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Research Paper

Copper–zinc superoxide dismutase-mediated redox regulation of bortezomib resistance in multiple myeloma

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ABSTRACT

Multiple myeloma (MM) is an incurable B-cell malignancy. The proteasome inhibitor bortezomib (BTZ) is a frontline MM drug; however, intrinsic or acquired resistance to BTZ remains a clinical hurdle. As BTZ induces oxidative stress in MM cells, we queried if altered redox homeostasis promotes BTZ resistance. In primary human MM samples, increased gene expression of copper–zinc superoxide dismutase (CuZnSOD or SOD1) correlated with cancer progression, high-risk disease, and adverse overall and event-free survival outcomes. As an *in vitro* model, human MM cell lines (MM.1S, 8226, U266) and the BTZ-resistant (BR) lines (MM.1SBR, 8226BR) were utilized to determine the role of antioxidants in intrinsic or acquired BTZ-resistance. An up-regulation of CuZnSOD, glutathione peroxidase-1 (GPx-1), and glutathione (GSH) were associated with BTZ resistance and attenuated prooxidant production by BTZ. Enforced overexpression of *SOD1* induced BTZ resistance and pharmacological inhibition of CuZnSOD with disulfiram (DSF) augmented BTZ cytotoxicity in both BTZ-sensitive and BTZ-resistant cell lines. Our data validates CuZnSOD as a novel therapeutic target in MM. We propose DSF as an adjuvant to BTZ in MM that is expected to overcome intrinsic and acquired BTZ resistance as well as augment BTZ cytotoxicity.

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Introduction

Multiple myeloma (MM) is the second most prevalent hematological malignancy in the United States [1]. The currently available frontline chemotherapeutic drugs have shown excellent disease remissions and improved the relative 5-year survival rate [2]. Unfortunately, the majority of MM patients rapidly develop drug-resistant disease and incurs uniform mortality [3]. The proteasome inhibitor bortezomib (BTZ, Velcade) is a frontline MM drug that provides excellent remissions, however, resistance to BTZ can arise [4]. Interestingly, BTZ treatment increases oxidative stress in certain cancers including MM [5–8] and induces ROS-mediated c-Jun NH_2-terminal kinase (JNK) activation and MM cell apoptosis [9]. However, the effects of BTZ on antioxidant defense systems and therapy resistance in MM remains unclear. A deeper understanding on unique biochemical targets in drug-resistant MM disease can therefore provide avenues for designing innovative drug combinations to improve survival and remission rates in MM.

Clinical studies suggest that MM patients have increased systemic oxidative stress [10–13]. An up-regulation of cellular antioxidant defense systems can offer protection against endogenous and therapy-induced oxidative stress [14]. Superoxide dismutases (SODs) constitute an important part of the free-radical scavenger system and function as the first line of defense against oxidative damage by catalyzing the dismutation of superoxide anions ($O_2^{\bullet -}$), yielding hydrogen peroxide (H_2O_2) and O_2 [15,16]. Copper–zinc superoxide dismutase (CuZnSOD or SOD1) comprises ~90% of the total SOD levels in mammalian cells and is mainly localized in the cytoplasm as well as the nucleus, peroxisomes, and the intermembrane space of the mitochondria [17,18]. Emerging evidence suggests that SOD1 may regulate cancer progression and oxidative stress resistance via different mechanisms [19,20] and serve as a novel target for cancer therapy [17,21]. Resistance to the

Abbreviations: ATN-224, choline tetrathiomolybdate; BR, BTZ resistant; BTZ, bortezomib; CI, confidence interval; Cu, copper; CuZnSOD, copper–zinc superoxide dismutase; DDC, diethyldithiocarbamate; DHE, dihydroethidium; DSF, disulfiram; EFS, event free survival; ER, endoplasmic reticulum; GEP, gene expression profile; GPx, glutathione peroxidase; GSH, glutathione; GSSG, glutathione disulfide; GST, glutathione S-transferase; H_2O_2, hydrogen peroxide; H_2DCF-DA, carboxy-2′,7′-dichlorodihydrofluorescein diacetate; MFI, mean fluorescence intensity; MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; MnSOD, manganese superoxide dismutase; NAC, N-acetylcysteine; NF-κB, nuclear factor-kappa B; NPC, Normal plasma cells; NSF, normalized survival fraction; PDI, protein disulfide isomerase; PE, plating efficiency; PSMB5, proteasome β5 subunit; $O_2^{\bullet -}$, superoxide; Os, overall survival; ROS, reactive oxygen species; SOD, superoxide dismutase; UPR, unfolded protein response

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prooxidant drug imexon has been linked to increased CuZnSOD protein levels in MM [22].

In the current study, we analyzed the gene expression level of CuZnSOD in primary human samples and found a positive correlation between *SOD1* expression and MM disease progression and prognostic clinical outcome. In MM cell line model, a concerted up-regulation of CuZnSOD and the H_2O_2-detoxifying enzyme glutathione peroxidase (GPx-1) was linked to BTZ resistance. The copper chelating drug disulfiram (DSF, Antabuse) was utilized to inhibit CuZnSOD activity; DSF is a clinically approved drug for aversion therapy in alcoholics and is being repurposed as an anti-cancer drug [23]. We demonstrate that DSF reversed BTZ resistance and increased BTZ cytotoxicity in MM and provide the preclinical rationale to combine DSF with BTZ for improving therapy responses in MM.

Methods

Microarray analysis of SOD1 expression and clinical prognosis in primary human samples

The gene expression profiling (GEP) data of total therapy (TT) 2 trial was analyzed for transcriptional expression of CuZnSOD. Human samples of normal plasma cells (NPC, $n=22$), or monoclonal gammopathy of undetermined significance (MGUS, $n=44$), or MM patients with overt MM disease requiring therapy ($n=351$) were run on the Affymetrix U133Plus2.0 microarray (Santa Clara, CA) [24,25]. These data are deposited in the NIH Gene Expression Omnibus (accession number GSE2658). *SOD1* expression was also analyzed in MM patients treated under an NIH-sponsored clinical trial (UARK 98-026) utilizing induction regimen followed by melphalan-based tandem auto-transplantations, consolidation chemotherapy, and maintenance treatment. In this study, the 70-gene model was used to identify high-risk and low-risk group of MM patients where high-risk group comprised of patients with shorter durations of complete remission, overall survival (OS), and event-free survival (EFS) [24]. Cox proportional hazard models were used to estimate OS and EFS hazard ratios and 95% confidence interval (CI) for *SOD1* as a continuous variable. *SOD1* expression was categorized by high and low using the upper (Q4) and lower quartiles (Q1, Q2, and Q3) and Kaplan–Meier curves were created (Biostatistics Core, UI).

Cell culture and development of BTZ-resistant MM cell lines

Human MM cell lines RPMI-8226 (8226), MM.1S, and U266B1 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The properties of these cell lines are outlined in Supplementary Table 1. All cell lines were routinely grown in RPMI 1640 medium (Gibco, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin (Gibco), 100 mg/ml streptomycin (Gibco), and 50 μM β-mercaptoethanol at 37 °C and 5% CO_2. The BTZ-resistant (BR) MM.1S subline (MM.1SBR) was established by stepwise increasing BTZ (LC laboratories, Woburn, MA) concentration over a period of 3 months; using a similar approach we have successfully established the BTZ-resistant 8226 subline (8226BR) [26]. These BR cells were adapted to a final concentration of 20 nM BTZ. Stable genotype of BR cells was confirmed by BTZ washout experiment for 2 weeks followed by dose response assays with BTZ.

