The Nuclear Factor (Erythroid-derived 2)-like 2 and Proteasome Maturation Protein Axis Mediate Bortezomib Resistance in Multiple Myeloma*

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Background: Acquired proteasome inhibitor resistance emerges in myeloma patients through incompletely understood mechanisms. Activation of nuclear factor (erythroid-derived 2)-like 2 (NRF2) and proteassemblin (POMP) was linked to bortezomib resistance, while their inhibition reversed resistance. NRF2/POMP axis contributes to bortezomib resistance. NRF2/POMP axis inhibition can be translated to the clinic to reverse bortezomib resistance and induce chemosensitization.

Results: Resistance to the proteasome inhibitor bortezomib is an emerging clinical problem whose mechanisms have not been fully elucidated. We considered the possibility that this could be associated with enhanced proteasome activity in part through the action of the proteasome maturation protein (POMP). Bortezomib-resistant myeloma models were used to examine the correlation between POMP expression and bortezomib sensitivity. POMP expression was then modulated using genetic and pharmacologic approaches to determine the effects on proteasome inhibitor sensitivity in cell lines and in vivo models. Resistance cell lines were found to overexpress POMP, and while its suppression in cell lines enhanced bortezomib sensitivity, POMP overexpression in drug-naive cells conferred resistance. Overexpression of POMP was associated with increased levels of nuclear factor (erythroid-derived 2)-like (NRF2), and NRF2 was found to bind to and activate the POMP promoter. Knockdown of NRF2 in bortezomib-resistant cells reduced POMP levels and proteasome activity, whereas its overexpression in drug-naive cells increased POMP and proteasome activity. The NRF2 inhibitor all-trans-retinoic acid reduced cellular NRF2 levels and increased the anti-proliferative and pro-apoptotic activities of bortezomib in resistant cells, while decreasing proteasome capacity. Finally, the combination of all-trans-retinoic acid with bortezomib showed enhanced activity against primary patient samples and in a murine model of bortezomib-resistant myeloma. Taken together, these studies validate a role for the NRF2/POMP axis in bortezomib resistance and identify NRF2 and POMP as potentially attractive targets for chemosensitization to this proteasome inhibitor.

Significance: Inhibition of the function of the ubiquitin-proteasome pathway with the proteasome inhibitor bortezomib is an accepted standard of care for the treatment of relapsed and/or refractory multiple myeloma both alone (1, 2) and as part of rationally designed combination regimens (3). In addition, bortezomib-based therapies have been incorporated into the front-line setting for patients with newly diagnosed myeloma (4–7) and are being considered in other settings as well, including as part of maintenance therapy (8). Indeed, together with other advances, such as the development of immunomodulatory agents, bortezomib has contributed to a doubling in the overall survival of myeloma patients over the last decade (9–12). Myeloma cells may be especially sensitive to proteasome inhibitors because protein turnover capacity is reduced during plasma cell differentiation (13). This increases proteasome load relative to capacity, thereby triggering cellular stress and enhancing reliance on the unfolded protein response for survival, which is easily overwhelmed by proteasome inhibitors through their rapid induction of ubiquitin-protein conjugates. Indeed, the ratio of proteasome load to capacity may determine apoptotic sensitivity to bortezomib, with plasma cells having a high load...
and/or low capacity showing sensitivity (14). However, even in patients whose disease initially responds very well to bortezomib, resistance eventually develops in the majority, thereby limiting the reuse of regimens that were previously successful (15–17).

Initial studies in leukemia cell lines described a role for overexpression of the β5 proteasome subunit targeted by bortezomib and showed that shRNA-mediated knockdown of β5 to some extent restored bortezomib sensitivity (18–20). Also, mutations in the β5 subunit’s bortezomib binding pocket were implicated in acquired bortezomib resistance (18–20). However, free β5 subunits are catalytically inactive and contain a pro-sequence that would preclude bortezomib binding (21, 22), and because the latter is responsible for assembly of the catalytically active subunit (21, 22), some maturation protein (POMP, proteassemblin) (21, 22), confers resistance, and POMP activation was associated with induction of an upstream transcription factor, nuclear factor, erythroid 2-like 2 (NRF2), and with enhanced proteasome activity. Finally, suppression of either NRF2 or POMP using either short hairpin (sh) RNAs or a pharmacologic agent restored sensitivity in cell lines, primary plasma cells, and an in vivo myeloma model.

### Experimental Procedures

#### Cell Lines and Primary Samples

Drug-naive and bortezomib-resistant myeloma cell lines were developed and maintained as described previously (27). Cell line authentication was performed by our cell line characterization core using short tandem repeat profiling. Bortezomib was removed from the culture for at least 7 days prior to all experiments, unless indicated otherwise, to negate the possibility that proteasome inhibitor-induced oxidative stress was impacting NRF2 and POMP expression. Primary plasma cells were purified from bone marrow aspirates collected from patients under an approved protocol from the Institutional Review Board at the Second Affiliated Hospital of Soochow University after informed consent was obtained in compliance with the Declaration of Helsinki. The clinical history, including prior treatments, of the patients whose samples were used is shown in Table 1.

#### Viability Assays

Proliferation and viability assays with bortezomib (Selleck Chemical, Houston, TX) and all-trans-retinoic acid (ATRA) (Sigma) were performed as described previously (28). Briefly, cell lines or primary samples were treated with the indicated compound for a minimum of 24 h, unless otherwise indicated, followed by the addition of the tetrazolium reagent WST-1. Colorimetric detection of metabolic activity was then obtained on a Victor3V plate reader (PerkinElmer Life Sciences). Data were normalized to vehicle controls, which were arbitrarily set at 100% viability, and all data points are represented as the mean with the standard deviation (S.D.).

