,17} Three ER-resident regulatory switches control the UPR leading to increased expression of proteins involved in ER biogenesis and capacity, redox pathways, and protein folding. ER stress activates an ER-membrane bound protein kinase IRE1 to splice XBP1 mRNA, to produce a key transcription factor of ER biogenesis. In addition, ATF6, an ER-anchored transcription factor, is cleaved and translocates to the nucleus. These two transcription factors regulate ER-resident proteins, chaperones, and protein transporters thereby building the capacity to deal with protein load. The third UPR switch is PERK, an ER-resident kinase that phosphorylates eIF2 α leading to reduced protein synthesis while increasing the translation of select mRNAs in particular of the transcription factor ATF4.¹⁷ Failure to restore ER homeostasis ultimately leads to cell death.¹⁸ Several effectors of UPR-mediated cell death have been identified and include caspase-4,¹⁹ CHOP,²⁰ and the BH3-only proteins BIM²¹ and NOXA.²²

Proteasome inhibition has also been reported to lead to oxidative stress.^{13,22-24} This effect is not unique to bortezomib but rather appears to be a consequence of the accumulation of misfolded proteins that would normally be degraded through the proteasome.^{19,25} The ensuing attempts at protein folding generate high levels of reactive oxygen species (ROS).¹⁹ Given that ROS scavengers protect from and depletion of glutathione increases bortezomib-induced cell death in vitro, it is apparent that ROS contribute to the cytotoxic effect of bortezomib.^{22,26,27} Oxidative stress activates transcription of genes containing the so called antioxidant response element (ARE).²⁸ A key regulator of this oxidative stress response is the transcription factor NRF2, which in unstressed cells is sequestered in the cytoplasm by the adaptor protein KEAP1.²⁹ KEAP1 can be oxidized by ROS resulting in conformational changes that release NRF2. In addition, the UPR transducer PERK phosphorylates a threonine residue in the KEAP1 binding domain of NRF2 leading to its dissociation from KEAP1 and nuclear translocation.³⁰

Bortezomib inhibits proteasome activity in all cells but its clinical activity is limited to a few select malignancies.^{4,9} Likewise, activation of the UPR and/or generation of ROS have been described in many different tumor cell lines in vitro. However, in vitro sensitivity of the respective cell lines does not predict clinical tumor response. Given the central role of the protein ubiquitination pathway in cell signaling and cellular function ranging from protein secretion and cell growth to starvation, it is possible that the cellular reaction to proteasome inhibition differs in vitro and in vivo. Thus, it remains ill-defined what mechanisms determine bortezomib-induced cytotoxicity in vivo.

We chose gene expression profiling to systematically study the effect of proteasome inhibition on the tumor biology of MCL in vitro and in patients during treatment. We hypothesized that resistant tumors would show preferential upregulation of homeostatic responses enabling them to withstand proteasome inhibition. Surprisingly, we found the opposite: in response to proteasome inhibition sensitive tumors showed strong upregulation of NRF2 target genes, of proteasome components, and protein ubiquitination gene expression programs, whereas resistant cells showed minimal gene expression changes. Furthermore, we discovered that increased expression of NRF2 target genes at baseline correlated with decreased sensitivity to proteasome inhibition.

Materials and Methods

Cell lines and Patient samples

The mantle cell lymphoma (MCL) cell lines HBL-2, Jeko-1, JVM-2, Mino, NCEB-1, REC-1, SP-53, UPN1, and Z-138 were grown in RPMI 1640 (Cellgro, Herndon, VA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 2 mM L-glutamine, 100 units/ml of penicillin G and 100 mg/ml of streptomycin (Gibco, Long Island, NY) as previously reported.³¹ Granta-519 cell line was grown in Dulbecco's modified Eagle's medium instead of RPMI (Cellgro). Peripheral blood samples from five previously untreated patients with leukemic disease, who were treated on clinical studies at the NCI were collected before (0 hours), at 6 and 24 hours after the first and at 24 hours after the second injection of bortezomib (1.5 mg/m², days 1, 4, 8 and 11). Written informed consent for all research collections was obtained. Diagnosis was confirmed by flow cytometry and all cases had t(11;14) translocation. Clinical response to bortezomib treatment was determined by the decrease in absolute lymphocyte count. Peripheral blood mononuclear cells were separated by gradient centrifugation using Lymphocyte Separation Medium (MP Biomedicals, Solon, OH), and CD19+ cells (>99% tumor cells, <1% normal B-cells) were purified by magnetic activated cell sorting using CD19 microbeads (Miltenyi, Auburn, CA). CD19⁺ cells were aliquoted and stored as pellets at -80 °C.