Cell titer blue (CTB) viability assay

Cells were seeded in a black, clear bottom 96-well plates at a density of 1×10^4 cells/100 μl media for 24 h. Cells were then exposed to BTZ (5, 15, 30 nM) and/or N-acetylcysteine (NAC, 5 mM, Sigma-Aldrich, St. Louis, MO), and/or DSF (5 μM, Sigma-Aldrich) for 48 h after which 20 μl of the redox sensitive dye (resazurin, Promega, Madison, WI) was added. Plates were incubated at 37 °C for 2.5 h and cell viability was analyzed by measuring fluorescence ($\lambda_{ex}=560$ nm, $\lambda_{em}=590$ nm, Infinite M200 plate reader, Tecan US, Inc.). All treatments were performed in triplicate and the mean ± SD was determined.

Clonogenic survival assay

Cells were seeded overnight in 6-well plates (1×10^5 cells/ml), and treated with BTZ (5, 15, 30 nM) and/or DSF (5 μM, Sigma-Aldrich) for 24 h. Clonogenic cell survival was determined using the limiting dilution method [27]. The plating efficiency (PE), survival fractions, and normalized survival fraction (NSF) was calculated for each cell population as described before [28,29]. Clonogenic assays for 8226, 8226BR and U266 cell lines were not performed as they displayed poor plating efficiency (data not shown).

20S proteasome activity

Cells were grown at a density of 7.5×10^5/ml for 24 h. Cells were collected, washed with cold PBS, and lysed in 50 mM Hepes, 5 mM EDTA, 150 mM NaCl, and 1% Triton X-100 (pH 7.5). Protein estimation was done using Bradford reagent. 20S proteasome activity was determined by using an activity kit (EMD Millipore, Temecula, CA) that uses a 20S substrate (LLVY) conjugated to the fluorophore 7-amino-4-methylcoumarin (AMC). The 20S proteasome activity was measured by AMC fluorescence ($\lambda_{ex}=380$ nm, $\lambda_{em}=460$ nm) and expressed as relative fluorescence intensity (RFI). Assay was calibrated using standard solutions of AMC and lactacystin was used to determine assay specificity.

Antioxidant enzyme assays

For measurement of antioxidant enzyme activities, cells were seeded in media at a density of 7.5 10^5 cells/ml for 24 h. Cells were then washed with cold phosphate buffered saline (PBS, 137 mM NaCl, 10 mM phosphate and 2.7 mM KCl, pH 7.4). Whole cell homogenates were made by adding DETAPAC buffer (50 mM potassium phosphate buffer pH=7.8 with 1.34 mM diethylenetriaminepenta–acetic acid) to cell pellets following one freeze–thaw cycle. *Superoxide dismutase (SOD):* This assay is based on the reduction of nitroblue tetrazolium (NBT) modified by Spitz and Oberley [30]. NaCN (5 mM, 30 min) was added to measure MnSOD activity. CuZnSOD activity was determined by subtracting MnSOD activity from the total SOD activity. Activity data are presented as units (U) of SOD activity per milligram of protein. *Glutathione peroxidase (GPx)-1*: GPx-1 activity was determined by incubating cell lysates with GSH, glutathione reductase (GR), and NADPH in a cuvette for 5 min at room temperature (RT). 2.5 mM H_2O_2 was added to the cuvette and oxidation of NADPH was measured at 340 nm for 5 min. 1 unit of GPx activity is defined as 1 μM of NADPH oxidized per min and is expressed as milliunits (mU) per milligram of protein [31]. Effective GPx activity was calculated as described by Li et al. [32]. *Catalase:* Catalase activity was determined by measuring the decay of H_2O_2 at 240 nm in potassium phosphate buffer and expressed as milli-k units (mkU) per milligram of protein [33].

Glutathione (GSH) assay

Cells were seeded in media at a density of 7.5×10^5 cells/ml for 24 h. Cells were then pelleted (800 g for 5 min at 4 °C), rinsed once with cold PBS and re-suspended in 5% sulfosalicylic acid (SSA,

Sigma-Aldrich). The 5,5′-dithiobis-2-nitrobenzoic (DTNB) acid recycling assay was used to quantify GSH and oxidized GSH (GSSG) levels in supernatants [34]. Briefly, supernatants were treated with 2-vinylpyridine (Sigma-Aldrich) for 2 h to measure GSSG, or left alone for total GSH estimations. NADPH, DTNB, ddH_2O, GR, and sample/blank were mixed in a 1 ml cuvette; absorbance was measured for a span of 2.5 min. Sample data were normalized to protein content as determined by bicinchoninic acid protein assay.

Western blot analysis

Cells were grown at a density of 7.5×10^5/ml for 24 h. Cells were collected, washed with cold PBS, and lysed in radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors [26,28,29]. Protein estimation was performed using Bradford reagent. Equal amounts of protein were electrophoresed in a 12% reducing SDS-PAGE gel. Proteins were transferred to PVDF membranes; non-specific binding was blocked with 5% non-fat milk in TBST buffer (4 mM Tris base, 10 mM NaCl, pH 7.5, 0.1% Tween-20), and incubated with the indicated primary antibody at 4 °C overnight. Antibodies against CuZnSOD (gift from Dr. Oberley, University of Iowa, IA), poly ADP-ribose polymerase (PARP, full-length, Cell Signaling Technology, Danvers, MA), and actin (Cell Signaling Technology) were used. Blots were then incubated for 1 h at RT with HRP-tagged secondary antibody and developed using an enhanced chemiluminescence assay (Thermo Scientific, Waltham, MA). Bands were visualized by autoradiography and protein expression was quantified using ImageJ 1.38 × software (http://rsbweb.nih.gov/ij/index.html).

Detection of intracellular reactive oxygen species (ROS)

Steady-state levels of intracellular prooxidants were measured using fluorescent probe dye carboxy – 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA, Molecular probes, Eugene, OR) as described before [28,29]. After treatment, cells were collected, washed, and labeled with oxidation-sensitive H_2DCF-DA probe (10 μg/ml, 15 min at 37 °C) in PBS. As a positive control for H_2DCF-DA oxidation, cells were treated with menadione (100 μM for 2 h, Sigma-Aldrich); menadione is a quinone-containing compound that forms semiquinones and increases ROS levels [35]. After labeling cells were kept on ice and analyzed using a FACScan flow cytometer (Becton Dickinson, λ_{ex}=495 nm, λ_{em}=530 nm). Intracellular levels of prooxidants (presumably $O_2^{\bullet -}$) were estimated using the redox-sensitive fluorescent probe dihydroethidium (DHE, Molecular probes) [28,29]. Briefly, cells were collected, washed, and labeled with DHE (10 μM, 40 min at 37 °C) in PBS containing 5 mM pyruvate. For positive control, cells were incubated with antimycin A (10 μM, Sigma-Aldrich) along with the probe; treatment with antimycin A generates mitochondrial $O_2^{\bullet -}$ by inhibiting electron transport between cytochrome b and c [36]. After labeling the cells were kept on ice and analyzed using a FACScan flow cytometer (λ_{ex}=405 nm, λ_{em}=585). For measuring H_2DCF-DA and DHE oxidation, the mean fluorescence intensity (MFI) of 10,000 cells was analyzed in each sample and corrected for by unlabeled cells. The MFI was normalized to control for each cell line to determine the relative MFI.