#### Immunoblotting

Cells were harvested and lysed in 1× Lysis Buffer (Cell Signaling Technology, Danvers, MA), followed by resolution on gradient gels (Thermo Fisher Scientific, Carlsbad, CA), transferred to nitrocellulose (Bio-Rad), and probed with...
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the indicated antibodies. Primary anti-POMP, anti-NRF2, anti-Kelch–like ECH-associated protein 1 (KEAP1) and anti-cleaved caspase 3 antibodies were from Cell Signaling Technology (Beverly, MA); the 20S proteasome β5 subunit (PSMB5) antibody was from Santa Cruz Biotechnology, and anti-β-actin was from Sigma. Densitometric quantitation was obtained using ImageJ software (National Institutes of Health, rsbweb.nih.gov) and normalized to β-actin and either vehicle-treated or wild-type controls, which were arbitrarily set to 1.

Real Time RT-PCR—Real time PCR was carried out as described previously, with some modifications (28). Briefly, total RNA was isolated from cultured cells or tumor tissues using TRIZol (Thermo Fisher Scientific), and cDNA was synthesized using a High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Quantitative (q) real time PCR was performed using the TaqMan Gene Expression Master Mix and the POMP, NRF2 (FAM™), β-actin (VIC™), proteasome β5 subunit, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, VIC®) TaqMan gene expression assays as multiplexed, triplicate samples on a StepOnePlus PCR system (Applied Biosystems). Relative quantification was done using the comparative Cq method after normalization to the internal GAPDH control, where all samples were then normalized to wild-type or vehicle controls.

POMP and NRF2 Silencing—Six lentivirus-based shRNAs targeted to POMP, eight lentivirus-based shRNAs targeted to NRF2, or a nonspecific scrambled control (Sigma) were transfected with the packaging vectors psPAX2 and pMD2.G into 293T cells by calcium chloride to produce the lentiviruses. Two days later, the supernatants were collected, filtered, concentrated, and used for experiments or frozen at –80 °C. KAS-6/1 bortezomib-resistant (V10R) and OPM-2 V10R cells were transduced by using lentiviruses with Polybrene (8 μg/ml, Sigma) and infected cells were selected with 2 μg/ml puromycin. The expression of POMP or NRF2 was determined by Western blot analysis and real time PCR. Two of the lentivirus-based shRNAs targeted to POMP, constructs 3 and 5, and two for NRF2, constructs 6 and 8, were validated for further studies. POMP shRNA lentiviral vectors contained two target-specific constructs, CCGGGGTCATATGGTCCGCTCAAATCTGAGTTTACGGAGCAATAATAGCCTTTTGG and CCGGC-TATTTGAGGATATTTTCTCGAGGAAATATCTTCAAATCAGTTTTT. NRF2 shRNA lentiviral vectors also contained two target-specific constructs, CCGGACCTTATCTGGAAGTTCTCGAGAAACTGTGGATATATTATCGAGATATTGTTT and CCGGGCCGACCATCTACCAAACTCACTGAGATATTATCGAGATATTGTTT. Sequences from POMP construct 3 were then also used in some transient transfection assays to knock down POMP without subsequent antibiotic selection. Non-targeting shRNAs (KO-N) or shRNAs targeting POMP (KO-3) were introduced by electroporation using the Neon® transfection system (Thermo Fisher Scientific).

POMP and NRF2 Expression—pCMV6-XL5 vectors containing POMP or NRF2 cDNAs were purchased from OriGene (Rockville, MD). POMP or NRF2 was subcloned into the lentiviral vector transfer plasmid pCDH-CMV-MCS-EF1-coGFP to generate pCDH-CMV-POMP-EF1-coGFP or pCDH-CMV-NRF2-EF1-coGFP. The recombinant pCDH-CMV-POMP-EF1-coGFP vector, pCDH-CMV-NRF2-EF1-coGFP vector, or the control vector pCDH-CMV-MCS-EF1-coGFP was transfected with the packaging vectors psPAX2 and pMD2.G into 293T cells by calcium chloride to produce lentiviruses. KAS-6/1 and OPM-2 cells were infected with control or either POMP- or NRF2-expressing lentiviruses, and expression was verified by qPCR and Western blotting.

Proteasome Activity Assays—Chymotrypsin-like proteasome activity was assayed in a total volume of 200 μl using 96-well plates performed according to the manufacturer’s instructions (Promega, Madison, WI). Briefly, Proteasome-Glo™ cell-based reagent was prepared by reconstituting the luciferin detection reagent, Proteasome-Glo™ cell-based buffer, and the Suc-LLVY-Glo™ substrate was then added to an equal volume of samples containing 15,000 cells and incubated for a minimum of 5–10 min before luminescence measurements.

Chromatin Immunoprecipitation (ChiP)—Cells were first cross-linked with 2% paraformaldehyde for 10 min at 37 °C and sonicated. DNA-protein complexes were isolated with a ChiP assay kit (EMD Millipore, Billerica, MA) according to the manufacturer’s instructions with antibodies against NRF2 (Abcam, Cambridge, MA). The precipitated DNA was purified and quantified by real time PCR. Primers used were as follows: 5′-CCTCCAACCTCATCTTATCT-3′ (forward) and 5′-CTGAACTAGCTGGGACTACA-3′ (reverse). The results were normalized relative to the input control.

Luciferase Assay—Luciferase reporter assays were performed using the LightSwitch Dual Assay System (SwitchGear Genomics, Carlsbad, CA) according to the manufacturer’s instructions. KAS-6/1 and KAS-6/1 V10R cells were transiently transfected in triplicate with either empty-luciferase or POMP-luciferase, along with a Cypridina TK control construct and empty pCMV6-XL5 vector or pCMV6-XL5-NRF2 by electroporation using the Neon® transfection system (Thermo Fisher Scientific). The Renilla luciferase/Cypridina luciferase ratio was calculated to normalize for transfection efficiency.

