Response of myeloma to the proteasome inhibitor bortezomib is correlated with the unfolded protein response regulator XBP-1

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ABSTRACT

Background
Multiple myeloma, a malignancy of the antibody-secreting plasma cells, remains incurable by current therapy. However, the proteasome inhibitor bortezomib and other new drugs are revolutionizing its treatment. It remains unclear why myelomas are peculiarly sensitive to bortezomib, or what causes primary or acquired resistance. The 'unfolded protein response' is necessary for folding and assembly of immunoglobulin chains in both normal and malignant plasma cells, as well as for the disposal of incorrectly folded or unpaired chains via the ubiquitin-proteasome pathway. We tested the hypothesis that levels of transcription factor *XBP-1*, a major regulator of the unfolded protein response, predict response to bortezomib.

Design and Methods
Expression of *XBP-1* and other regulators of the unfolded protein response were measured in myeloma and other cancer cell lines and two cohorts of patients with refractory myeloma and correlated with sensitivity/response to bortezomib. Bortezomib-resistant myeloma cell lines were derived and the effects on expression of unfolded protein response regulators, immunoglobulin secretion, proteasome activity and cross-resistance to cytotoxic drugs and tunicamycin determined. The consequences of manipulation of *XBP-1* levels for sensitivity to bortezomib were tested.

Results
Low *XBP-1* levels predicted poor response to bortezomib, both *in vitro* and in myeloma patients. Moreover, myeloma cell lines selected for resistance to bortezomib had down-regulated *XBP-1* and immunoglobulin secretion. Expression of ATF6, another regulator of the unfolded protein response, also correlated with bortezomib sensitivity. Direct manipulation of XBP-1 levels had only modest effects on sensitivity to bortezomib, suggesting it is a surrogate marker of response to bortezomib rather than a target itself.

Conclusions
The unfolded protein response may be a relevant target pathway for proteasome inhibitors in the treatment of myeloma and its regulator XBP-1 is a potential response marker. *(The BIR study was registered with Australian Clinical Trial Registry Number 12605000770662)*

Key words: myeloma, bortezomib, unfolded protein response, XBP-1.

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The online version of this article has a Supplementary Appendix.

Introduction

Even with autologous stem cell transplantation, the percentage of long-term survivors of myeloma is currently only 35-40%.[1] The median survival time is 4 to 5 years.[2-4] The relapse rate is high and relapsed myeloma is particularly resistant to conventional chemotherapy. Proteasome inhibitors are a new class of drugs which is particularly effective in myeloma compared with in most other cancers. Bortezomib was the first of the class approved for clinical use. In phase II/III clinical trials, 35-50% of relapsed and refractory myelomas were sensitive to bortezomib.[5-8] The peculiar sensitivity of myeloma to this drug is not well understood, but the unfolded protein response (UPR) is a convincing target.[9] Bortezomib is a specific and reversible inhibitor of chymotryptic activity of the 26S proteasome, the major cellular pathway of protein degradation and, as a consequence, its biological effects are numerous. Attention to its anticancer action has focused on nuclear factor-κB, p53, cyclin-dependent kinase inhibitors p21 and p27, apoptotic pathways, growth factor signaling, the interaction of myeloma cells and bone marrow stroma, angiogenesis, and stress responses.[10-14] However, none of these effects has so far proven to be related to response to the drug and the mechanism by which bortezomb kills myeloma cells has remained elusive. Similarly, the causes of primary and acquired resistance to the drug are unknown.

Plasma cells, the normal counterparts of myeloma, are specialized, terminally differentiated secretory B lymphocytes capable of prodigious antigen-specific immunoglobulin production. The UPR is essential for the folding of both heavy and light immunoglobulin chains and their assembly.[15-17] The UPR is also required for disposal of irreversibly misfolded polypeptides via the ubiquitin-proteasome pathway.[18] Proteasome inhibition increases the accumulation of misfolded proteins and hence invokes the UPR at the same time as disrupting it. It is, therefore, possible that dependence on the UPR renders myeloma sensitive to proteasome inhibitors. There is some *in vitro* evidence that sensitivity of myeloma cell lines to bortezomib is related to a high level of immunoglobulin production,[9] although serum immunoglobulin levels have not predicted response in clinical trials.

The transcription factor *XBP-1* is a major regulator of the UPR, is expressed at high levels in myelomas compared with in other cancers and is indispensable for plasma cell development.[19-22] *Xbp-1*-deficient mice lack plasma cells and have impaired immunoglobulin production.[21] Myeloma cell lines in which *XBP-1* expression was knocked down had higher apoptotic indices and reduced survival.[23] Active XBP-1 is generated by unconventional extra-nuclear splicing of its mRNA by endoribonuclease IRE1, in response to exposed hydrophobic moieties on misfolded or unfolded proteins in the endoplasmic reticulum. Spliced *XBP-1* mRNA encodes an active transcription factor for downstream stress response genes including *ERDJ3, ERDj4, p58^{IPK}, EDEM, HEDJ, EDEM, RAMP4* and *protein disulfide isomerase-P5*.[24,25] Unspliced *XBP-1* mRNA encodes an inactive or dominant negative protein lacking the transactivation domain.

In this study, we related *XBP-1* expression to primary sensitivity or resistance of myeloma to bortezomib both *in vitro* and in patients, and with acquired resistance to bortezomib *in vitro*.

Design and Methods

XBP-1 *assays*

Spliced and unspliced *XBP-1* mRNA differ by a 26-bp intron homologous to adjacent sequences, complicating the use of specific primers or Taqman probes to distinguish the two forms directly. Hence, total *XBP-1* cDNA was amplified with primers spanning the intron; the relative abundance of the two forms of the mRNA was determined by quantification of the respective polymerase chain reaction (PCR) products.