Quantitative real-time PCR (qPCR)

For qPCR analysis, total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA) and quantified. cDNA was synthesized from 400 ng of total RNA, using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) and subjected to qPCR analysis with the following primers (5′→3′, sense and antisense respectively): *SOD1,* TGGTGTGGCCGATGTGTCTA and TTCATGGACCACCAGTGTGC, amplicon length 88 bp; *SOD2*, GCCTGCACTGAAGTTCAATGG and GCTTCCAGCAACTCCCCTTT, amplicon length 105 bp; *CAT*, TTCGGTTCTCCACTGTTGCTG and AATTTCACTGCAAACCCACGA, amplicon length 76 bp; *GPX1*, AACGATGTTGCCTGGAACTTTG and GAAGCGGCGGCTGTACCT, amplicon length 79 bp; and *18S*, CCTTGGATGTGGTAGCCGTTT and AACTTTCGATGGTAGTCGCCG, amplicon length 105 bp. Primers were designed using the Universal Probe Library Assay Design Center software (Roche, Basel, Switzerland). The assay was performed using synthesized cDNA (20 ng), primers (100 μM each), and 2 × SYBR Green/ROX PCR master mix. Results were analyzed using ABI 7500 software v2.0.5. The C_T values for the target genes in all of the samples were normalized on the basis of the abundance of the 18S transcript, and the fold difference (relative abundance) was calculated using the formula $2^{-\Delta\Delta}C_T$ as previously described [26,29].

Caspase-3 activity

Apoptosis was measured using a caspase-3 fluorescence assay (Cayman Chemical, Ann Arbor, MI) as previously described [26,28]. Caspase-3 activity is expressed as units per milligram of total protein.

Statistical analysis

GraphPad Prism 6.04 software (GraphPad Software, San Diego, CA) was used for data handling, analysis, and presentation. Statistical significance was determined using either two-tailed unpaired *t* test or one-way ANOVA with Tukey post-test with confidence interval 95%. For calculating the half maximal inhibitory concentration (IC_{50}) with 95% confidence intervals, the nonlinear regression log (BTZ) vs. normalized response variable slope option was used. For Kaplan–Meier survival curves, all statistical tests were two-sided and assessed for significance with the SAS 9.3 software package (Cary, NC). $P<0.05$ was considered to be statistically significant.

Results

Retrospective analysis of GEP data from NPC, MGUS, and MM clinical trials showed that *SOD1* expression increases in MGUS and MM cells (NPC < MGUS < MM; $P<0.05$; Fig. 1A). Specifically, MGUS (7340 ± 365, raw expression values) and MM (10,775 ± 202) showed a 1.6- and 2.4-fold increase compared to NPC (4486 ± 205) in *SOD1* expression, respectively (Fig. 1A). When gene expression was analyzed in high-risk and low-risk group of MM patients; high-risk patients exhibited 1.4-fold increased *SOD1* expression relative to low-risk patients (13,838 ± 450 and 10,243 ± 210, respectively, $P<0.05$) (Fig. 1B). Next, survival analysis methods were used to estimate and compare the impact of the *SOD1* expression on the OS and EFS for MM patients. When looking at *SOD1* as a continuous variable, the death risk increased 1.3 times or 32% (CI=1.2, 1.6, $P<0.05$) and the risk of an event increased 1.2 times or 17% (CI=1.0,1.4, $P<0.05$) with each 3789 unit increase in *SOD1*. For OS, the risk of death for MM patients with high *SOD1* levels was 1.5 times higher than for patients with low levels (Fig. 1C). Similarly, for the EFS, the risk of an event for MM patients with high *SOD1* level was noted as 2.0 times greater than for patients with low levels (Fig. 1D). To further validate our finding we queried the TT3 data set (thalidomide+BTZ), however, unlike the results from the TT2 cohort, a correlation between *SOD1* expression and survival was not present. These results may be explained by the short median follow-up of 12 months compared to a median follow-up of 36 months for the TT2 cohort, suggesting that the effects from *SOD1* occurs later in disease progression.

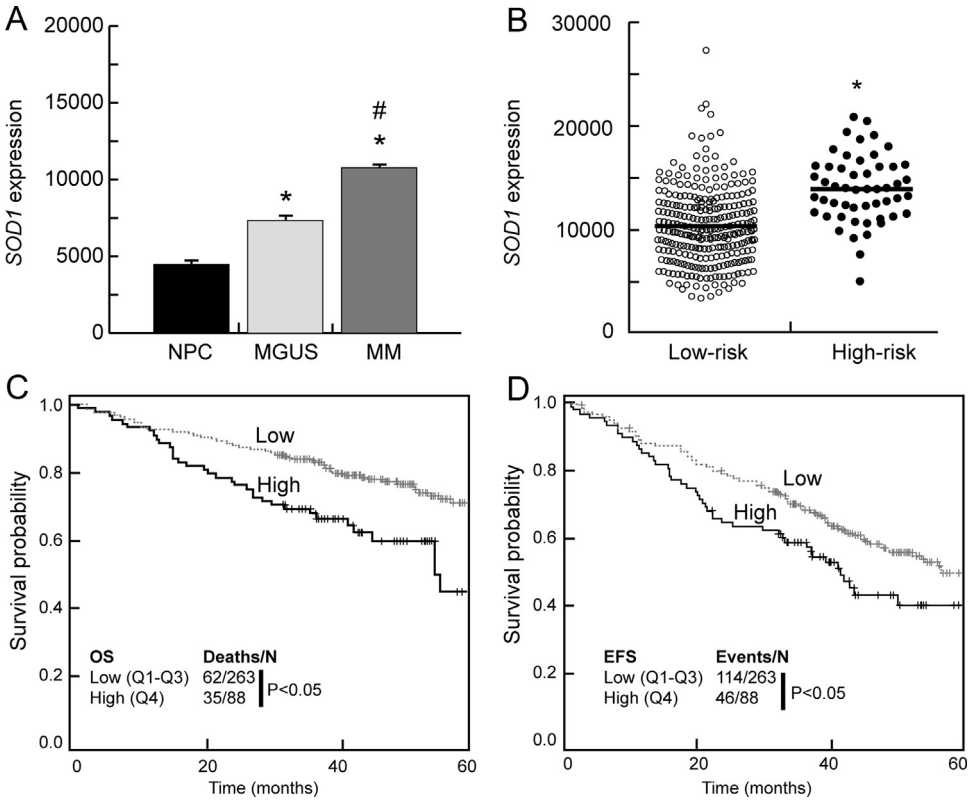

Fig 1. Increased *SOD1* expression in MM associates with disease progression and poor prognosis. GEP data for *SOD1* was compared from a single probeset (Affymetrix ID 200642) using (A) NPC ($n=22$), MGUS ($n=44$), or MM patients samples ($n=351$), (B) MM patients with low-risk ($n=254$) vs. high-risk ($n=47$) disease. $^{*}P<0.001$ vs. NPC or low risk MM, $^{\#}P<0.005$ vs. MGUS. Kaplan–Meier curves were generated for (C) overall survival and (D) event free survival.