Electrophoretic Mobility Shift Assay—DNA-protein binding assays were carried out with nuclear extract from KAS-6/1 V10R cells with 3′-biotinylated synthetic complementary oligonucleotides (Sigma). The sequence of the oligonucleotide used was 5′-CTCCAGGCTAGGTGACACGGAAGA-3′, and the labeled oligonucleotides were annealed by mixing equal molar amounts of the two single-stranded oligonucleotides, heating to 95 °C for 5 min, followed by ramp cooling to 25 °C over a period of 45 min. Nuclear extracts were prepared using the nuclear/cytosol fractionation kit (BioVision, Carlsbad, CA) following the manufacturer’s instructions. Binding reactions were carried out for 20 min at room temperature in the presence of 50 ng/μl poly(dl-dC), 0.05% Nonidet P-40, 5 mM MgCl2, 10 mM EDTA, and 2.5% glycerol in 1× binding buffer using 20 fmol of biotin end-labeled target DNA and 4 μg of nuclear extract. Additionally, 4 pmol of unlabeled probe was added to some binding reactions as a specific competitor DNA. Assays were loaded onto native 4% polyacrylamide gels pre-electrophoresed for 60 min in 0.5× Tris borate/EDTA and electrophoresed at 100 V before being transferred onto a positively charged nylon membrane in 0.5× Tris borate/EDTA at 100 V for 30 min.
Transferred DNAs were cross-linked to the membrane at 120 mJ/cm² and detected using horseradish peroxidase-conjugated streptavidin according to the manufacturer’s instructions using the LightShift chemiluminescent EMSA kit (Thermo Fisher Scientific).

**Xenograft Modeling**—Bortezomib-resistant KAS-6/1 cells (7 × 10⁶ cells/mouse) were subcutaneously xenografted into 6-week-old non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice (NOD.Cg-Prkdcscid IL2rgtm1Wjl)/SzJ; The Jackson Laboratory, Bar Harbor, ME) with Matrigel (BD Biosciences, San Jose, CA) under a protocol approved by the institutional Animal Care and Use Facility. The mice were randomized into four groups with five subjects in each cohort, and treatments were administered by intraperitoneal injection using peanut oil as a carrier three times weekly, starting on day 7 post-implantation. Tumors were monitored by caliper measurement, and tumor volume was determined using the equation volume = 0.4l × w². The CONTRAST statement in PROC MIXED procedure in SAS (SAS Institute, Inc., Cary, NC) was used to compare the tumor growth rates between each pair of groups. The tumor volume was log-transformed to satisfy the normality assumption of the models. Tumors were removed for qPCR or Western blot assays at the indicated time point. Pairwise differences between the combination group (bortezomib + ATRA) versus ATRA alone, combination versus bortezomib, combination versus control, bortezomib versus control, and ATRA versus control were examined using the ESTIMATE statement in PROC MIXED for each time point. Statistically significant determinations were made by calculation of the probability of χ².

**Results**

**Bortezomib-resistant Cells Overexpress POMP**—Previous studies from our group determined that bortezomib-resistant myeloma cells exposed to proteasome inhibitors showed a more rapid recovery of the chymotrypsin-like proteasome activity (27). We considered the possibility that this could be due to more rapid assembly of new proteasomes and increased proteasome capacity, and analysis of gene expression profiling data comparing bortezomib-resistant cells with their sensitive counterparts revealed up-regulation of POMP (data not shown). To further validate these findings, we performed qPCR comparing bortezomib-resistant (V10R) RPMI 8226, OPM-2, ANBL-6, and KAS-6/1 cells with their wild-type (WT), vehicle-treated, and drug-naive counterparts passaged in parallel. Bortezomib-resistant cells consistently showed enhanced POMP levels may be associated with bortezomib resistance.

**POMP Modulates Bortezomib Sensitivity**—Because a number of mechanisms may be simultaneously activated to confer bortezomib resistance in myeloma cell lines, we sought to confirm that changes in POMP were alone sufficient to modulate sensitivity. We therefore generated KAS-6/1 V10R cells infected with lentiviral vectors expressing either a control, non-targeting (NT) shRNA, or one of two different shRNAs that successfully suppressed POMP (KO-3 and KO-5) (Fig. 2A). When these cells were then treated with either vehicle or bortezomib, compared with the parental KAS-6/1 V10R and NT controls, the KO-3 and -5 cells with lower levels of POMP were consistently more sensitive to proteasome inhibition (Fig. 2B). Moreover, the resistance to bortezomib in V10R cells almost fully reversed to the levels of KAS-6/1 wild-type (WT) cells (Fig. 2B), which was associated with inhibited proteasome chymot...