Total RNA was extracted from myeloma cells using isophasic guanidine isothiocyanate:phenol (Tri Reagent, MRC) and treated with DNase I (Ambion). RNA quality was checked on a Bioanalyzer 2100 (Agilent) and RNA quantified by fluorescence (Ribogreen, Invitrogen). First strand cDNA synthesis was performed with 1 μg RNA from myeloma cell lines or 1-10 ng RNA from patients' myeloma cells with SuperScript III™ (Invitrogen) and mixed oligo dT and random hexamer primers. Duplicate cDNA were prepared for each sample. Two quantitative real-time PCR reactions were performed for each of the cDNA, yielding four data points per sample. A Stratagene MX3000P instrument was used with the following cycling conditions: initial denaturation and activation of the polymerase at 94°C for 8 min, followed by 35 cycles of 30 s at 94°C, 64°C and 72°C, and 20 s at 85°C. The PCR reaction volume was 50 μL, consisting of 1.25 units AmpliTaq Gold Polymerase, 0.2 mM of each dNTP, 50 mM KCl, 10 mM Tris HCl pH 8.3, 2.5 mM $MgCl_2$, 140 nM of each primer, 3% dimethyl sulfoxide (DMSO) and Sybr I Green 1:25,000 (Invitrogen). *XBP-1* primers were: forward 5'-GGAGTTAAGACAGCGCTTGG-3' and reverse 5'-GTCAATACCGCCAGAATCC-3', at positions 461 and 613 respectively of GenBank sequence NM_005080. They span the intron and amplify spliced and unspliced *XBP-1* mRNA with similar efficiencies (~100%). Acquisition of fluorescence was at 85°C, at which any primer dimers were denatured. *XBP-1* mRNA levels were normalized to the level of *β-actin* mRNA as this had the least variable expression in human myeloma cell lines compared with other housekeeping genes tested (*BCR, RPL5a, α-tubulin, 18S rRNA and ubiquitin*) and its level of expression was closest to that of *XBP-1*. β-actin primers were: forward 5'-ACCAACTGGGACGACATGGAGAAAA-3' and reverse 5'-CGCACGATTTCCCGCTCGGC-3'; amplification parameters as for *XBP-1*. Copy numbers of both genes were derived from standard curves based on plasmid-cloned templates diluted to known concentrations. PCR products were sequenced and the assay validated by northern analysis of myeloma cell lines. *GAPDH* was used as the housekeeping gene for analysis of cell lines that had been treated with bortezomib, because bortezomib reduced *β-Actin* mRNA expression. *β-Actin* was used as the housekeeping gene for the clinical samples because biopsies were taken prior to bortezomib treatment.

The ratio of spliced:unspliced XBP-1 PCR products was determined using a separate PCR analysis carried out under identical conditions but with individual reactions stopped in log phase at a fluorescence threshold of 30,000 on the Stratagene MX3000P, to allow for different starting quantities of template. The PCR products were quantified by microelectrophoresis on an Agilent Bioanalyzer 2100P. There was a small, systematic underestimation of the spliced fraction, which was corrected by reference to a standard curve derived from known ratios of spliced:unspliced *XBP-1* plasmid templates amplified in parallel.

Cell lines and cell culture

Human cell lines used in this study were the myelomas RPMI-8226, KMS-11, KMS-18, OPM2, H929 and U266, the lymphoblastoid CCRF-CEM, Jurkat & WL2,[26] the Burkitt lymphomas

Ambodi, Elijah, Ag879, Puy, Ramos and WW-1-BL, prostate carcinomas PC3, DU145 and LNCaP, lung adenocarcinoma A549, neuroblastoma SHEP, colon carcinoma Widr, cervical cancer HeLa, ovarian carcinoma 2008 and HEK293 (embryonic kidney cells). Myeloma, lymphoid and prostate cell lines were cultured in RPMI-1640 (Gibco) with 10% fetal calf serum, 50 μM 2-mercaptoethanol, and penicillin and streptomycin (complete medium). The other cell lines were cultured in Dulbecco's modified Eagle's medium plus 10% supplemented calf serum (Hyclone) and antibiotics as above. All cells were maintained in a humidified incubator at 37°C with a 5% CO_2 atmosphere.

Cytotoxicity assays

Cytotoxicity assays for non-myeloma cell lines were performed essentially as described elsewhere;[27] modifications were made to allow for the slow growth, non-adherence and dilution-sensitivity of myeloma lines. Briefly, cells were seeded at 10,000 per well in 96-well plates in RPMI-1640 without phenol red (which interferes with the detection of fluorescence). The drug was applied in a concentration series along the long axis of the plate. After 5 days of proliferation, cells were permeabilized *in situ* and DNA stained by adding 5% (v/v) of 21X lysis buffer (1X was 10 mM TrisHCl pH 7.4, 5 mM EDTA, 0.1% Triton X-100 and Sybr I Green (Invitrogen) 1:4000). The lysate was mixed thoroughly with a multi-channel pipette. The number of cells was determined by fluorescence, measured on a plate reader with 485 nm excitation and 535 nm emission filters. The IC_{50} is the concentration of drug that inhibits proliferation to 50% that of the untreated controls.