Overall, retrospective analysis of primary samples shows that increased *SOD1* expression portends poor prognosis in MM.

We used a well-established human MM cell line (MM.1S) to determine if BTZ-mediated cytotoxicity was primarily rendered via increased oxidative stress. The causal role of redox perturbations in cell killing was assessed using the non-specific thiol antioxidant N-acetylcysteine (NAC) [28,29]. BTZ treatment (15 and 30 nM) decreased cell viability and NAC abrogated BTZ-mediated cell killing, indicating that oxidative stress and BTZ sensitivity are potentially linked in MM (Fig. 2A). Based on the protective role of NAC on BTZ-mediated cytotoxicity in MM.1S cell line (Fig. 2A) and clinical data showing increased *SOD1* expression with poor MM prognosis (Fig. 1), we hypothesized that BTZ resistance in MM is associated with adaptation to oxidative stress via up-regulation of antioxidant defense systems. To test this hypothesis, we established an *in vitro* platform by utilizing human MM cell lines (MM.1S, 8226, and U266) and their BR counterparts (MM.1SBR and 8226BR); the 8226 and 8226BR pair has developed and published [26]. The dose response curves for BTZ for drug-naïve cells showed BTZ cytotoxicity pattern as MM.1S < 8226 < U266 with MM.1S being the most sensitive (Fig. 2B). When adaptive BTZ resistance was assessed, the MM.1SBR and 8226BR cells showed decreased BTZ cytotoxicity relative to drug naïve MM.1S and 8226 counterparts (Fig. 2B). Also, when exposed to BTZ (30 nM), the BR cells (adapted to 20 nM BTZ) showed a trend towards increased cytotoxicity relative to BTZ (15 nM) suggesting that the BR cells remain sensitive to BTZ treatment at higher BTZ concentrations. Clonogenic survival assays were performed to confirm a differential BTZ cytotoxicity in MM.1S and MM.1SBR cells. MM.1S cells showed significant clonogenic killing with BTZ (15 and 30 nM) while MM.1SBR cells were relatively resistant to BTZ (Fig. 2C).

Oxidative stress induces both necrotic as well as a more physiologically relevant apoptotic cell death [37]. To determine if BTZ-induced oxidative stress mediates apoptosis in MM cell lines, we analyzed the activation of caspase-3 and proteolytic cleavage of PARP after incubation with 15 nM BTZ for 24 h. BTZ treatment showed caspase-3 activation in MM.1S and 8226 cells (by approximately 2-fold) but not in BTZ-resistant cell lines (MM.1SBR, 8226BR, and U266, Fig. 2D). Interestingly, MM.1SBR cells displayed a 40% decrease in caspase-3 activity that may be due to adaptation of these cells to 20 nM BTZ. The caspase inhibitor (Ac-DEVD-CHO) completely abrogated BTZ-induced apoptosis showing the specificity of the caspase-3 assay (data not shown). To further confirm BTZ-mediated apoptosis in BR cells, PARP Western blot analysis was performed. BTZ treatment resulted in decrease PARP cleavage in BTZ adapted cells (MM.1SBR and 8226BR) relative to the drug naïve counterparts; also BTZ-mediated PARP cleavage was more prominent in MM.1S cells relative to 8226 cell lines (Fig. 2E). Overall, caspase-3 and PARP results indicate that BTZ treatment induces caspase-dependent apoptosis in BTZ-sensitive and not BTZ-resistant (intrinsic and adapted) MM cell lines.

Studies have shown that *in vitro* adaptation of cancer cell lines to BTZ renders resistance via multiple mechanisms [38] and frequently includes point mutations in the gene encoding the proteasome β5 subunit (PSMB5) [39–41], or shift from immunoproteasome β5 subunit (β5i) to mutant β5 subunit [39,42,43]. Also, sensitivity to proteasome inhibitors has been correlated with proteasome workload with MM cell lines with intrinsic sensitivity to BTZ displaying higher protein synthesis but low proteasome activity [44]. To further characterize the mechanism of BTZ resistance in MM cell line panel, we evaluated basal proteasome activity in the cell line panel. The drug naïve MM cell lines (MM.1S, 8226, and U266) displayed similar levels of endogenous proteasome activity (Fig. 2F). However, BTZ-resistant variants (MM.1SBR, 8226BR) showed decrease in basal proteasome activity compared to the their drug-naïve counterparts (Fig. 2F).