**FIGURE 1. Bortezomib resistance and POMP levels in myeloma cell lines.** A, bortezomib-sensitive (WT) and bortezomib-resistant (V10R) myeloma cell lines, including RPMI 8226 (8226), OPM-2, ANBL-6, and KAS-6/1 cells, were subjected to qPCR to detect POMP mRNA content, which was analyzed using the comparative Ct method and normalized to GAPDH as an internal control. POMP expression in drug-naive 8226 cells was arbitrarily set at 1.0, and data are provided from three independently performed experiments ± standard deviation. The Student’s paired t test was used to determine statistical significance (*, p < 0.05 versus WT). B, POMP protein levels were evaluated in these same cell lines by immunoblotting and compared with β-actin as a loading control. Densitometry was performed to quantify POMP levels, which were normalized to RPMI 8226 WT cells arbitrarily set to 1.0. A representative autoradiograph is shown from one of two independently performed experiments. C, POMP and β-actin levels are shown by Western blotting in primary plasma cells from four patients who were bortezomib-naive and three patients who were previously bortezomib-exposed and clinically bortezomib-refractory. Densitometry was performed to quantify POMP levels, which were normalized to MM1 cells arbitrarily set to 1.0.
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**NRF2 Regulates POMP Expression**—No direct inhibitors of POMP function have yet been described, and with the hope of finding an approach that could suppress POMP expression to sensitize bortezomib-resistant cells, we studied the POMP promoter and found a consensus binding site for NRF-2 within the −2833 to −2842 region. Also, a ChIP sequencing study in lymphoblastoid cells had suggested that the POMP promoter could be a target for NRF2 binding (29). To determine whether NRF2 indeed influenced POMP expression in myeloma cells, we first studied the bortezomib-resistant V10R cells by qPCR and found that, as had been the case for POMP (Fig. 1), they expressed higher levels of NRF2 mRNA than their wild-type counterparts (Fig. 3A). In KAS-6/1 cells, for example, NRF2 levels were increased almost 4-fold in the resistant versus the sensitive cells. Moreover, this resulted in higher levels of NRF2 protein expression, as determined by Western blotting comparing the V10R and WT cells (Fig. 3B). For example, again in the KAS-6/1 models, NRF2 levels were increased by 4-fold in the bortezomib-resistant cells. To determine whether NRF2 levels were increased in primary samples, Western blotting was performed on CD138⁺ plasma cells from the same four bortezomib-naive patients and three bortezomib-resistant patients used earlier. The latter showed a relatively higher NRF2 expression level (Fig. 3C), supporting the hypothesis that higher NRF2 levels may be associated with higher POMP levels and bortezomib resistance.

NRF2, along with KEAP1, are parts of a signaling pathway that is important in cell defense and survival, including in response to anti-oxidant stress (30). Because POMP has also been linked to anti-oxidant defenses (31), this was another rea-

**FIGURE 2. Influence of POMP on bortezomib sensitivity.** A, KAS-6/1 bortezomib-resistant cells (KAS-6/1 V10R) were infected with lentiviral vectors expressing a scrambled sequence, non-targeting shRNA (KO-NT), or one of two different shRNAs targeting POMP (KO-3 and KO-5). The success of POMP knockdown was verified with Western blotting and compared with β-actin as a loading control. Autoradiometry was performed to quantify POMP levels, and cells were normalized to KAS-6/1 V10R cells arbitrarily set to 1.0. A representative autoradiograph from one of two independent experiments is shown. B, cells described in A and KAS-6/1 drug-naive cells (KAS-6/1 WT) were then exposed to bortezomib for 24 h at the indicated concentrations, and viability was determined with the tetrazolium reagent WST-1. Data presented are from three independently performed experiments and are presented as the mean ± S.D. (⁎, p < 0.05 versus KAS-6/1 V10R or KO-NT). C, protein expression of the cells described in A was examined as described under “Experimental Procedures.” Data are from three independent experiments and are presented as the mean ± S.D. (⁎, p < 0.05 versus KAS-6/1 V10R or KO-NT). D, protein expression of the cells described in B were normalized to OPM-2 V10R cells arbitrarily set to 1.0. A representative autoradiograph from one of two independent experiments is shown. H, cells described in G and OPM-2 drug-naive cells (OPM-2 WT) were then treated with lentiviral vectors without a cDNA insert (OE-control) or the cDNA for POMP (OE-POMP). The success of POMP overexpression was verified with Western blotting and compared with β-actin as a loading control. Autoradiometry was performed to quantify POMP levels, which were normalized to KAS-6/1 WT cells arbitrarily set to 1.0. A representative autoradiograph from one of two independent experiments is shown. E, cells described in D were then exposed to bortezomib for 24 h at the indicated concentrations, and viability was determined with the tetrazolium reagent WST-1. Data presented are from three independently performed experiments and are presented as the mean ± S.D. (⁎, p < 0.05 versus KAS-6/1 WT or OE-control). F, protein activity in the cells described in D was examined as described under “Experimental Procedures.” Data are from three independent experiments and are presented as the mean ± S.D. (⁎, p < 0.05 versus KAS-6/1 WT or OE-control). G, OPM-2 bortezomib-resistant cells (OPM-2 V10R) were infected with lentiviral vectors expressing a scrambled sequence, non-targeting shRNA (KO-NT), or one of two different shRNAs targeting POMP (KO-3 and KO-5). The success of POMP knockdown was verified with Western blotting and compared with β-actin as a loading control. Autoradiometry was performed to quantify POMP levels, and cells were normalized to OMP-2 V10R cells arbitrarily set to 1.0. A representative autoradiograph from one of two independent experiments is shown. H, cells described in G and OPM-2 drug-naive cells (OPM-2 WT) were then treated with lentiviral vectors without a cDNA insert (OE-control) or the cDNA for POMP (OE-POMP). The success of POMP overexpression was verified with Western blotting and compared with β-actin as a loading control. Autoradiometry was performed to quantify POMP levels, which were normalized to OPM-2 WT cells arbitrarily set to 1.0. A representative autoradiograph from one of two independent experiments is shown. K, cells described in J were then exposed to bortezomib for 24 h at the indicated concentrations, and viability was determined with the tetrazolium reagent WST-1. Data presented are from three independently performed experiments and are presented as the mean ± S.D. (⁎, p < 0.05 versus OPM-2 WT or OE-control). L, protein activity in the cells described in J was examined as described under “Experimental Procedures.” Data are from three independent experiments and are presented as the mean ± S.D. (⁎, p < 0.05 versus OPM-2 WT or OE-control). M, KAS-6/1 cells with POMP overexpressed (OE-POMP cells) were transiently transfected with non-targeting shRNAs (OE-shRNA-control) or shRNAs targeting POMP (OE-shRNA-POMP). The cells and KAS-6/1 wild-type cells (KAS-6/1 WT) were then exposed to bortezomib for 24 h at the indicated concentrations, and viability was determined with the tetrazolium reagent WST-1. Data presented are from three independently performed experiments and are presented as the mean ± S.D. (⁎, p < 0.05 versus OE-POMP and OE-shRNA-control).
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**FIGURE 3.** Bortezomib resistance and NRF2 levels in myeloma cell lines. A, bortezomib-sensitive (WT) and bortezomib-resistant (V10R) myeloma cell lines, including RPMI 8226 (8226), OPM-2, ANBL-6, and KAS-6/1 cells, were subjected to qPCR to detect NRF2 mRNA content, which was analyzed using the comparative \( C_{\Delta \Delta T} \) method and normalized to GAPDH as an internal control. \( \Delta \) NRF2 expression in drug-naive 8226 cells was arbitrarily set at 1.0, and representative data are shown from one of three independent experiments along with the standard deviation (*, \( p < 0.05 \) versus WT). B, NRF2 protein levels were evaluated in these same cell lines by immunoblotting and compared with \( \beta \)-actin as a loading control. Densitometry was performed to quantify NRF2 levels, which were normalized to 8226 WT cells arbitrarily set to 1.0. A representative autoradiograph is shown from one of two independently performed experiments. C, NRF2 protein levels were evaluated in the primary myeloma cells by immunoblotting and compared with \( \beta \)-actin as a loading control. Densitometry was performed to quantify NRF2 levels, which were normalized to the MM1 sample arbitrarily set to 1.0. A representative autoradiograph is shown from one of two independently performed experiments.