Patients

The first cohort included 17 patients with relapsed or refractory myeloma all treated with bortezomib on days 1, 4, 8 and 11 of a 21-day cycle, for a minimum of two cycles, between 2004 and 2007. Nine patients were treated at the Royal Prince Alfred Hospital with a dose of 1.3 mg/m^2; three of these patients concurrently received dexamethasone. Eight patients were treated as part of the multicenter Bortezomib Induction and Re-induction (BIR) study, a pilot study to explore the tolerability and efficacy of bortezomib as part of induction and post-transplant therapy in multiple myeloma; these patients had persistent disease 8 weeks after high-dose melphalan and autologous stem cell transplantation and were then treated with bortezomib (1.0 mg/m^2) and a maintenance dose of prednisone (50 mg, on alternate days). The study was approved by the Sydney South West Area Health Service Ethics Committee, Eastern Zone.

A second set of 25 sepcimens consisted of bone marrow samples from the Victorian Cancer BioBank; these were pre-treatment biopsies from myeloma patients treated with bortezomib as a single agent or in combination with corticosteroids. Patients treated concurrently with other drugs were excluded. The project was approved by the Biobank Ethics Committee and Tissue Management Research Committee at the Peter MacCallum Cancer Centre.

Purification of myeloma cells

Buffy coat cells from bone marrow biopsies were cryopreserved prior to analysis. Thawed cells were washed in phosphate-buffered saline (PBS). A small aliquot of each sample was fixed in 4% formaldehyde in PBS and permeabilized with 0.5% saponin in PBS. The remaining cells were stained with fluorochrome-antibody conjugates anti-CD38-PE and anti-CD14-APC (Becton Dickinson) and DAPI (Fluka) as a vital dye. The fixed, permeabilized aliquots were stained for CD38 and cytoplasmic kappa and lambda light chains (FITC conjugates, DAKO). Stained cells were sorted on a Becton-Dickinson FACSAria. The fixed sample was used to verify correct gating of the myeloma population - brightest for CD38 and cytoplasmic light chain. The corresponding live cell population gated was $CD38^{bright}$, $CD14^{neg}$ (to exclude non-myeloma monocytic cells) and $DAPI^{neg}$. Yields were 5,000-500,000 cells, which were stored at -70°C until RNA extraction.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblot analysis

Cells were lysed by three freeze-thaw cycles in 0.1% Triton-X 100, 10 mM Tris HCl pH 8.0, 10 mM $MgCl_2$, 2 mM $CaCl_2$ and a protease inhibitor cocktail (Sigma). The lysate was clarified by centrifugation at 14,000 g at 4°C. Proteins were quantified by the Bradford assay (BioRad). Lysates were mixed 1:1 with 2X Laemmli buffer, boiled for 3 min and fractionated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. Proteins were transferred to a polyvinylidene fluoride membrane (Millipore) by electroblotting. Membranes were blocked in 5% blotto [5% w/v skimmed milk powder in PBST (PBS plus 0.1% v/v Tween-20)] for 1 h then incubated with monoclonal antibodies in 1% blotto in PBST to detect phosphorylated eIF2-α (119A11, Cell Signaling Technology), BiP (C50B12, Cell Signaling Technology), ATF6 (70B1413.1, Imgenex), or GAPDH (sc-47724, Santa Cruz Biotechnology). Membranes were washed three times for 15 min in PBST. Specifically bound antibodies were then detected with horseradish peroxidase-conjugated secondary antibodies, visualized by enhanced chemiluminescence (West Pico, Pierce).

Immunoglobulin secretion

Cells were seeded at 10^6/mL in complete medium. After 24 h, the culture supernatant was clarified by centrifugation. Secreted immunoglobulin therein was assayed by a sandwich enzyme-linked immunosorbent assay (ELISA), essentially as described by Avery *et al.*[28] Capture antisera (SouthernBiotech) were goat $F(ab')_2$ anti-human IgG or goat $F(ab')_2$ anti-human IgL kappa, both at 1:1000. Detection antisera were biotinylated versions of the capture antisera. Standards were purified human IgG (Sigma, I8640), a dilution series in 1% bovine serum albumin in PBS.

Proteasome activity

Proteasome chymotryptic activity was assayed by cleavage of a pro-luminescent peptide-luciferin substrate – Proteasome Glo (Promega), according to the manufacturer's instructions. Luminescence was measured on a Wallach Victor plate reader.

Over-expression and knockdown of XBP-1

Unspliced and spliced *XBP-1* cDNA were cloned based on the sequence of GenBank/NM005080 and inserted into the retroviral vector pLZRS-IRES-EGFP. Human myeloma cell lines H929 and RPMI-8226 were transduced. Stably transduced cells were sorted by flow cytometry using green fluorescent protein (GFP) as the marker.

A set of five candidate retroviral short hairpin RNA (shRNA) for knockdown of *XBP-1* were obtained in lentiviral vectors as VSVG-pseudotype virus from the MISSION™ TRC-Hs 1.0 (Human) shRNA Target Set (Sigma). Transduced H929 and RPMI-8226 cells were selected by puromycin resistance (2 μg/mL).

Statistical analysis

The distribution of *XBP-1* levels was heavily skewed so hypothesis testing was performed on logarithms of the raw data, using two-tailed, unpaired t-tests. The spread of the data was less for the cell lines so Spearman's non-parametric R was used to quantify correlations. Analyses were performed with Graphpad Prism 4.