Gene expression profiling

Total RNA of CD19-selected tumor cells was isolated using Qiagen RNA isolation kit (Invitrogen, Carlsbad, CA) and 1 µg was used for cRNA amplification according to manufacturer's instructions. RNA yield and integrity of biotin-labeled cRNA were determined using the Agilent 2100 bioanalyzer (Agilent Technologies). For gene expression profiling, 20 µg of labeled RNA was fragmented and hybridized to Affymetrix Human Genome U133_2 chips (Affymetrix, Santa Clara, CA) for 16 hours, washed and stained on an Affymetrix fluidics station. Affymetrix GeneChip operating software (v1.4) was used to calculate signal intensity and present calls. Signal intensity values were transformed with an adaptive variance-stabilizing, quantile-normalizing transformation logarithm referred to as S10 (Symmetric Adapted transformed, base 10) available at <http://abs.cit.nih.gov/MSCLtoolbox/> (MSCL Analyst's toolbox software, Peter J. Munson, GeneLogic Workshop of Low Level Analysis of Affymetrix GeneChip Data, 2001).

Data analysis

Gene set enrichment analysis (v2.0) was performed using curated (C2) and motif (C3) gene sets from the Molecular Signature Database (v2.5; Broad Institute, Cambridge, MA). Gene sets were identified using a false discovery rate (FDR) of <0.1 (except for analysis of curated gene sets on MCL patients where a FDR of <0.2 was used), and a normalized enrichment score (NES) of >1.8 or <-1.8. Gene sets were ranked by NES. The leading edge genes, i.e. the genes that contribute most to the identification of the corresponding gene set, were averaged to a score that was used as a comparative measure of expression. Ingenuity Pathway Analysis was performed using the Benjamini-Hochberg method for multiple testing correction and a cutoff of FDR<0.05 was used (Ingenuity Systems®). Probesets identified were collapsed to genes by maximum expression value. Signaling pathways identified must contain at least three genes. Gene expression data were visualized using Cluster (v2.11) and TreeView (v1.6) (Eisen laboratory, Stanford, CA). Statistical significance was assessed by Mann-Whitney non-parametric U test using Prism 4 software (v4, GraphPad Software Inc, La Jolla, CA) or Student's T-test as indicated in the figure legends.

Protein extraction and Immunoblot analysis

Preparation of whole cell extracts was performed as described previously.³¹ Briefly, pelleted cells were resuspended in lysis buffer (50 mM Tris-HCl (pH 7.4), 1% Triton, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM Na₃VO₄ and a cocktail of protease and phosphatase inhibitors (Complete and PhosphoSTOP, Roche Diagnostics, Mannheim, Germany; Phosphatase inhibitor cocktail I, Sigma (Saint Louis, MO)) and vortexed. Cytoplasmic and nuclear protein extracts were prepared using a nuclear/cytosol fractionation kit (BioVision, Mountain View, CA) following the manufacturer's instructions with minor modifications. The cytosol extraction buffer was supplemented with protease and phosphatase inhibitors as described above. Proteins were quantified using the Bradford method (Bradford reagent; Bio-Rad, Hercules, CA). Proteins were separated using NuPAGE Bis-Tris gels (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes (iBlot Gel Transfer Stacks; Invitrogen) using the iBlot dry blotting system (Invitrogen). Following immunodetection with indicated primary and corresponding secondary antibodies, membranes were incubated with chemiluminescence horseradish peroxidase substrate (Millipore Corporation, Billerica, MA) and images were acquired with the Fujifilm LAS-4000 image reader (Fujifilm life science, Tokyo, Japan) and Multi Gauge V3.0 software (Fujifilm Life Science). Immunoblots were stained using antibodies to ATF3, ATF4 and NRF2 (Santa Cruz Biotechnology, Santa Cruz, CA), CHOP, TBP and MAFG (Abcam, Cambridge, MA), NOXA (Calbiochem, San Diego, CA) and p97 (Fitzgerald Industries International, Concord, MA). Horseradish peroxidase linked anti-mouse and anti-rabbit secondary antibodies were purchased from Amersham Biosciences (Little Chalfont, England).