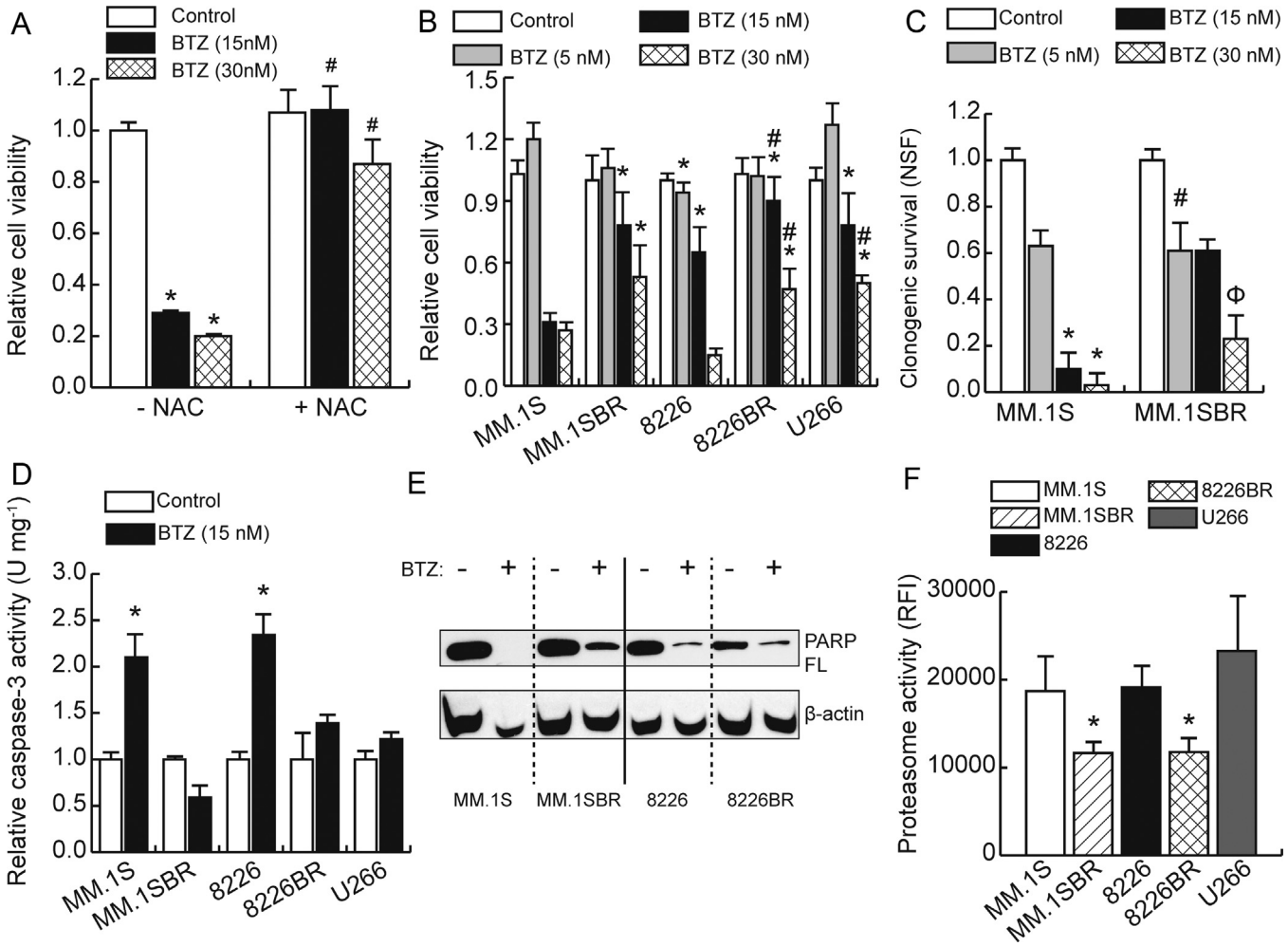

Fig 2. *In vitro* model for evaluating BTZ resistance in MM. Cell viability was assessed using CTB assay and normalized to untreated control cells. (A) MM.1S cells were treated with BTZ (15 or 30 nM) in the presence or absence of NAC (5 mM) for 48 h. $^{*}P <$ vs. control, $^{\#}P < 0.05$ vs. respective BTZ concentration. (B) Drug-naïve (MM.1S, 8226, U226) and BR variants (MM.1SBR, 8226BR) cells were treated with BTZ (5, 15, or 30 nM) for 48 h. $P < 0.05$ vs. MM.1S, $^{\#}P < 0.05$ vs. 8226. (C) Clonogenic survival of MM.1S and MM.1SBR cells treated with BTZ (5, 15, or 30 nM) for 24 h and normalized to untreated control cells. $^{*}P < 0.05$ vs. control MM.1S cells, $^{\#}P < 0.05$ vs. MM.1S at 15 nM BTZ, $^{\Phi}P < 0.05$ vs. MM.1SBR at 15 nM BTZ. MM cells were treated with/without BTZ (15 nM) for 24 h and (D) caspase-3 activity was determined, $^{*}P < 0.05$ vs. control for respective cell lines and (E) Western blot analysis of total protein lysates was performed with anti-PARP antibodies. FL indicates full length PARP. β-Actin levels were used as loading control. (F) Endogenous 20S proteasome activity was measured in cell lysates and expressed and RFI. $^{*}P < 0.05$ vs. drug naïve cells. All error bars depict the standard deviation of 3 independent experiments.

Cumulatively, Fig. 2 shows that BTZ-mediated cytotoxicity is primarily rendered by increased oxidative stress and the selected panel of MM cell lines provides a model system to study redox manipulation strategies in overcoming BTZ resistance in MM.

In mammalian cells, ROS-scavenging systems include enzymatic antioxidants (i.e. superoxide dismutases, glutathione peroxidases, catalase) as well as non-enzymatic antioxidants (i.e. glutathione and thioredoxin systems) [45]. We utilized the cell line model to determine if intrinsic- and adaptive-resistance to BTZ was associated with an altered expression of key cellular antioxidants. We first measured SOD activity in BTZ naïve and BTZ adapted cell lines (Fig. 3A). BTZ naïve cells displayed a positive

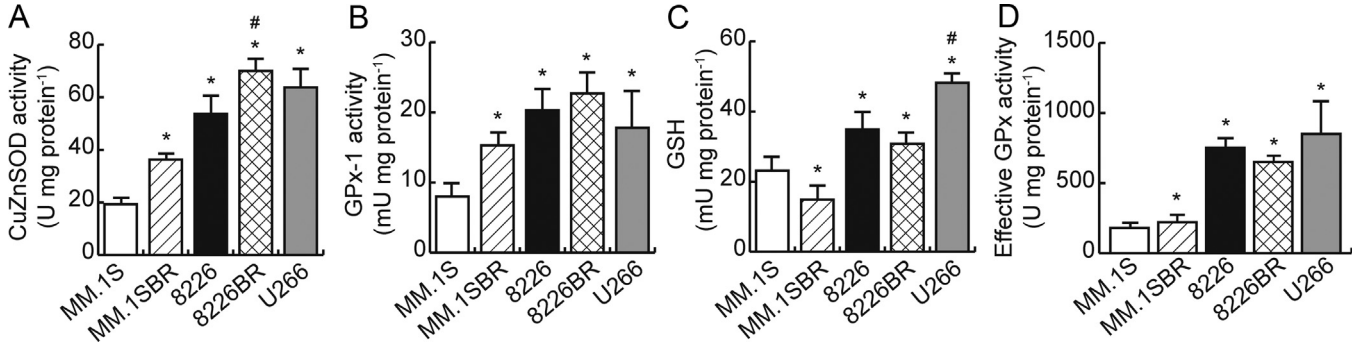

Fig 3. BTZ resistance is associated with altered endogenous antioxidant levels in MM cell lines. In drug naïve cells (MM.1S, 8226, and U266) and adapted BR cells (MM.1SBR and 8226BR), enzyme activities of (A) CuZnSOD, (B) GPx-1, and (C) GSH levels were measured. $^{*}P < 0.05$ vs. MM.1S, $^{\#}P < 0.05$ vs. 8226. (D) Effective GPx activity was calculated for MM cells using data shown in B and C. $^{*}P < 0.05$ vs. MM.1S. All error bars depict the standard deviation of 3 independent experiments.

correlation between CuZnSOD activity and intrinsic BTZ resistance (MM.1S < 8226 < U266, 19.3 ± 3.1, 53.7 ± 7.6, and 63.7 ± 8.1 U mg protein^{-1}, respectively). A similar increase in CuZnSOD activity was found in BR cells compared to parental cells where MM.1SBR (36.3 ± 3.5 U mg protein^{-1}) and 8226BR (70.0 ± 5.6 U mg protein^{-1}) cells showed 1.9- and 1.3-fold increase in CuZnSOD activity relative to MM.1S and 8226 cells, respectively. MnSOD activity did not alter significantly with intrinsic or acquired resistant to BTZ (Supplementary Fig. S1A).

We next measured GPx-1 activities in the MM cell line panel. Interestingly, 8226 and U266 cells displayed 2-fold increase in GPx-1 activity (21.8 ± 3.4 and 17.8 ± 5.1 mU mg protein^{-1}, respectively) compared to MM.1S cells (8.0 ± 2.1 mU mg protein^{-1}) and the BR cells showed a trend towards increased GPx-1 activity relative to drug naïve counterparts (Fig. 3B). Catalase activity was decreased in acquired BTZ-resistant cells compared to drug naïve cells (Supplementary Fig. S1B). We also compared the endogenous GSH levels in our panel of MM cell lines. A positive correlation between GSH levels and intrinsic BTZ resistance (MM.1S < 8226 < U266, 23.1 ± 3.4, 34.9 ± 4.1, and 48.2 ± 2.7 nmol mg protein^{-1}, respectively) was observed (Fig. 3C). The BR cells however did not show differences in GSH levels compared to their drug naïve counterparts (Fig. 3D). As GPxs reduce H_2O_2 to H_2O using GSH as the reducing agent, effective GPx activities were calculated and found to increase with intrinsic but not with acquired resistance to BTZ (Fig. 3D).