Results

Sensitivity of myeloma cell lines to bortezomib is associated with high XBP-1 *mRNA levels*

Six human myeloma cell lines were tested for bortezomib sensitivity by cytotoxicity assays and their *XBP-1* mRNA levels determined by PCR. As *XBP-1* is a stress response gene whose expression can vary significantly with cell density, medium depletion and other culture conditions, it was crucial to quantify *XBP-1* mRNA with the same aliquot of cells used for analysis of sensitivity to bortezomib. Total *XBP-1* mRNA levels were determined by real-time PCR with primers spanning the 26 bp intron. The ratios of spliced:unspliced *XBP-1* mRNA were determined by separate semiquantitative PCR in which the log-phase yields of spliced and unspliced PCR products were compared by microelectrophoresis and corrected by reference to standards.

Total *XBP-1* mRNA levels showed a strong inverse correlation with the IC_{50} values for bortezomib (Spearman's r = -0.89; Figure 1A) i.e. high levels of *XBP-1* were associated with sensitivity to the drug. Unspliced *XBP-1* levels showed a similar relationship (Figure 1B), as the unspliced transcript was the dominant form of the transcript in the myeloma cell lines tested and, indeed, most other tumor cell lines tested (*Online Supplementary Figure S1*). Spliced *XBP-1* mRNA was the minority species and its correlation with bortezomib sensitivity was weaker (r = -0.60; Figure 1C), due to an outlier. Although the ratio of spliced:unspliced *XBP-1* mRNA might also indicate the degree activation of the UPR, it was not related to bortezomib sensitivity (Figure 1D).

The relationship between *XBP-1* mRNA levels and sensitivity to bortezomib was much weaker in a panel of non-myeloma human lymphoid cell lines including Burkitt lymphomas, T lymphoblastoid lines, one lymphoplasmacytoid line and a panel of solid tumor cell lines including neuroblastoma, lung cancer, colon cancer, ovarian cancer, cervical cancer and prostate cancer (Figure 1E). It may, however, be noted that the levels of *XBP-1* mRNA in the non-myeloma cell lines were often much lower than in the myelomas, which again suggests that high *XBP-1* levels are indicative of sensitivity to bortezomib.

Other components of the UPR were examined by immunoblots. It is interesting that ATF6 levels were also higher in bortezomib-sensitive myeloma cell lines (Figure 1F) whereas neither BiP, the main molecular chaperone in the endoplasmic reticulum, nor phosphorylated eIF2a, were related to sensitivity. ATF6 is a regulator of the UPR with functions similar to XBP-1.

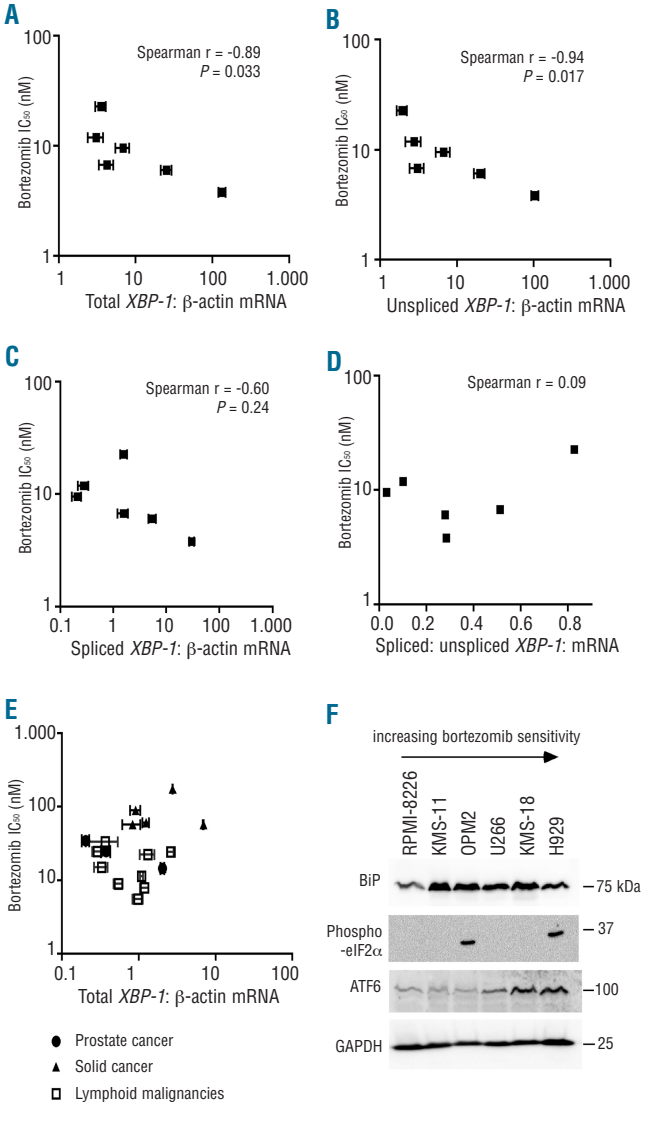

Figure 1. Relationship between bortezomib sensitivity and *XBP-1* mRNA levels. (A) Total, (B) unspliced, (C) spliced and (D) ratio of spliced to unspliced *XBP-1* mRNA in myeloma cell lines, determined by quantitative PCR, compared with levels in (E) lymphoid and solid tumors. *XBP-1* copy number was normalized to *β-actin* in panels A-C, E. Error bars are SEM, four replicates. (F) Immunoblot analysis of UPR proteins in six myeloma cell lines, related to relative bortezomib sensitivity.

XBP-1 *levels correlate with response of clinical myelomas to bortezomib*

A retrospective pilot study was conducted of 17 relapsed or refractory myeloma patients treated with bortezomib alone or in combination with a corticosteroid. The patients' characteristics are presented in Table 1. Briefly, responders and non-responders did not differ in terms of age, gender, isotype of myeloma, or the number of previous treatments. Myeloma cells were isolated from cryopreserved bone marrow biopsies collected prior to treatment with bortezomib. The time between bone marrow collection and bortezomib treatment ranged from 1 to

Table 1. Patients' characteristics.