**A**

![Graph showing NRF2 and POMP expression in different myeloma cell lines](image1)

**B**

| Cell Line   | NRF2   | \( \beta \)-Actin |
|-------------|--------|------------------|
| 8226        | 1.0    | 1.0              |
| OPM-2       | 3.1    | 4.5              |
| ANBL-6      | 4.5    | 6.9              |
| KAS-6/1     | 7.1    | 7.1              |
| WT          | 0.9    | 0.9              |
| V10R        | 3.4    | 3.4              |

**C**

| Cell Line   | NRF2   | \( \beta \)-Actin |
|-------------|--------|------------------|
| MM1         | 1.0    | 1.0              |
| MM2         | 0.9    | 0.9              |
| MM3         | 0.8    | 0.8              |
| MM4         | 0.7    | 0.7              |
| MM5         | 3.1    | 3.1              |
| MM6         | 2.0    | 2.0              |
| MM7         | 2.2    | 2.2              |

**Inhibition of NRF2 Sensitizes Bortezomib-resistant Cells**

The involvement of NRF2 in bortezomib resistance provided us with an avenue to suppress the NRF2/POMP pathway, because retinoic acid has been described to inhibit NRF2 activity (32). Activation of retinoic acid receptor \( \alpha \) (32). Because ATRA is a clinically relevant agent in this class, which is a standard of care for promyelocytic leukemia (33), we examined the possibility that it could be applied to bortezomib resistance. We exposed KAS-6/1 V10R cells to the indicated concentrations of ATRA, bortezomib, or both for 24 h, and we noted that bortezomib alone enhanced the levels of both NRF2 and POMP, although they decreased with exposure to ATRA alone. ATRA in combination with bortezomib also inhibited the levels of both NRF2 and POMP compared with single agent treatment with bortezomib (Fig. 6A). Notably, there was no associated change in the levels of KEAP1, which serves as an adaptor for the E3 ubiquitin ligase responsible for ubiquitination of NRF2 (34). Compared with the vehicle controls, the single agent ATRA or bortezomib treatments showed only a slight ability to...
reduce the viability of KAS-6/1 V10R cells (Fig. 6B), but the combination regimens were much more effective in this regard. ATRA and bortezomib together produced a greater level of apoptosis, as measured by the appearance of the cleaved, activated form of caspase 3 (Fig. 6C), and the enhanced activity of the combinations was associated with a greater reduction in the chymotrypsin-like proteasome activity (Fig. 6D). Importantly, ATRA showed similar effects in OPM-2 bortezomib-resistant cells, where it reduced NRF2 and POMP levels (Fig. 6E), enhanced the ability of bortezomib to reduce cell viability (Fig. 6F), induced caspase cleavage (Fig. 6G), and suppressed proteasome activity (Fig. 6H).

To examine the possibility that ATRA could enhance the action of bortezomib in drug-sensitive cells, we performed comparable experiments in KAS-6/1 and OPM-2 WT cells. Similar trends were observed in KAS-6/1 (Fig. 6, I–L) and OPM-2 cells (Fig. 6, M–P), in that ATRA in combination with bortezomib inhibited the levels of both NRF2 and POMP compared with single agent treatment with bortezomib and enhanced cell death. However, the level of enhanced cell death was smaller than that in the BR cells, in part because, as expected, bortezomib alone produced a much more dramatic effect.

**ATRA Enhances Bortezomib Activity against Primary Samples and in Vivo**—To contribute to the design of future clinical trials, we next examined the possibility that ATRA could enhance the efficacy of bortezomib against CD138<sup>+</sup> primary plasma cells from patients with multiple myeloma. In samples...
where bortezomib showed minimal activity, as defined by a less than 20% reduction in viability as a single agent, such as in MM8 and MM9 (Fig. 7A), addition of ATRA, which itself showed even less efficacy, showed an enhanced reduction in viability with the combination. The same was true in samples where bortezomib showed greater activity, such as MM10 through MM12, where again ATRA increased the ability of bortezomib to reduce viability. Finally, it was also of interest to validate these findings in vivo using a bortezomib-resistant xenograft model. Seven days after inoculation of KAS-6/1 V10R cells, subject mice were randomized to treatment with intraperitoneal injections of vehicle, bortezomib, ATRA, or the combination, and tumor volumes were determined from measurements performed by an investigator blinded to the treatment assignments. Bortezomib and ATRA alone did show some activity in this setting, but the bortezomib and ATRA combination regimen reduced tumor volume (Fig. 7B) compared with either agent alone. These differences reached statistical significance.
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Discussion