Polymerase chain reaction (PCR) for detection of XBP1 splicing

PCR analysis was performed to detect splicing of XBP1 using the following primers: forward 5'-TTACGAGAGAAAACATCATGGC-3' and reverse 5'-GGGTCCAAGTTGTCCAGAAATGC-3'.³² GAPDH primers were as follows: forward 5'-CCTGTTTCGACAGTCAGCCG-3' and reverse 5'-CGACCAAATCCGTTGACTCC-3'. 30 cycles were performed at 95 °C for 45 sec, 58 °C for 30 sec and 72 °C for 20 sec. PCR products were separated using a 2.5% agarose gel and imaged using ChemiImager 5500 (Alpha Innotech Cell Biosciences, Santa Clara, CA) and AlphaEase Software (v3.2.2).

Cell survival (MTT) assay

5×10⁴ cells per well were exposed to serial doubling concentrations of hydrogen peroxide (Sigma) for 24 hours in 96-well plates. 0.5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) reagent (Chemicon, Temecula, CA) was added 4 hours before the reaction was terminated by the addition of 0.01M HCl, 10% SDS. Absorbance was measured at the wavelengths of 570 nm (test) and 650 nm (reference) after overnight incubation.

Flow cytometry

To detect cell viability by flow cytometry, MitoTracker Green FM and MitoTracker Red CMX Ros were used (Invitrogen). Briefly, cells were incubated in media with 100 nM of each probe for 30 min at 37 °C, and 10,000 cells per tube were analyzed for data acquisition. To detect changes in mitochondrial transmembrane potential cells were stained with 20 nM 3,3-diethyloxycarbocyanine iodide (DiOC6[3]; Invitrogen) and the generation of reactive oxygen species was determined by staining cells with 2 μM dihydroethidine (Invitrogen) as described elsewhere.²²

Results

Bortezomib induces a transcriptional response in MCL cell lines

We have previously characterized mantle cell lymphoma (MCL) cell lines in regards to bortezomib sensitivity.³¹ A cutoff of 10 nM bortezomib was established to divide MCL cell lines into sensitive and resistant groups. Baseline proteasome activity and inhibition of the chymotrypsin-like activity by bortezomib was comparable between the two groups.³¹ As it is now well established that proteasome inhibition triggers a complex cellular response activating both pro- and anti-apoptotic pathways, we hypothesized that differences in the cellular stress response to proteasome inhibition could explain differential drug sensitivity. Specifically, we wished to test whether induction of a protective response could give rise to bortezomib resistance. To this end, we used gene expression profiling to characterize the stress response induced by bortezomib in MCL cells. First, we determined the kinetics of bortezomib-induced apoptosis in two representative sensitive (HBL-2 and Jeko-1) and two resistant (Mino and REC-1) MCL cell lines. Exposure to 10 nM bortezomib for less than 6 hours induced very little cell death even in sensitive cells and failed to induce any cell death in resistant cells up to 8 hours (Figure 1A). We next analyzed the transcriptional response to proteasome inhibition after short (3 and 6 hours) and long drug exposure (24 hours). Only few genes showed significant changes (at least 2-fold change, FDR <0.1) in expression at the early time points but at 24 hours widespread changes were present especially in the sensitive cell lines (Figure 1B). Expression of 553 genes changed significantly in sensitive and of 129 genes in resistant cell lines, respectively, including 73 genes that changed in both groups. Of 480 genes that changed only in the sensitive group, 236 were increased and 244 decreased. Of 56 genes that changed only in the resistant cell lines, 42 were increased and

14 decreased. Of 73 genes that changed in both groups, 61 genes were increased and 12 decreased (Figure 1C).

To better understand the biologic basis of this response we submitted all genes that changed significantly in response to bortezomib to Ingenuity Pathway Analysis. In the sensitive cell lines we identified 3 pathways induced by bortezomib, most prominently the protein ubiquitination and the NRF2-mediated oxidative stress response pathways ($P<0.0001$; Figure 1D). Twelve signaling pathways were found to be downregulated, most prominently the antigen presentation pathway ($P<0.0001$). Contrary to our initial hypothesis, no distinct pathways were identified among genes that changed in the resistant cell lines. Thus, bortezomib induced a transcriptional response involving protein ubiquitination and oxidative stress response pathways that was activated only in sensitive cells.