Characteristic	Responders (n=13)	Non responder (n=4)	*P*
Median age	58	56	0.65
Males/females	9/4	2/2	0.58
Myeloma type			
IgG/A/non-secretory	6/5/0	3/1/0	0.60
light chain disease	2	0	1
kappa/lambda	10/3	2/2	0.54
Median number of previous therapies	1	1	0.86
Concurrent corticosteroid	9	2	0.58
Bortezomib dose 1.0/1.3 (mg/m²)	8/5	2/2	1

33 months. Myeloma cells were purified by meticulously standardized flow cytometry. Responses to bortezomib treatment at week 3 of cycle 2 were categorized according to criteria of the European Group for Blood and Marrow Transplantation;[29] the cohort consisted of complete, partial, minimal and non-responders. Briefly, a complete response was defined as a negative result on immunofixation, a partial response as a greater than 50% reduction of paraprotein and a minimal response as a 25-49% reduction of paraprotein; non-responders were patients who had progressive disease and those who did not satisfy plateau or minimal response criteria.

The purified myeloma cells showed progressively less total *XBP-1* mRNA according to the level of response, from complete response to partial response to minimal response to no response (Figure 2A). Indeed, non-responders all had lower *XBP-1* levels than all the responders (P<0.0001). The results were similar when considering unspliced or spliced *XBP-1* (Figure 2B,C). This suggests that *XBP-1* levels prior to bortezomib treatment might be useful predictors of response in the clinical setting if the myeloma cells can be efficiently purified. The ratio of spliced:unspliced *XBP-1* mRNA was, however, unrelated to response (Figure 2D), consistent with the *in vitro* results.

An independent analysis was performed on a second set of samples from myeloma patients from Victoria, Australia. A similar trend was evident, albeit weaker (Figure 2E). This set of samples consisted of banked marrow biopsies obtained from more diverse sources and included samples from a higher proportion of patients (21/25) treated with corticosteroids (in some cases at a high-dose) concurrently with bortezomib, which partially accounts for the presence of responders with low *XBP-1* levels.

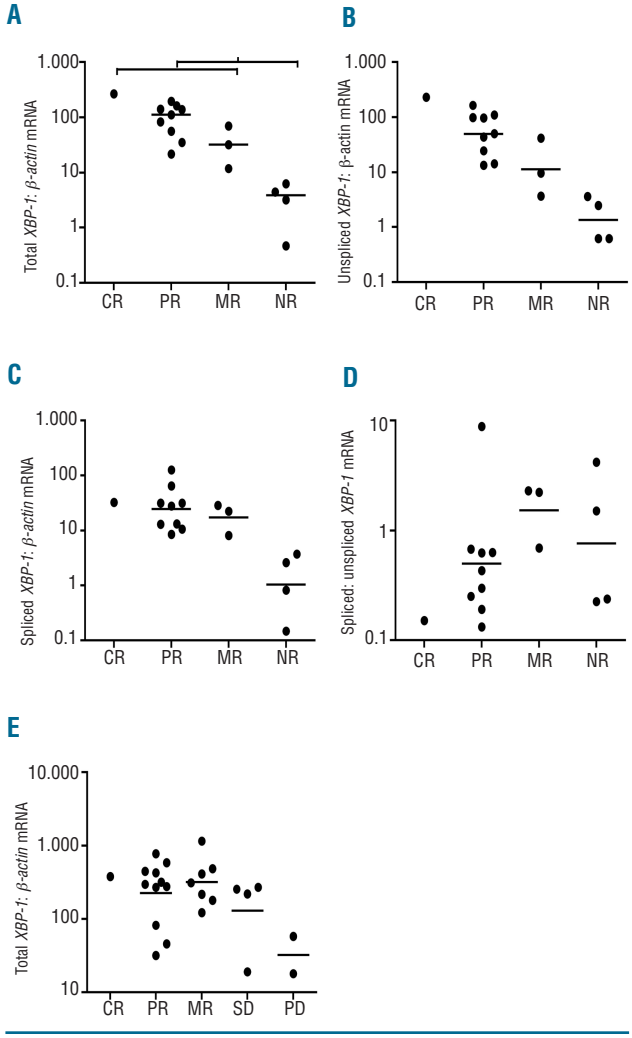

Figure 2. Clinical response to bortezomib and *XBP-1* levels. (A) Total, (B) unspliced, (C) spliced, and (D) spliced:unspliced *XBP-1* mRNA levels in myeloma cells extracted from bone marrow biopsies taken pre-treatment. Patients' responses were stratified according to European Blood and Marrow Transplantation group (EBMT) criteria: CR: complete response; PR: partial response; MR: minimal response; NR: no response. (E) Victorian Tissue Bank samples, whose responses were assessed according to the International Myeloma Working Group uniform response criteria.