The proteasome inhibitor bortezomib is an important part of the standard of care for myeloma patients (1–7), and carfilzomib, a second generation irreversible inhibitor, has recently been approved in the relapsed and refractory setting (35). Following the lead of bortezomib, carfilzomib is being further developed as part of rationally designed regimens for patients with either relapsed disease (36, 37) or newly diagnosed myeloma (38). Moreover, proteasome inhibitors with novel properties are being developed, such as marizomib, which may inhibit all three of the major proteolytic activities of the proteasome, as well as orally bioavailable inhibitors, including ixazomib and oprozomib (39). In this light, and considering the contribution of this class of drugs to the improving outcomes in myeloma (9–12), it seems reasonable to expect that they will remain part of the standard of care for this disease for many years to come. However, due perhaps in part to their incorporation into the treatment of newly diagnosed patients, resistance to proteasome inhibitors is an emerging clinical problem, especially because such patients have a poor prognosis. Indeed, bortezomib-refractory patients who were also relapsed following, refractory to, or ineligible to receive immunomodulatory agents have been reported to have a median survival of less than 1 year (40). This indicates a strong need to better understand the mechanisms underlying bortezomib resistance because this could lead to the design of regimens to overcome this phenotype, which would extend the utility of these drugs and, more importantly, if validated, prolong patient survival.

This study has identified POMP as a modulator of bortezomib resistance in myeloma, because its overexpression was seen in resistant cell lines and primary samples (Fig. 1). POMP suppression with shRNAs restored sensitivity, although its overexpression in drug-naive cells was sufficient to induce resistance (Fig. 2). Also, starting with the observation that NRF2 was induced in bortezomib-resistant cells as well (Fig. 3), we have documented that NRF2 controls POMP levels in myeloma through an impact on transcription from the POMP promoter (Fig. 4). Notably, overexpression or suppression of either POMP (Fig. 2) or NRF2 (Fig. 5) had a consistently greater differential impact on bortezomib sensitivity in KAS-6/1 cells than it did in OPM-2 cells. Interestingly, OPM-2 cells expressed higher basal levels of both POMP and NRF2, and this may explain the differential effects in these cell lines. A high basal level of POMP and NRF2 could blunt the impact of a further overexpression, although a fixed reduction of either would leave higher levels in OPM-2 cells than in KAS-6/1 cells, thereby blunting the impact of shRNAs. These findings are consistent with a recent study that linked activation of NRF2 by tert-butylhydroquinone and other approaches to increased POMP expression and pluripotency in human embryonic stem cells (41). Moreover, antioxidants and oxidative stress have

(Fig. 7C), supporting the possibility that this approach could be translated to the clinic to overcome bortezomib resistance. We next tested whether treatment with bortezomib and ATRA changed the expression of POMP or the 20S proteasome β5 subunit targeted by bortezomib expression at day 32. ATRA alone inhibited the mRNA levels of both POMP (Fig. 7D, left panel) and the β5 proteasome subunit (PSMB5; Fig. 7D, right panel) compared with the vehicle controls, whereas bortezomib alone stimulated expression of these two genes. In contrast, the addition of ATRA to bortezomib significantly reduced POMP and β5 expression compared with bortezomib alone, and these returned to levels comparable with those seen in vehicle-treated controls. Finally, both POMP and β5 expression at the protein level changed in a pattern consistent with that of their mRNAs (Fig. 7E).