Bortezomib induces oxidative and ER-stress response pathways in vitro

To further dissect the transcriptional response we performed gene set enrichment analysis on bortezomib-induced gene expression changes (Supplemental Table 1 and 2). Several gene sets controlled by specific transcription factors were strongly upregulated in sensitive but less so in resistant cell lines (Figure 2A). Biologic functions controlled by these transcription factors involve the response to oxidative stress (NRF2, NFE2, TCF11_MAFG, and BACH2), the UPR (NRF2 and XBP1), and heat shock (HSF and HSF1). The only gene sets significantly downregulated by bortezomib relate to cell cycle and are controlled by E2F transcription factors (Supplemental Table 1).

Next we interrogated bortezomib-induced gene expression changes relative to the activity of signaling pathways and the effects of chemical and genetic perturbations on cell biology. We again identified gene sets indicating an oxidative stress response including genes upregulated by H_2O_2 in breast cancer cell lines and genes in the ARE-NRF2 pathway. We also observed downregulation of genes whose expression is known to be suppressed by oxidizing agents (Figure 2B and Supplemental Table 2). In addition, two gene sets of the proteasome pathway were upregulated. Thus, this independent analysis using gene set enrichment analysis confirmed the key pathways identified by Ingenuity Pathway Analysis. Most gene sets linked to cell proliferation were downregulated in good agreement with the reduced expression of E2F-controlled genes (Supplemental Table 2) and reflects decreased proliferation of bortezomib-treated cells in agreement with reports that it can induce cell cycle arrest.^{33,34} Changes in expression of these gene sets were again more striking in sensitive than in resistant cell lines (Figure 2B). In the oxidative stress pathway the transcription factor NRF2 regulates numerous detoxifying and antioxidant genes.³⁵ Li and colleagues established a specific NRF2 signature in a genetic mouse model.³⁶ To compare the NRF2 response between different cell lines we derived an expression average of the NRF2 signature genes, which increased 15-fold in response to bortezomib in sensitive but only 2-fold in resistant cell lines ($P<0.0063$; Figure 2C and D).

The transcriptional changes in MCL cell lines indicated engagement of both ER and oxidative stress pathways in response to proteasome inhibition. Consistent with UPR activation primarily in sensitive cells, we found bortezomib-induced IRE1 phosphorylation and XBP1 splicing in Jeko-1, but not in Mino cells (Figure 3A). Activation of a second UPR pathway controlled by PERK upregulates the transcription factors ATF4, ATF3, and CHOP. In keeping with their ability to promote apoptosis, nuclear accumulation of these proteins correlated with bortezomib-induced apoptosis (Figure 3B). Next we measured production of reactive oxygen species (ROS) in response to bortezomib in two sensitive and two resistant cell lines. Bortezomib caused a striking increase in ROS production as measured with dihydroethidium in the sensitive cell lines. The increase in ROS was paralleled by a decrease in mitochondrial membrane potential indicating cell death. In contrast, resistant cell lines

showed only minimal increase in ROS and little or no cell death (Figure 3C). The time-dependent increase in ROS production and its correlation with bortezomib sensitivity are in agreement with the strong induction of an NRF2 signature in sensitive cell lines. Finally, to directly assess the response to oxidative stress these four cell lines were treated with hydrogen peroxide and viability was analyzed by MTT assay. Again, bortezomib resistant cell lines were less affected by oxidative stress than sensitive cell lines (Figure 3D).

Bortezomib-induced transcriptional response in vivo

We wished to assess whether a stress response similar to that what we have defined in our model in vitro is also involved in the antitumor activity of bortezomib in MCL patients. We were able to study five patients with leukemic MCL during their first cycle of bortezomib single-agent therapy (1.5 mg/m², days 1, 4, 8, and 11). Patients MCL1-4 continued treatment with six cycles of combination chemotherapy starting on day 21. Patient MCL5 could not continue treatment due to intercurrent illness. Patients MCL1, MCL3 and MCL4 had classic and MCL2 and MCL5 blastoid morphology (full patient characteristics in supplemental Table 5). Two patients, MCL1 and MCL3 achieved a rapid and near complete reduction of circulating tumor cells in response to one cycle of single-agent bortezomib, whereas MCL2 showed no response at all (Figure 4A). The other two patients had an intermediate response with a delayed onset in MCL4, and a moderate treatment effect in MCL5. Tumor cells obtained pre-treatment and on days 2 and 5, corresponding to 24 hours and 96 hours after the first bortezomib injection, respectively, were analyzed by gene expression profiling. First, we identified 2004 probesets representing 1606 genes that showed at least a 2-fold change on days 2 or 5 compared to pre-treatment in at least one of the five patients. Unsupervised hierarchical clustering grouped samples in good accordance with the therapeutic effect of bortezomib (Figure 4B).