Down-regulation of XBP-1, ATF6 and immunoglobulin synthesis in bortezomib-resistant myeloma cell lines

Bortezomib-resistant sub-lines of the KMS-11 myeloma cell line were derived from long-term adaptation to continuous exposure to increasing concentrations of bortezomib, ultimately tolerating 3- to 4-fold the starting IC_{50}. These resistant sub-lines showed no loss of sensitivity of the proteasome itself to bortezomib, as indicated by inhibition of proteolysis of a fluorescent substrate probe (Figure 3A). Thus the resistance could not be due to mutation of the proteasome. However, in multiple independently derived resistant sub-lines, total *XBP-1* mRNA levels were substantially reduced compared to those of the bortezomib-sensitive parent cell line KMS-11 (Figure 3B); the greater the resistance to bortezomib, the greater the reduction in *XBP-1* levels. This reduction was not a short-term artifact of exposure to bortezomib as it persisted for at least 48 h after bortezomib had been washed off. The down-regulation of total *XBP-1* mRNA in bortezomib-resistant myeloma lines was accompanied by a reduction in the proportion of the spliced form of the transcript (Figure 3C), which encodes the active XBP-1 protein. Together, these results suggest a marked suppression of elements of the UPR accompanying acquisition of resistance to bortezomib. Immunoblot analysis of other UPR components (Figure 3D) indicated that resistant sub-lines also had reduced expression of the transcription factor ATF6, which is responsible for activation of chaperones in the UPR, a function similar to that of XBP-1. The expression of phosphorylated-eIF2α was consistently up-regulated in the bortezomib-resistant sub-lines (Figure 3D), suggesting overall down-regulation of protein synthesis.

Indeed, immunoglobulin production was suppressed in the bortezomib-resistant myeloma sub-lines, as determined by ELISA (Figure 3E) or immunoblot analysis *(data not shown)*. This suggests that myeloma cells adapt to bortezomib by reducing immunoglobulin synthesis, which would reduce dependence on, and permit down-regulation of, other elements of the UPR.

Bortezomib-resistant cell lines are sensitized to agents that induce the unfolded protein response

Some components of the UPR have been implicated in resistance to conventional chemotherapy. Our results suggest that elements of the UPR are down-regulated in acquired bortezomib resistance. If this is true, then bortezomib resistance might be accompanied by sensitization to conventional chemotherapy and agents that induce the

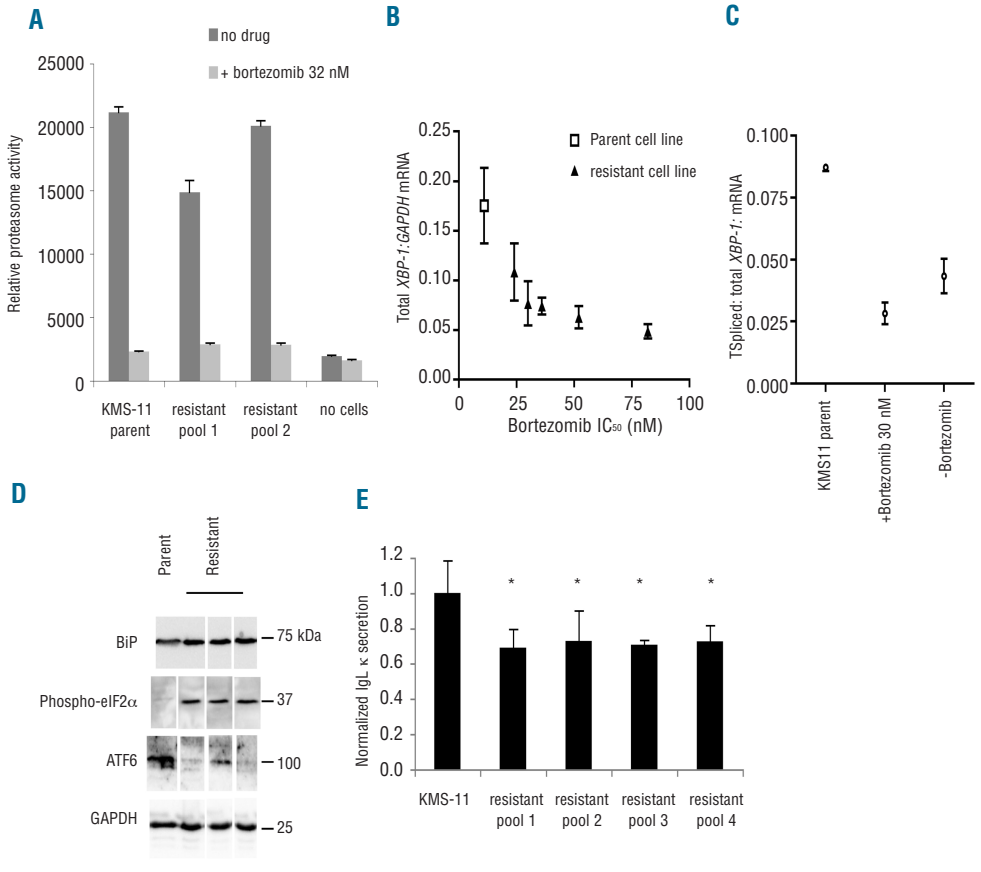

Figure 3. Analysis of bortezomib-resistant myeloma cell lines. (A) Proteasome activity and its inhibition by bortezomib in parent KMS-11 myeloma cells and two bortezomib-resistant sublines, as indicated by proteolysis of a fluorescent substrate. (B) Reduced total *XBP-1* mRNA levels, determined by quantitative PCR, in bortezomib-resistant KMS-11 myeloma sub-lines, determined by cytotoxicity assay. Error bars in panels A and B are SEM of three replicates. (C) Proportion of spliced *XBP-1* mRNA in parent and resistant sublines, 48 h after bortezomib was removed. (D) Immunoblot analysis of UPR proteins in bortezomib-sensitive KMS-11 myeloma cells (parent cell line) and three bortezomib-resistant sublines. *GAPDH* is a loading control. (E) Reduction in kappa light chain secretion in bortezomib-resistant sublines compared to the KMS11 parent cell line. Mean reductions in four experiments are shown; error bars are SE; **P*<0.05. Levels secreted by the parent cell line depended on experimental conditions but were ~ 1 μg/mL.