We performed qPCR analysis for endogenous mRNA expression of various antioxidant enzymes. In agreement with activity data, compared to MM.1S cells, the intrinsic BTZ-resistant lines (8226 and U266) exhibited increased *SOD1* and *GPX1* expression (Supplementary Fig. 1C). Interestingly, with MnSOD profiling, *SOD2* expression increased in both 8226 and U266 cell lines (Supplementary Fig. 1C) but no changes in the protein expression (data not shown) or enzyme activity were observed (Supplementary Fig. 1A). Overall, our results show that BTZ resistance (both intrinsic and acquired) correlates with increased CuZnSOD levels that are accompanied by increased GPx-1 levels. MM cell lines with intrinsic BTZ resistance also showed increased GSH levels and are in agreement with another published report in MM where manipulation of GSH metabolism with buthionine sulfoximine (a potent and specific inhibitor of glutamate cysteine ligase) sensitized 8226 and U266 cell lines to BTZ [6].

Our published studies show that MM cells are more susceptible to oxidative stress-induced cell death relative to normal cells (bone marrow stromal cells and hematopoietic stem/progenitor cells) [28,29]. Based on the up-regulation of antioxidant expression in BTZ-resistant MM cell lines (Fig. 3) we hypothesized that an increased removal of ROS ($O_2^{\bullet -}$ and H_2O_2) may promote BTZ resistance via counteracting BTZ-mediated oxidative stress and cytotoxicity in MM. For this, we compared the BTZ-mediated changes in the steady-state prooxidant levels in MM cell lines with intrinsic and adaptive BTZ resistance. Using oxidative sensitive fluorescent probes (H_2DCF-DA and DHE) the steady-state levels of prooxidants were measured after BTZ treatment (15 nM, 24 h) and compared to controls. In MM.1S cells, BTZ treatment resulted in 2-fold increase in H_2DCF-DA oxidation (Fig. 4A) and a 1.5-fold increase in DHE oxidation (Fig. 4B). Notably, BTZ treatment did not perturb the intracellular redox equilibrium of MM.1SBR, 8226, 8226BR and U266 cells. These results indicate that cellular adaptation to oxidative stress, presumably via the up-regulated expression of antioxidant pathways, is linked to BTZ resistance.

As increased CuZnSOD gene expression and enzyme activity is associated with poor clinical prognosis and *in vitro* resistance to BTZ, we hypothesized that targeting *SOD1* would selectively induce MM cell killing by increasing their susceptibility to ROS-inducing chemotherapies. To test this hypothesis we first determined if *SOD1* overexpression would decrease BTZ cytotoxicity in MM cells. For this, the MM.1S cell line was transduced with Ad-*SOD1* and increased CuZnSOD protein (~2.5 fold, Fig. 5A) and activity levels (~1.5 fold, Fig. 5B) were confirmed. Clonogenic assays show that relative to MM.1S cells, enforced overexpression of CuZnSOD expression inhibits BTZ cytotoxicity and increases clonogenic survival (~2.5 fold, Fig. 5C). These results suggest CuZnSOD as a molecular target to increase BTZ cytotoxicity in MM.

We next determined if pharmacological inhibition of CuZnSOD activity sensitizes MM cell lines to BTZ treatment. For this we used the small molecule inhibitor disulfiram (DSF) that binds to copper to form DSF–Cu complexes; in cancer cells DSF has been shown to increase oxidative stress-induced cytotoxicity. DSF treatment (5 μM, 24 h) inhibited CuZnSOD activity by ~65% in MM.1SBR and ~40% in both 8226BR and U266 cells (Fig. 6A); no DSF-mediated alterations to MnSOD activity were noted (data not shown). Next, DSF was combined with BTZ and cell viability was determined. For all cell lines, combination of DSF with BTZ induced more potent cytotoxicity compared to treatments with DSF or BTZ alone (Fig. 6B). Clonogenic assays in MM.1S/MM.1SBR pair confirmed that combination treatment of DSF with BTZ was highly effective in inducing cell killing of MM.1S cells and significantly restored BTZ cytotoxicity in MM.1SBR cells (Fig. 6C). Furthermore, DSF enhanced BTZ cytotoxicity in the Ad-*SOD1*-transduced BTZ-sensitive

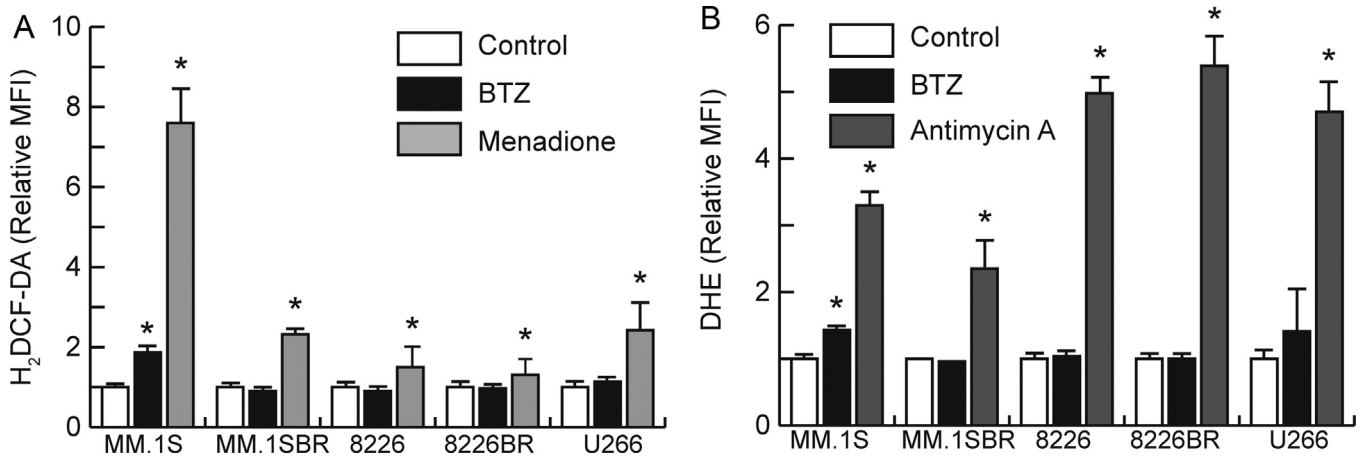

Fig 4. BTZ-mediated ROS production is suppressed in BTZ resistant cells. MM cell lines were cultured with/without BTZ (15 nM) for 24 h and (A) H_2DCF-DA or (B) DHE oxidation was determined by flow cytometry. Untreated cells were used to quantify basal oxidation while either menadione or antimycin A was used as a positive control for oxidative stress. Error bars represent the standard deviation of 3 independent experiments. *$P < 0.05$ vs. control group.