For subsequent analyses we considered the two groups of samples identified by hierarchical clustering separately. Between both groups a total of 246 genes showed at least a 2-fold change, 240 of these were unique to MCL1 and MCL3, five were only identified in the MCL2, MCL4, MCL5 group, and one gene was found in both (Figure 4B). By Ingenuity Pathway Analysis, genes upregulated by bortezomib indicated activation of protein ubiquitination and NRF2-mediated oxidative stress response pathways ($P < 0.001$; Figure 4C). Thus, in accordance with observations in cell lines, induction of a strong transcriptional response correlated with the antitumor effect of bortezomib and the accumulation of the BH3-only protein NOXA in circulating tumor cells of patients MCL1 and MCL3 (Figure 4D).

Upregulation of proteasome and oxidative stress pathways in vivo correlates with clinical response

We used gene set enrichment analysis to further dissect the gene expression changes in primary tumor samples during bortezomib therapy in vivo. The top three gene sets induced by bortezomib comprised the oxidative stress-related genes controlled by transcription factors TCF11_MAFG, NRF2, and NFE2 that we have previously identified to be upregulated in sensitive MCL cell lines (Figure 5A and Supplemental Table 3). Likewise, we found overrepresentation of functional gene sets indicating engagement of oxidative stress and proteasome pathways (Figure 5A and Supplemental Table 4). Next we analyzed changes in expression of proteasome subunits. Most subunits were strongly induced in MCL1 and MCL3, while PSMB8-10 subunits encoding components of the immunoproteasome were slightly decreased (Figure 5B). A similar change in the expression of proteasome subunits was also seen in cell lines, but differences between sensitive and resistant cells were less striking (data not shown). In accordance with gene expression changes, bortezomib induced protein expression of the $\beta 5$ subunit (PSMB5) only in

circulating tumor cells of these two patients (Figure 5B). In contrast to the cell lines, XBP1 and ATF6 gene sets were not significantly enriched in primary tumor samples. Consistently, we did not detect XBP1 splicing in primary tumors (data not shown).

Strong induction of NRF2 target gene expression in MCL1 and MCL3 but not in the other patients indicated a correlation between oxidative stress and clinical activity of bortezomib (Figure 5C). Consistently, NRF2 protein increased in circulating tumor cells of patients with excellent clinical response, but not in the other patients (Figure 5D). Finally, we aimed to identify genes differentially expressed between sensitive and resistant tumors at baseline. Twenty-six genes were more highly expressed in MCL1 and MCL3 compared to 75 genes whose expression was higher in MCL2, MCL4, and MCL5 (Figure 6A). Among the 75 genes more highly expressed in resistant tumors, NRF2-controlled antioxidant genes and genes of the ubiquitin proteasome pathway were significantly overrepresented (FDR<0.01 by Ingenuity Pathway Analysis). Comparison of individual samples by Student's t-test confirmed overexpression of the NRF2 signature average and of proteasome subunits in tumors with inferior clinical response (Figure 6B and C).

Discussion

Bortezomib inhibits the proteasome, a universal pathway essential for all cells. To improve therapeutic strategies it is crucial to understand the underlying mechanisms for its selective cytotoxicity against tumor cells. Here we used gene expression profiling to characterize the stress response of MCL cells during bortezomib treatment in vitro and in vivo. We discovered a prominent transcriptional response indicating activation of the UPR and oxidative stress pathways. Unexpectedly, genes encoding detoxifying and antioxidant molecules were strongly upregulated in sensitive but not in resistant cells. Contrary to our expectation we could not detect a reactive homeostatic response in resistant cells that might enable these cells to tolerate proteasome inhibition. However, we discovered that high baseline expression of NRF2 target genes in untreated cells correlated with drug resistance in vitro and in vivo. Thus, our data implicate high cellular antioxidant capacity as a mechanism of bortezomib resistance. In contrast, acute upregulation of the antioxidant response in sensitive cells was not sufficient to restore homeostasis. The strong reactive upregulation of NRF2 target genes then appears as a correlate of an overwhelming cytotoxic insult. However, we cannot rule out that under certain circumstances NRF2 may contribute to the induction of apoptosis. ATF4 that can activate antioxidant genes as well as pro-apoptotic mechanisms serves as an example of such a dual role.^{15,37}