Table 2. Cross-resistance of bortezomib-resistant cell lines to doxorubicin, melphalan and tunicamycin.

	Bortezomib (nM)	*P*	Doxorubicin (nM)	*P*	Melphalan (μM)	*P*	Tunicamycin (μM)	*P*
KMS11	12.7±1.0		75±3.3		10.9±0.8		0.47±0.021	
KMS11 B1	100±3.2	<0.0001	37±4.0	0.00030	7.5±0.9	0.0020	0.24±0.018	0.015
RF	6.9±0.8		0.5±0.053		0.4±0.1		0.49±0.055	
KMS11 B2	87.9±14.9	<0.0001	69.7±6.8	0.35	6.5±1.4	0.0066	0.35±0.014	0.0093
RF	6.7±0.8		0.94±0.094		0.6±0.1		0.71±0.044	

Figures are mean IC_{50} or resistance factor (RF), ± SEM, n = 3 or 4; P values are from two-tailed, paired t-tests comparing log IC_{50} values. KMS11 B1 and KMS11 B2 are two independently derived pools of bortezomib-resistant cells.

UPR. Doxorubicin and melphalan are commonly used in the treatment of myeloma. Doxorubicin intercalates DNA and also generates reactive oxygen species that can damage proteins. Melphalan alkylates DNA and also proteins. Tunicamycin induces endoplasmic reticulum stress by inhibiting glycosylation of nascent proteins. Two independent pools of bortezomib-resistant myeloma cells and their parent cell line were, therefore, tested for sensitivity to melphalan, doxorubicin and tunicamycin. The resistant lines were modestly sensitized to melphalan and tunicamycin and possibly also to doxorubicin (Table 2), as predicted.

Modulation of XBP-1 *levels has little effect on sensitivity to bortezomib*

XBP-1 mRNA levels in H929 and RPMI-8226 cells were knocked down by ectopic expression of shRNA in lentiviral vectors. H929 cells had high starting levels of *XBP-1* and the knockdown resulted in decreased sensitivity to bortezomib although this was not statistically significant (Figure 4A). RPMI-8226 cells had a low initial level of *XBP-1* and knockdown to even lower levels had no effect on sensitivity to bortezomib.

The consequences of over-expressing either the spliced or unspliced form of *XBP-1* were also investigated in RPMI-8226 cells, chosen for their low levels of the endogenous mRNA. High-level over-expression of each form was achieved (Figure 4B). However, transfectants of unspliced *XBP-1* also showed marked elevation of spliced *XBP-1*, likely because there was a larger pool available for splicing by IRE-1. Similarly, when spliced *XBP-1* was over-expressed, the levels of the unspliced form also increased. The transcription factor derived from the spliced form of *XBP-1* has been shown to act on its own promoter, thus increasing transcription of the unspliced mRNA.[23] Thus, despite altering the ratio of unspliced:spliced mRNA, the net effect on the relative influence of active *versus* inactive/dominant negative

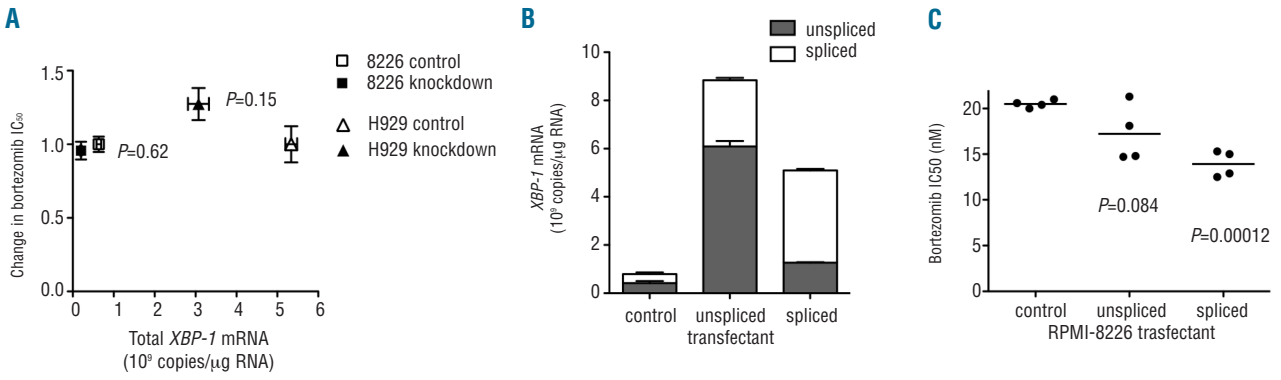

Figure 4. **Manipulation of *XBP-1* in myeloma cell lines and effects on bortezomib sensitivity. (A) Knockdown of *XBP-1* and consequences for bortezomib sensitivity. (B) Over-expression of unspliced or spliced *XBP-1*. (C) The effect of overexpression of *XBP-1* on bortezomib sensitivity. Total *XBP-1* mRNA was normalized to the total RNA. IC_{50} values were determined by cytotoxicity assay.**

XBP-1 proteins was uncertain. Both manipulations sensitized RPMI-8226 cells to bortezomib, but the changes were modest and statistically significant only for transfectants of the spliced form (Figure 4C).

Discussion

Proteasome inhibitors and other new drugs are revolutionizing the treatment of myeloma. When the choice of several agents is available, markers associated with drug response provide the opportunity for optimizing combination chemotherapy for individual patients, avoiding needless side effects, and time and expense lost because of suboptimal regimens. Response markers highlight the relevant biological pathways affected by a drug and the changes which mediate drug resistance, thus indicating the way to better drugs and new drug targets.