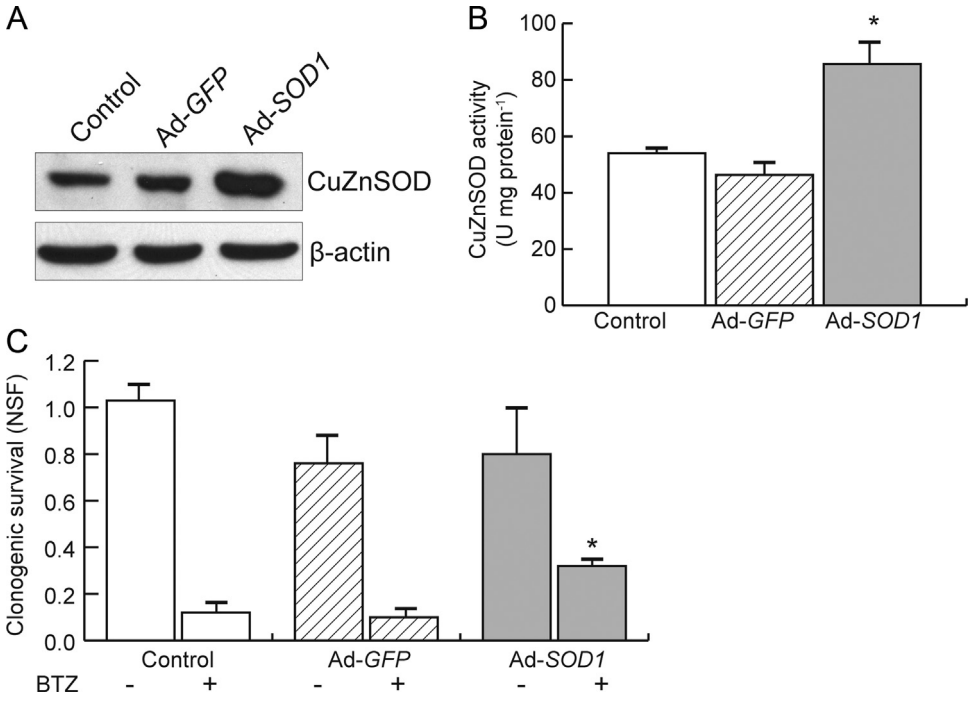

Fig 5. Overexpression of *SOD1* promotes BTZ resistance. MM.1S cell line was transduced with recombinant adenovirus (MOI = 100) expressing *GFP* or *SOD1*. CuZnSOD expression was determined by (A) Western blot and (B) activity assays. (C) Clonogenic survival of MM.1S cells (control or cells overexpressing *GFP* or *SOD1*) after BTZ treatment (15 nM, 24 h). $^{*}P < 0.05$ vs. BTZ treatment on control or *GFP* expressing cells. Error bars represent the standard deviation of 3 independent experiments.

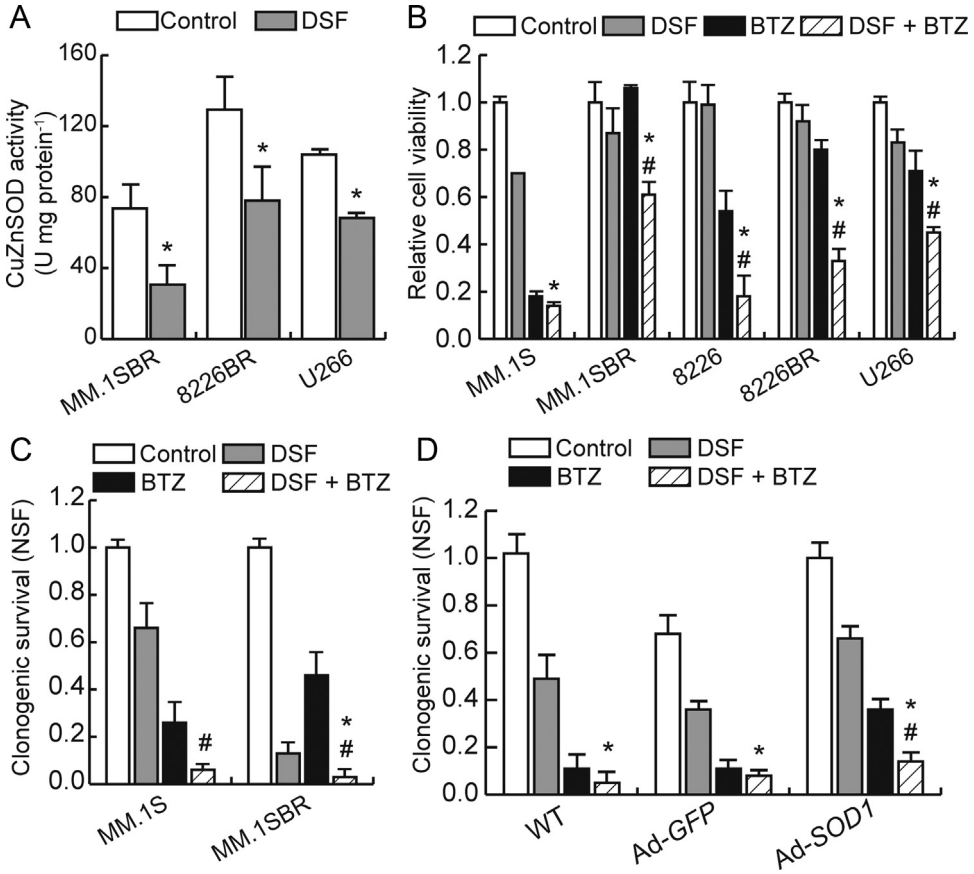

Fig. 6. DSF augments BTZ cytotoxicity in MM cell lines. (A) MM cell lines were treated with DSF (5 μM, 24 h) and CuZnSOD activity was measured. $^{*}P < 0.05$ vs. control cells. Cell lines were treated with DSF (5 μM) and/or BTZ (15 nM) and (B) cell viability was measured at 48 h. $P < 0.05$ vs. DSF, $^{\#}P < 0.05$ vs. DSF, or (C) clonogenic survival assays were performed. $^{*}P < 0.05$ vs. BTZ, $^{\#}P < 0.05$ vs. DSF. (D) Clonogenic survival of MM.1S cells that were either transduced with Ad-*SOD1* or Ad-*GFP* or left alone (wild type, WT), and treated with DSF and/or BTZ. $^{*}P < 0.05$ vs. DSF, $^{\#}P < 0.05$ vs. BTZ. All error bars represent the standard deviation of 3 independent experiments.

MM.1S cell line (Fig. 6D). Overall, these results provide evidence that CuZnSOD is the target for DSF-mediated BTZ sensitization in MM.

Discussion

Compared to normal cells, cancer cells frequently exhibit altered oxidative metabolism and intrinsic oxidative stress [46]. Therefore, redox-directed therapies that increase prooxidant levels, deplete low molecular weight antioxidants, or inhibit antioxidant enzymes have offered avenues to selectively induce cytotoxicity in cancer cells [14]. MM patients show parameters of oxidative stress [10–13] and manipulation of cellular redox parameters to augment therapeutic responses have provided encouraging results in B-cell malignancies [47]. Our published results show that oxidative stress-inducing drug combinations result in selective cytotoxicity in MM cells [28,29]. Notably, a correlation between cancer cell adaptation to oxidative stress and chemo-resistance has been documented [48]. Studies have shown that overexpression of catalase promotes chemo-resistance in lymphoma cells [49] and resistance to imexon drug in MM is linked to increased CuZnSOD protein levels [22]. In this study we report for the first time, a role of cellular antioxidant network in therapy resistance in MM.