The BH3-only protein NOXA is induced in response to bortezomib in many cell types in vitro. Here, we show for the first time NOXA upregulation in primary tumor cells in MCL patients undergoing treatment with bortezomib. In keeping with its function as a terminal effector of bortezomib cytotoxicity,^{22,31} NOXA was upregulated only in cells that also demonstrated a stress response by gene expression profiling. While originally described as a p53 target gene, p53 is not necessary for NOXA induction in response to proteasome inhibition.²² Recently, Wang and colleagues demonstrated an alternative pathway of NOXA activation involving epigenetic modification of the promoter combined with transcriptional activation by ATF3 and ATF4 dimerization.¹⁵ As predicted by these studies, we found ATF3 and ATF4 upregulation in sensitive but not in resistant cells. Whereas ATF3 mRNA increased as part of the bortezomib-induced transcriptional response, ATF4 mRNA was largely unchanged, in keeping with its regulation through a translational mechanism downstream of PERK.¹⁷

For many cell types disruption of protein and ER homeostasis has been linked to induction of the UPR and bortezomib-induced cytotoxicity in vitro.¹⁸ This effect may be particularly

important in secretory cells and has been proposed as an explanation for the high sensitivity of plasma cells to proteasome inhibition.^{4,14} In vitro we observed bortezomib-induced upregulation of ATF6, XBP1, and NRF2 gene signatures indicating activation of all three arms of the UPR. This was confirmed by the demonstration of IRE1 phosphorylation, XBP1 splicing, and increased protein levels of ATF4 in bortezomib-treated cell lines. In contrast, in vivo we observed primarily an NRF2 gene expression response and no activation of the IRE1/XBP1 arm of the UPR. Thus, while bortezomib induces both UPR and oxidative stress responses in vitro, our results suggest that in vivo oxidative stress may dominate, at least in some settings. Given that UPR and oxidative stress response are modulated by several cellular factors including biosynthetic load, amino acid starvation, and redox homeostasis,³⁸ all of which can differ significantly between in vitro and in vivo environments, such differences are certainly plausible. It is also notable that proteasome inhibition with bortezomib consistently induces NOXA but not BIM,^{22,31} whereas the apoptotic response of the UPR in response to classic pharmacologic or pathophysiologic ER stressors has been associated with BIM upregulation.²¹ Taken together, these data indicate that bortezomib-induced cytotoxicity differs in several aspects from classic UPR-associated mechanisms.

We found a prominent antioxidant response in response to bortezomib both in vitro and in vivo. Oxidative stress is closely linked with ER stress. On one hand, ROS induce protein modifications that may lead to disruption of protein homeostasis and increased demands on protein degradation, on the other hand protein folding is one of the major sources of intracellular ROS.^{19,25} ER and oxidative stress also overlap in the activation of NRF2; ROS directly inactivate KEAP1, which binds and sequesters NRF2 in the cytoplasm, while PERK-mediated phosphorylation of NRF2 inhibits KEAP1 binding.³⁷ Inhibition of KEAP1 binding stabilizes NRF2 and allows its translocation to the nucleus, where it regulates genes involved in detoxification, redox homeostasis, and proteome maintenance. NRF2 thereby promotes cellular homeostasis in the face of oxidative and ER stress. In vitro, bortezomib increased ROS levels significantly in sensitive cells, but only minimally in resistant cells. We were unable to show a bortezomib-dependent increase in ROS in primary tumor cells because fresh cells were not prospectively analyzed for ROS and the freeze-thaw process in DMSO interfered with the analysis. However, we detected increased NRF2 protein expression in tumor cells isolated directly from patients.