FIGURE 5. NRF2, POMP, and proteasome activity. A, KAS-6/1 bortezomib-resistant cells were transfected with lentiviral vectors expressing a scrambled sequence, non-targeting shRNA (KO-NT), or one of two different shRNAs targeting and suppressing NRF2 (KO-6 and KO-8). Knockdown of NRF2, and its impact on downstream POMP, was examined by Western blotting and compared with β-actin as a loading control. Densitometry was performed to quantify NRF2 and POMP levels, which were normalized to KAS-6/1 V10R cells arbitrarily set to 1.0. A representative autoradiograph from one of two independent experiments is shown. B, proteasome activity of the cells described in A was examined as detailed under “Experimental Procedures.” Data are from three independent experiments and are presented as the mean ± S.D. (*, p < 0.05 versus KAS-6/1 V10R or KO-NT). C, cells described in A and KAS-6/1 bortezomib-sensitive (KAS-6/1 WT) cells were then exposed to bortezomib for 24 h at the indicated concentrations, and viability was determined with the tetrazolium reagent WST-1. Data presented are from three independently performed experiments and are presented as the mean ± S.D. (*, p < 0.05 versus KAS-6/1 V10R or KO-NT). D, KAS-6/1 bortezomib-sensitive (KAS-6/1 WT) cells were transfected with control lentiviral vectors (KAS-6/1 OE-control) or lentiviral vectors containing the NRF2 cDNA (KAS-6/1 OE-NRF2). Expression of NRF2 and POMP was examined with Western blotting and compared with β-actin as a loading control. Densitometry was performed to quantify NRF2 and POMP levels, which were normalized to KAS-6/1 WT cells arbitrarily set to 1.0. A representative autoradiograph from one of two independent experiments is shown. E, proteasome activity in the cells described in D was examined as under “Experimental Procedures.” Data are from three independent experiments and are presented as the mean ± S.D. (*, p < 0.05 versus KAS-6/1 WT or KAS-6/1 OE-NRF2). G, OPM-2 bortezomib-resistant cells were transfected with lentiviral vectors expressing a scrambled sequence, non-targeting shRNA (KO-NT), or one of two different shRNAs targeting and suppressing NRF2 (KO-4 and KO-8). Knockdown of NRF2, and its impact on downstream POMP, was examined by Western blotting and compared with β-actin as a loading control. Densitometry was performed to quantify NRF2 and POMP levels, which were normalized to OPM-2 V10R cells arbitrarily set to 1.0. A representative autoradiograph from one of two independent experiments is shown. H, proteasome activity of the cells described in G was examined as described under “Experimental Procedures.” Data are from three independent experiments and are presented as the mean ± S.D. (*, p < 0.05 versus OPM-2 V10R or OPM-2 V10R KO-NT). I, cells described in G and OPM-2 bortezomib-sensitive (OPM-2 WT) cells were then exposed to bortezomib for 24 h at the indicated concentrations, and viability was determined with the tetrazolium reagent WST-1. Data presented are from three independently performed experiments and are presented as the mean ± S.D. (*, p < 0.05 versus OPM-2 V10R or OPM-2 V10R KO-NT). J, OPM-2 bortezomib-sensitive (OPM-2 WT) cells were then exposed to bortezomib for 24 h at the indicated concentrations, and viability was determined with the tetrazolium reagent WST-1. Data presented are from three independently performed experiments and are presented as the mean ± S.D. (*, p < 0.05 versus OPM-2 OE-NRF2).
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**FIGURE 6. ATRA and bortezomib sensitivity.** A, KAS-6/1 bortezomib-resistant cells (KAS-6/1 V10R) were exposed to the indicated concentrations of ATRA, bortezomib, or both for 24 h, and expression of NRF2, KEAP1, and POMP was examined by Western blotting. A representative autoradiograph from one of two independent experiments is shown. Densitometry was performed to quantify NRF2, KEAP1, and POMP levels, which were normalized to the indicated concentrations of ATRA, bortezomib, or both for 24 h. Cellular viability measurements were then performed using the WST-1 assay as described under "Experimental Procedures." All data points were normalized to the vehicle control, which was arbitrarily set at 100% viability. Mean viability values are provided from three independently performed experiments ± S.D., and the Student’s paired t test was used to determine statistical significance (*, p < 0.05). B, KAS-6/1 bortezomib-resistant cells were treated with the indicated concentrations of ATRA, bortezomib, or both for 24 h. Cellular viability measurements were then performed using the WST-1 assay as described under "Experimental Procedures." All data points were normalized to the vehicle control, which was arbitrarily set at 100% viability. Mean viability values are provided from three independently performed experiments ± S.D., and the Student’s paired t test was used to determine statistical significance (*, p < 0.05). C, levels of apoptosis were determined in cells treated as described in B by Western blotting to detect the cleaved fragment of caspase 3, with β-actin as a loading control. A representative autoradiograph from one of two independent experiments is shown. Densitometry was performed to quantify the cleaved caspase 3 level, which was normalized to the vehicle control arbitrarily set to 1.0. D, proteasome chymotrypsin-like activity was measured as described under "Experimental Procedures" in cells treated as above. All data points were normalized to the vehicle control, which was arbitrarily set at 100% activity. Mean proteasome activity values are provided from three independent experiments ± S.D., and the Student’s paired t test was used to determine statistical significance (*, p < 0.05). OPM-2 bortezomib-sensitive cells (OPM-2 WT) were exposed to the indicated concentrations of ATRA, bortezomib, or both for 24 h, and expression of NRF2, KEAP1, and POMP, cellular viability, and abundance of cleaved caspase 3 and proteasome chymotrypsin-like activity are shown in I–L, respectively. E, OPM-2 bortezomib-resistant cells (OPM-2 V10R) were exposed to the indicated concentrations of ATRA, bortezomib, or both for 24 h, and expression of NRF2, KEAP1, and POMP was tested by Western blotting, all relative to β-actin as a loading control. A representative autoradiograph from one of two independent experiments is shown. Densitometry was performed to quantify NRF2, KEAP1, and POMP levels, which were normalized to the vehicle control arbitrarily set to 1.0. F, OPM-2 bortezomib-resistant cells were treated with the indicated concentrations of ATRA, bortezomib, or both for 24 h. Cellular viability measurements were then performed using the WST-1 assay as described under "Experimental Procedures." All data points were normalized to the vehicle control, which was arbitrarily set at 100% viability. Mean viability values are provided from three independently performed experiments ± S.D., and the Student’s paired t test was used to determine statistical significance (*, p < 0.05). G, levels of apoptosis were determined in cells treated as described in E by Western blotting to detect the cleaved fragment of caspase 3, with β-actin as a loading control. A representative autoradiograph from one of two independent experiments is shown. Densitometry was performed to quantify the cleaved caspase 3 levels, which were normalized to the vehicle control arbitrarily set to 1.0. H, proteasome chymotrypsin-like activity was measured as described under "Experimental Procedures" in cells treated as above. All data points were normalized to the vehicle control, which was arbitrarily set at 100% activity. Mean proteasome activity values are provided from three independent experiments ± S.D., and the Student’s paired t test was used to determine statistical significance (*, p < 0.05). OPM-2 bortezomib-sensitive cells (OPM-2 WT) were exposed to the indicated concentrations of ATRA, bortezomib, or both for 24 h. Expression of NRF2, KEAP1, and POMP, cellular viability, expression of cleaved caspase 3, and proteasome chymotrypsin-like activity are shown in M–P, respectively.