Three independent lines of evidence support an association between *XBP-1* levels and sensitivity to bortezomib: their correlation in myeloma cell lines, the corresponding relationship in clinical myeloma, and the down-regulation of *XBP-1* and immunoglobulin production in myeloma cell lines with acquired resistance to bortezomib. These findings are consistent with the high levels of *XBP-1* in myeloma and the fact that this malignancy is markedly more sensitive to bortezomib than are other cancers. The relationship between *XBP-1* levels and sensitivity to bortezomib was weak in other tumor cell lines examined, which had lower levels of *XBP-1*. Reliance on the UPR to support active immunoglobulin synthesis may be the principal reason for the sensitivity of myelomas, as a class, to bortezomib whereas other factors would be more important in cells not so dependent on the UPR.

Results from both cohorts of myeloma patients indicated that low *XBP-1* levels were associated with non-response, despite the limitations of these pilot studies. The samples from the Victorian cohort in particular were drawn from a wide variety of biopsy sources from patients who had been given different treatment regimens, many including steroids that mask the response to bortezomib. The intervals between biopsy and bortezomib treatment were highly variable, the handling of the biopsies was not controlled and there was likely technical variability between operators during the processing of the biopsies. Given that evidence for a relationship between *XBP-1* levels and bortezomib sensitivity was nevertheless obtained, prospective studies are warranted with larger cohorts, employing standardized time points and procedures for the collection and handling of biopsies, treatment and assessment of treatment response.

Bagratuni *et al.* recently showed in a large study that spliced *XBP-1* predicts outcome in patients treated with thalidomide.[30] Yet, despite considerable interest in the effects of proteasome inhibitors on the UPR, *XBP-1* had not previously been identified as a predictor of response for myelomas in, for example, several microarray studies completed so far.[31-33] There are a number of factors that might have contributed to this discrepancy. The very low *XBP-1* levels in non-responders could be masked by higher *XBP-1* expression in other, contaminating cells from the marrow biopsies, placing a premium on adequate purification of the myeloma cells. Flow cytometry is arguably superior to panning in this respect. We were fortunate to have access to a small group of patients treated with bortezomib alone or with only steroids added. Such patients are already uncommon, as the response rate to combination chemotherapy with bortezomib is much higher than to the drug as a single agent.[34-38] Analysis of bortezomib-resistant myeloma cell lines supports the view that the target pathway of the drug is the UPR. The reduced *XBP-1* level in resistant cells was accompanied by down-regulation of ATF6, another regulator of the response. The up-regulation of phosphorylated eIF2α, which inhibits protein synthesis, and the reduced secretion of immunoglobulin point to dependence on the UPR for immunoglobulin synthesis as an Achilles-heel rendering myelomas sensitive to proteasome inhibitors.

Other cancers with an active UPR, such as prostate, breast and pancreas cancers, are also relatively sensitive to bortezomib. The original screening of the NCI tumor cell line panel identified prostate cancer as particularly sensitive[39] and it is interesting that although only three prostate cell lines were examined in this study, the relationship between *XBP-1* and bortezomib sensitivity held for that small group (Figure 1E). Bortezomib is also an effective treatment for mantle cell lymphoma[40] and Waldenstrom's macrogobulinemia.[41] While the reason for the sensitivity of mantle cell lymphoma to bortezomib is unknown, it is similar to myeloma in having a mature B-cell immunophe-

notype and strong expression of immunoglobulins, in this case surface Ig. It would, therefore, be of interest to test whether *XBP-1* levels are significantly related to response in the context of clinical trials of bortezomib for cancers other than myeloma.

It is particularly interesting that resistance to bortezomib was accompanied by increased sensitivity to three other drugs. Thus, multidrug sensitivity, rather than multidrug resistance, may follow bortezomib treatment. If this were indeed to be the case, it would have important implications for the order in which bortezomib is employed *vis* other treatment options. It might also explain, in part, the high response rates obtained by combining cytotoxic drugs with bortezomib.[35,36]

The changes in sensitivity to bortezomib which followed manipulation of *XBP-1* levels by over-expression or knockdown were in line with the correlation observed in other contexts. Even so, the modest scale of the effects suggests that *XBP-1* does not determine sensitivity to proteasome inhibitors directly but is, rather, a surrogate marker of sensitivity. XBP-1 is a major regulator of the UPR so its normal level may reflect a corresponding degree of cellular dependence on the UPR. Changing *XBP-1* mRNA levels need not affect that dependence strongly. This interpretation remains subject to the caveat that levels of the spliced and unspliced forms of *XBP-1* could not be altered independently and the net effect was, therefore uncertain.

In summary, in the context of myeloma, high *XBP-1* mRNA levels appear to be associated with sensitivity to bortezomib and low levels with resistance. *XBP-1* levels constitute a potential bortezomib response marker, whose clinical utility needs to be confirmed in larger, controlled studies in patients. The value of these levels for predicting response to combination therapy or cross-resistance to other drugs also warrants investigation. The view that myeloma is inherently sensitive to proteasome inhibitors because of its dependence on the UPR is satisfying, being consistent with the known high levels of XBP-1 in plasma cells, myeloma and myeloma cell lines, the particular sensitivity of myeloma to the drugs, and the new observations presented here. This model suggests that elements of the UPR may be appropriate targets for new drugs for myeloma and also that drugs that induce the UPR will sensitize cells to proteasome inhibitors.

Authorship and Disclosures

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