In cancer biology NRF2 has many at times even contradictory functions.^{39,40} NRF2 can protect cells from the carcinogenic effects of ROS. Natural compounds that increase NRF2 are therefore being studied as cancer preventing agents. On the other hand, high NRF2 expression is associated with more aggressive tumors, higher proliferation rate, and even resistance to chemotherapy. Here, we describe that high baseline activity of NRF2 in MCL cell lines as well as in primary tumors cell is associated with bortezomib resistance. In support of a protective role of NRF2 against bortezomib-induced cytotoxicity a genome wide RNAi screen in colon cancer identified knockdown of NRF2 as a synthetic lethal hit.⁴¹ NRF2 expression has also been linked with in vitro sensitivity to other chemotherapeutic agents in solid tumor cell lines.^{42,43} Whether treatment resistance is due to the overall enhanced ability of cells to deal with toxic insults or one distinct NRF2 target gene is undefined. While NRF2 upregulates proteasome subunits,⁴⁴ we have previously shown that proteasome capacity and the degree of inhibition of the chymotrypsin-like activity by bortezomib does not differ between sensitive and resistant cells.³¹ The basis for increased NRF2 activity in a subset of MCL is unclear but could reflect differences in cellular differentiation. We recently reported that partial plasma cell differentiation of MCL cells is associated with bortezomib resistance.⁴⁵ Given that increased expression of genes involved in protein folding and redox homeostasis are critical elements of plasma cell differentiation,^{19,25,46} this could explain an increased ability to cope with oxidative stress and account for reduced sensitivity to bortezomib.

This study is limited to the analysis of patients with leukemic disease supported by analysis of cell line models. Our findings thus await confirmation in nodal disease, and in a larger group of patients. Nevertheless, we have made several novel observations that direct further studies. The correlation of increased cellular antioxidant capacity with bortezomib resistance could yield predictive markers of treatment success and identifies avenues for synergistic treatment approaches. The relative contribution of NRF2 to bortezomib resistance, a possible proapoptotic role, and the mechanism of its upregulation in a subset of MCL require further study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Relevance

Mantle cell lymphoma is incurable with conventional chemotherapy. Bortezomib, the first proteasome inhibitor approved for the treatment of hematologic malignancies, is active in up to 50% of patients with otherwise refractory disease. The molecular mechanism of selective anti-tumor activity of bortezomib and determinants of resistance to proteasome inhibition are ill-defined. Gene expression profiling has become a powerful method to dissect disease heterogeneity. We used gene expression profiling to investigate the cellular response to proteasome inhibition in vitro and in vivo. Our data indicate that induction of oxidative stress is key to bortezomib-induced apoptosis and clinical response. Moreover, we find evidence that high basal antioxidant capacity is associated with reduced responsiveness and inferior clinical outcome to bortezomib treatment. The correlation of cellular antioxidant capacity with bortezomib resistance could yield predictive markers of treatment success and identifies avenues for synergistic treatment approaches.

Figure 1. Bortezomib induces a transcriptional stress response in MCL cells

(A) Apoptosis was measured after exposure to 10 nM bortezomib in sensitive (HBL-2 and Jeko-1) and resistant cell lines (Mino and REC-1). After the indicated times of drug exposure cells were washed and re-plated in drug-free medium. Cell viability was assessed at 24 hours by flow cytometry using mitotracker probes. (B) Number of genes showing >2-fold change in expression after bortezomib exposure compared to untreated control (FDR < 0.1). (C) Heatmap of genes regulated by bortezomib at 24 hours. Cell lines: G, Granta-519; H, HBL-2; J1, Jeko-1; J2, JVM-2; S, SP-53; U, UPN-1; Z, Z-138; M, Mino; N, NCEB-1; R, REC-1 are arranged in columns, genes in rows, the relative expression is color coded according to the scale shown. Yellow bar indicates genes regulated only in sensitive cell lines; black bar, genes regulated only in resistant cell lines; green bar, genes regulated in both groups. (D) Ingenuity Pathway Analysis of gene expression changes after 24 hours of drug exposure (FDR < 0.01).

Figure 2. Gene set enrichment analysis identifies an integrated stress response in sensitive MCL cell lines

Gene sets regulated in bortezomib-treated cells (Supplemental Table 1 and 2) at 24 hours are summarized. (A, B) The average fold change mRNA expression of the most significantly enriched genes for each set (leading edge genes) are displayed: (A) motif-based gene sets, (B) curated gene sets. Asterisk indicates the p-value of the comparison between sensitive and resistant cell lines (Student's t-test). (C) Heatmap of the fold change mRNA expression of experimentally validated NRF2 signature genes³⁶ ranked by the average fold change in sensitive cell lines. Cell lines: G, Granta-519; H, HBL-2; J1, Jeko-1; J2, JVM-2; S, SP-53; U, UPN-1; Z, Z-138; M, Mino; N, NCEB-1; R, REC-1. (D) The average fold change mRNA expression of the NRF2 signature at 24 hours is shown (P=0.0063, Mann Whitney U-test).