been shown to enhance proteasome subunit expression through signaling pathways involving NRF2 (42, 43). However, because overexpression of POMP was by itself sufficient to induce bortezomib resistance in drug-naïve cells (Fig. 2), this suggests that the compendium of NRF2-regulated genes was not required for this phenotype and that POMP may be rate-limiting. In addition, this observation is especially interesting because NRF1 was previously implicated in the recovery of mammalian cells from proteasome inhibition by up-regulating proteasome subunit expression (44). Together, these findings
suggest that NRF1 and NRF2 may work in a coordinated fashion, with the former inducing proteasome subunits and the latter enhancing proteasome assembly, both of which would be needed to restore full proteasome function. Because knockdown of NRF2 reduced proteasome activity, and its overexpression enhanced proteasome capacity (Fig. 5), we then studied the NRF2 inhibitor ATRA, which sensitized resistant cells to bortezomib, and also to some extent enhanced bortezomib efficacy in sensitive cells, although to a much lesser extent (Fig. 6). The lesser impact of ATRA in sensitive cells was expected, as bortezomib shows strong activity against drug-naive myeloma models, and baseline levels of POMP in sensitive cells are lower (Fig. 1). Notably, ATRA consistently reduced levels of both NRF2 and POMP in bortezomib-naive and -resistant cells either alone or in combination with bortezomib. Our finding of increased activation of NRF2 is consistent with the data of Stessman et al. (45), who found in mouse and human cell line models of myeloma that bortezomib resistance produced a gene signature enriched for downstream targets of this transcription factor, although they did not look at what downstream NRF2 effectors could be involved.

ATRA with bortezomib enhanced activity against primary plasma cells and, in our in vivo studies, against a murine model of bortezomib resistance (Fig. 7). We used a subcutaneous xenograft model in these studies, which probably best represents myeloma with an extramedullary plasmacytoma. This has been associated with a poor clinical prognosis in myeloma patients (46) and may be linked to bortezomib resistance (47), but it does not fully recapitulate a physiologically relevant bone marrow microenvironment. Thus, studies in a systemic
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myeloma model or a humanized model providing bone cells, immune cells, and the appropriate cytokine milieu (48) could provide further insights into the utility of ATRA as an approach to resensitize to bortezomib. In our in vivo modeling, ATRA reduced POMP mRNA and protein levels, which was expected based on its impact on the NRF2-POMP axis. Also of interest was that β5 subunit protein and mRNA expression levels were suppressed by ATRA. The reduction of β5 protein could be due to the short half-life of the β5 precursor (49), whose turnover could be enhanced when it cannot be incorporated into proteasomes because of reduced POMP levels. Alternatively, or in addition to that, ATRA may have a direct effect on the PSMB5 gene to reduce promoter transcription and thereby protein levels, which would provide another mechanism for it to enhance the activity of bortezomib. Additional studies will therefore be needed to fully elucidate the effects of ATRA on proteasome biogenesis pathways. However, because inhibition of NRF2 and POMP using shRNAs was sufficient to enhance the efficacy of bortezomib, at least part of ATRA’s sensitization likely is due to its effect on the NRF2/POMP axis.

POMP is a proteasome assembly chaperone that is involved in the addition of subunits to a pre-formed ring of seven subunits (50) and generates a hemi-proteasome once the β ring assembly is completed. Two of these hemi-proteasomes are then combined to form the 20S core particle, which contains all of the proteolytic activities of the proteasome (21, 22). In addition, POMP can bind to endoplasmic reticulum membranes to facilitate proteasome assembly close to one of the major sites at which proteasomes function (51), but POMP is ultimately cleaved by the proteasome once the latter is activated (21, 22). A number of studies have previously shown that transient inhibition of the proteasome produces up-regulation of proteasome subunit synthesis (52, 53), as cells attempt to restore normal protein homeostasis. POMP is also up-regulated under such conditions, but it has not been completely clear whether this was due to coordinate regulation of POMP with proteasome subunits or whether this was simply because POMP degradation was suppressed by proteasome inhibition. Our data show that POMP overexpression can be a genetically stable, acquired phenotype in proteasome inhibitor resistance, because these cells were free of bortezomib treatment for as long as 8 weeks or more. Also, in that POMP overexpression or suppression was by itself sufficient to confer resistance or sensitization to bortezomib, respectively, our findings indicate that POMP alone, aside from any impact on NRF2, is a mediator of bortezomib sensitivity. Thus, our cell lines may serve to some extent as models of what is seen clinically, because retreatment with bortezomib, even in patients who had all previously responded well to this agent, produces response rates of only 50–60% (54, 55), indicating a rapid acquisition of resistance. Moreover, the involvement of POMP may provide some indication of why these patients have a poor overall prognosis, because both NRF2 (56) and POMP (30) have been linked to cellular defense mechanisms against electrophilic and oxidative stress. In that other drugs used against myeloma work in part by generating reactive oxygen species, including alkylating agents and anthracyclines, activation of the NRF2/POMP axis may reduce sensitivity to these other drug classes as well.

Finally, our translational studies suggest that strategies targeting and suppressing the NRF2/POMP axis may be attractive ones to enhance bortezomib sensitivity in drug-naïve patients and to restore some sensitivity in drug-resistant patients. Approaches that should be successful in this regard include the use of NRF2 inhibitors or of agents that would induce KEAP1, which would contribute to turnover of NRF2 (57) and thereby reduce POMP levels. In this work, we have validated ATRA as one such strategy, and this is clinically relevant, because ATRA is already in use against acute promyelocytic leukemia (33). A regimen of ATRA with bortezomib could therefore be piloted first in phase I to determine its safety and then to examine its ability to overcome resistance to this proteasome inhibitor in larger and preferably randomized phase II or III studies.

Author Contributions—B. L. designed and performed the majority of the experiments, analyzed the data, prepared the figures, and wrote a draft of the manuscript. J. F. facilitated access to primary samples. J. F., P. C., X. G., and Y. L. assisted with some experiments and were involved in data analysis and manuscript preparation and provided statistical analyses of mouse xenograft modeling. I. K., H. W., and X. D.-Z. performed in vivo experiments. H. W. generated lentiviral constructs. R. Z. O. provided research guidance, supervised the work herein, and proofed the manuscript.

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