Using the frontline anti-MM drug BTZ, that perturbs redox homeostasis in MM [5,6], we have evaluated the role of the antioxidant network in intrinsic and acquired BTZ resistance. To emulate intrinsic BTZ-resistance seen in MM patients, we have utilized a well-established panel of human MM cell lines (MM.1S, 8226, U266) that display differential sensitivity towards BTZ [50,51]. Also, to understand the role of the antioxidant network in BTZ resistance, we adapted MM cell lines to 20 nM BTZ (8226BR and MM.1SBR) as BTZ concentration ranges from 1 to 100 nM in patients within the first 24 h [52]. Many groups have taken a similar approach of exposing MM cell lines to serially increased drug concentrations to develop cells with acquired resistance to proteasome inhibitors [39,42,53,54]. We noted a decrease in basal proteasome activity in MM.1SBR and 8226BR cells that could be linked to decreased BTZ sensitivity compared to their drug-naïve counterparts. A similar decrease in proteasome activity was reported for BTZ adapted MM cell lines 8226.BR and ANBL-6.BR over their parental counterparts 8226 and ANBL-6 cells, respectively [55]. Different mechanisms of BTZ resistance have been suggested including *PSMB5* mutations, however, these proteasome subunit mutations and proteasome activity differences have not been fully confirmed in primary MM samples [56–58]. In the present study, we have not analyzed proteasome mutations and composition as a determinant in acquired resistance to BTZ. Also, the cross-resistance profile of MM.1SBR and 8226BR cells remains to be analyzed towards various proteasome inhibitors.

One of the mechanisms proteasome inhibitors can induce cytotoxicity is through the generation of ROS [59]. Hence, modulation of antioxidants to overcome resistance towards proteasome inhibitors in cancer cells is logical. In preclinical models of neurodegeneration, increased GPx or thioredoxin expression promoted cytoprotection against proteasome inhibitors [60–62]. In amyotrophic lateral sclerosis, loss of CuZnSOD activity has been suggested to contribute to formation of protein aggregates that are degraded by proteasome pathway [63]. Our results show that BTZ resistance is associated with increased CuZnSOD activity in MM. The drug naïve MM cell lines (MM.1S, 8226, U266) exhibit a differential expression of CuZnSOD protein [22] with highest mRNA and activity levels in the U266 cell line. A protective role of CuZnSOD in BTZ-resistant MM cells may be attributed to (i) BTZ-mediated inhibition of the 26S proteasome activity which increases cellular oxidative stress [64] and/or (ii) drug resistance may be associated with defects in mitochondrial respiratory chain resulting in a more robust production of $O_2^{\bullet -}$ [65]. In BTZ-resistant cell lines, we noticed increased expression of both *SOD1* and *SOD2*, however only SOD1 activity was increased. The mitochondrial manganese-containing superoxide dismutase (MnSOD, SOD2) is an established oxidative stress-inducible gene that plays a critical role in the development and progression of cancer [66]. An inducible expression of *SOD1* is feasible as multiple ROS-inducible transcription factor binding sites are mapped in the *SOD1* promoter region [67]. Also, both CuZnSOD and MnSOD activity are influenced by post-translational mechanisms and could partially explain a more prominent role of CuZnSOD in BTZ resistance in MM [68].

Studies have shown that a balance between the first and second step antioxidant enzymes is critical in maintaining survival against oxidative stress [69]. A coordinated increased expression of CuZnSOD with H_2O_2-metabolizing enzymes (i.e. GPxs and catalase) can aid in maintaining the net redox state of the malignant cells generated from intracellular metabolism and/or chemotherapy. Interestingly, increased *SOD1* in trisomy 21 is accompanied by increased GPx activity [70]. In this report we show that BTZ-resistant MM cells exhibit a concerted up-regulation of CuZnSOD and GPx-1. GPx-1 activity was measured as it is the ubiquitously expressed isoform of GPx that contributes to most of the cellular GPx activity [71]. Also, studies have shown that CuZnSOD overexpression results in a compensatory increase in GPx-1 [70,72,73] and transfection of GPx-1 can overcome CuZnSOD mediated cytotoxicity [74,75]. It has been shown that secreted GPxs (GPx7 and GPx8) are located in the endoplasmic reticulum (ER) and associate with the peroxide-producing Ero1α to facilitate protein disulfide isomerase (PDI) in oxidative refolding of a reduced denatured protein [76]. It remains to be seen if an increased expression of secreted GPxs in BTZ resistant cells is linked to less severe BTZ-mediated unfolded protein response (UPR) and cytotoxicity.

BTZ-resistant cells also showed increased GSH levels which constitute the most abundant cellular thiol redox buffer and provides electrons for enzymes such as GPxs [77]. Also, multidrug and radiation-resistance in tumors, as compared with normal tissues, appears to be associated with higher GSH levels in cancer cells [78]. Notably, like other prooxidant drugs i.e. imexon and arsenic trioxide [22,79], BTZ treatment has also been shown to decrease intracellular GSH levels [6] and could account for the decrease in GSH levels seen in the BR cells relative to their drug-naïve counterparts. Also, in the present study we have not assessed the role of thioredoxin-dependent peroxidases that may be involved in metabolizing H_2O_2 and other hydroperoxides in MM cell lines with acquired BTZ-resistance.

Copper is one of the essential trace elements for all organisms and plays a crucial role in redox reactions via regulating cuproenzymes like CuZnSOD [80]. Copper chelators like thiocarbamates (DSF and its metabolite diethyldithiocarbamate, DDC), and the choline tetrathiomolybdate (ATN-224) has been tested for cancer therapy [21,81,82]. DSF is used for the treatment of alcoholism for the past five decades and shown to have anti-cancer activity via increasing oxidative stress. DSF can also target other pathways i.e. inhibition of drug efflux pump P-glycoprotein, p53-dependent apoptosis, ubiquitin–proteasome system, aldehyde dehydrogenases, and canonical NF-κB signaling [83,84]. Our study shows that DSF-mediated increases in BTZ cytotoxicity in MM can be attributed to inhibition of CuZnSOD activity as enforced overexpression of *SOD1* rescued BTZ cytotoxicity in MM cells. We postulate that DSF-mediated inhibition of CuZnSOD activity relative to GPx-1 activity could result in accumulation of superoxide radical that act as primary as well as secondary ROS, damaging macromolecules and inducing cytotoxicity in MM cells.

In this study, we have not combined DSF with exogenous copper as (i) DSF alone shows potent cytotoxicity with no 20S proteasome activity in MM cell lines [85,86], and (ii) our goal is to integrate DSF with anti-MM drugs in the clinic in the near future that may not require copper supplementation. Our published studies have shown that BTZ displays acceptable toxicity pattern towards normal hematopoietic cells [87] as also reported for DSF [88]. Also, DSF has been successfully combined with cisplatin in clinical trials [89]. We therefore postulate that inclusion of DSF with other frontline anti-MM drugs would augment cytotoxic effects of oxidative stress inducing drugs without unacceptable toxicity towards normal hematopoiesis.

Taken together, our data suggest a role of up-regulated antioxidant network in intrinsic and acquired BTZ resistance in MM. We propose that combination of a well-tolerated and clinically established drug DSF can be further developed for combination therapies in MM to circumvent and potentially inhibit BTZ resistance and improve survival outcomes of MM patients.

Conflicts of interest

The author(s) declare that they have no competing interests.

Author contributions

Conception and design: K. Salem, A. Goel.
Development of methodology: K. Salem, A. Goel.
Acquisition of data: K. Salem, M. L. McCormick, E. Wendlandt.
Analysis and interpretation of data: K. Salem, F. Zhan, A. Goel.
Manuscript preparation: K. Salem, A. Goel.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2014.11.002.

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