Figure 3. Bortezomib induces endoplasmic reticulum and oxidative stress responses in sensitive but not resistant MCL cell lines

(A) Jeko-1 and Mino cells were treated with 10 nM bortezomib (B) or 2 μ M thapsigargin (T) as positive control. Activation of IRE1 by phosphorylation (p-IRE1) was assessed by immunoblot and splicing of XBP1 mRNA by RTPCR. PCR products of unspliced (XBP1u) and spliced (XBP1s) are indicated. A higher migrating band represents a hybrid amplicon.⁴⁷ Cell viability determined by flow cytometry using mitotracker probes is shown. (B) Induction of ER stress-related transcription factors ATF3, ATF4 and CHOP was analyzed in nuclear protein extracts by immunoblotting. TATA box binding protein (TBP) and p97 were used as loading controls in the immunoblots. (C) The generation of ROS was measured using dihydroethidium (DHE+; gray bars) and loss of mitochondrial membrane potential ($\Delta\Phi_m$; black bars) indicative of cell death was determined using 3,3'-dihexyloxycarbocyanine iodide (DiOC6[3]) by flow cytometry. (D) Cell viability by MTT assay after 24 hours exposure to increasing concentrations of hydrogen peroxide (H_2O_2). Mean and standard deviation of a representative experiment using triplicates is shown.

Figure 4. Upregulation of the NRF2-mediated oxidative stress response and NOXA protein in tumor cells from patients with leukemic MCL correlate with clinical response to bortezomib (A) Absolute lymphocyte (ALC) and absolute neutrophil counts (ANC) in MCL patients treated with single-agent bortezomib injected on days 1, 4, 8, and 11. (B) Unsupervised hierarchical clustering analysis of all genes, having at least a 2-fold change in expression during treatment in at least one patient, identified two separate clusters of patients. Bortezomib responsive genes showing an absolute change of at least 2-fold during treatment ($P < 0.01$ compared to pre-treatment). Genes are ranked by the average fold change in MCL1 and MCL3. Yellow bar indicates genes significantly changed in MCL1 and MCL3 only; black bar, genes identified in MCL2, MCL4 and MCL5; green bar, genes identified across all samples. (C) Ingenuity Pathway Analysis of bortezomib responsive genes. Pathways identified with $\text{FDR} < 0.05$ are shown. (D) Immunoblot of NOXA induction in CD19-selected tumor cells before and after 96 hours of bortezomib treatment in vivo. p97 was used as loading control.

Figure 5. Bortezomib induces an NRF2 signature and NRF2 protein in tumor cells from leukemic MCL

Gene sets regulated by bortezomib (Supplemental Table 3 and 4) in tumor cells in vivo at 96 hours are summarized. (A) The average fold changes in expression of the most significantly enriched genes for each gene set (leading edge genes) are shown: (left) motif-based gene sets, (right) curated gene sets. Asterisk indicates p-value of the comparison between the two groups of patients (Student's t-test). (B) Heatmap of fold change mRNA expression of proteasome subunits in response to bortezomib at 96 hours. Immunoblot of β 5 subunit (PSMB5) protein expression in CD19-selected tumor cells. p97 was used as loading control. (C) Heatmap of the fold change expression of experimentally validated NRF2 signature genes³⁶ ranked by the average fold change in MCL1 and MCL3 at 96 hours of bortezomib treatment in vivo. (D) The averaged fold change mRNA expression of the NRF2 signature genes is shown ($P=0.034$, Student's t-test). Immunoblot of NRF2 protein expression in CD19-selected tumor cells before and 96 hours of bortezomib treatment in vivo. p97 was used as loading control.

Figure 6. Basal expression of NRF2 signature antioxidant genes in primary MCL tumor cells determine clinical response to bortezomib

(A) Heatmap of probesets differentially expressed between MCL1 and MCL3 and the patients MCL2, MCL4 and MCL5 (>1.3-fold difference). Genes in the NRF2 signature (asterisk) and proteasome subunits are indicated. The averaged mRNA expression of NRF2 signature genes (B) and of all proteasome subunits (C) was computed for each tumor sample at baseline and compared by Student's t-test.