Cancer Research

High Basal Nuclear Levels of Nrf2 in Acute Myeloid Leukemia Reduces Sensitivity to Proteasome Inhibitors

Stuart A. Rushworth[1], Kristian M. Bowles[2], and David J. MacEwan[1]

Abstract

Proteasome inhibitors such as bortezomib exhibit clinical efficacy in multiple myeloma, but studies in acute myeloid leukemia (AML) have been disappointing to date. The apparent failure in AML likely reflects a lack of biological understanding that might clarify applications of proteosome inhibitors in this disease. Here we show that AML cells are considerably less sensitive than control noncancerous cells to bortezomib-induced cytotoxicity, permitting most bortezomib-treated AML cells to survive treatment. We traced reduced bortezomib sensitivity to increased basal levels of nuclear Nrf2, a transcription factor that stimulates protective antioxidant enzymes. Bortezomib stimulates cytotoxicity through accumulation of reactive oxygen species (ROS) but elevated basal levels of nuclear Nrf2 present in AML cells reduced ROS levels, permitting AML cells to survive drug treatment. We further found that the Nrf2 transcriptional repressor Bach1 is rapidly inactivated by bortezomib, allowing rapid induction of Nrf2-regulated cytoprotective and detoxification genes that protect AML cells from bortezomib-induced apoptosis. By contrast, nonmalignant control cells lacked constitutive activation of Nrf2, such that bortezomib-mediated inactivation of Bach1 led to a delay in induction of Nrf2-regulated genes, effectively preventing the manifestation of apoptotic protection that is seen in AML cells. Together, our findings argue that AML might be rendered sensitive to proteasome inhibitors by cotreatment with either an Nrf2-inhibitory or Bach1-inhibitory treatment, rationalizing a targeted therapy against AML. *Cancer Res; 71(5); 1999–2009. ©2011 AACR.*

Introduction

Acute myeloid leukemia (AML) comprises a heterogeneous group of clonal disorders of hematopoietic progenitors, showing genetic instability and characterized by proliferation and differentiation of abnormal cells, causing accumulation of immature myeloid cells in bone marrow and blood. Five-year survival varies from 15% to 70% depending on clinical and biological factors, with overall AML survival rates for all patients diagnosed with the disease being less than 20% (1). Furthermore, with 75% of patients diagnosed after the age of 60, current intensive therapeutic strategies are generally limited to a minority of younger, fitter patients. There is a significant unmet need for better tolerated, more widely applicable targeted anti-AML therapy (2).

Recently, a number of potential new systemic anticancer therapies (SACT) have emerged that are targeted to specific signaling pathways or cellular processes aimed at blocking proliferation and inducing apoptosis (3). Many potential SACTs are being evaluated but their roles, efficacies, and modes of action as either single agents or combined with other drugs are yet to be fully defined. One such pathway which is being targeted is the ubiquitin-proteasome system that controls protein turnover and many cellular processes, including cell cycle, gene regulation, and oxidative stress responses. NF-E2–related factor 2 (Nrf2) and nuclear factor-κB (NF-κB) are 2 transcription factors regulated by the ubiquitin-proteasome system (4, 5). Both NF-κB and Nrf2 activate survival pathways known to play important roles in protecting malignant cells from SACT cytotoxicity (6, 7). AML cells show aberrant or constitutive NF-κB activation (8), with a central step being phosphorylation and proteasome-dependent degradation of its inhibitory proteins (IκBs), possible targets for proteasome inhibitors in aberrant elevated NF-κB found in certain cancers.

The role of Nrf2 in AML-resistance to proteasome inhibition has not been investigated. Nrf2 functions to rapidly change the sensitivity of a cell's environment to oxidants and electrophiles by stimulating the transcriptional activation of over a hundred cytoprotective genes, including antioxidants ferritin, glutathione-*S*-reductase (GSR), and glutamyl cysteine ligase modulator (GCLM) and catalytic (GCLC), phase I drug oxidation enzyme NAD(P)H:quinone oxidoreductase 1 (NQO1), and cytoprotective enzyme heme oxygenase-1 (HO-1) genes (9). With respect to HO-1, other transcription factors including NF-κB and AP-1 are also involved in its expression (10–12). Under normal physiologic conditions, the inhibitor of Nrf2,

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Keap1, mediates ubiquitin-26S proteasomal degradation of Nrf2. Oxidative and electrophilic stresses such as reactive oxygen species (ROS) or SACTs, impairs Keap1-mediated proteasomal degradation of Nrf2, causing Nrf2 activation and subsequent nuclear translocation (10). Nuclear Nrf2 forms a heterodimer complex with Maf proteins which bind the antioxidant response element (ARE) located in the enhancer regions of Nrf2-inducible genes. Therefore, side effects of proteasome inhibition may be increased cellular Nrf2 leading to its activation, and upregulation of cytoprotective proteins. However, a regulatory process exists to control nuclear Nrf2 activation in the form of the transcriptional repressor Bach1, bound to ARE enhancer regions in cells naïve to oxidative stress to block Nrf2 binding. Bach1 becomes deactivated and translocates to the cytosol, upon pro-oxidant stimuli (13).

We recently showed in AML that high basal NF-κB levels regulate expression of HO-1 and that upon NF-κB inhibition, HO-1 levels increase and protect AML cells from apoptosis via inhibition of ROS formation (12). This study was undertaken to define the role of HO-1 and Nrf2 in response to the only clinically approved proteasome inhibitor, bortezomib. Bortezomib is already used for effective treatment of multiple myeloma and mantle cell lymphoma and is well tolerated clinically. With regards to AML, a small number of clinical trials have been conducted with proteasome inhibitors revealing relatively disappointing results (14–16). The present studies were undertaken to investigate the mechanism by which treating AML with proteasome inhibitors alone is ineffective.

Materials and Methods

Materials

AML-derived cell lines THP-1 and HL60 were obtained from ECACC where they are authenticated by DNA-fingerprinting. In the laboratory they are used at low passage number for a maximum of 6 months postresuscitation, testing regularly for *Mycoplasma* infection. Antibodies were sourced from Assay Designs (HO-1), Abcam (Bach1), Santa Cruz Biotechnology (all others). Control, HO-1, Nrf2, Bach1, and Keap1 siRNA were from Applied Biosystems. Dead cells were removed from samples using Dead-Cert Nanoparticles. Other reagents were from Sigma-Aldrich unless indicated.

Cell culture

Primary AML cells were obtained under local ethical approval (LREC ref 07/H0310/146). For primary cell isolation peripheral blood mononuclear cells (PBMC) and monocytes were isolated from healthy donors as described (12). Selection of human hematopoietic stem cells were isolated from PBMCs using a $CD34^+$ selection kit (Miltenyi Biotec). For all experiments at least 3 different donors were used to obtain the results presented. AML samples, less than 80% blasts expressing CD34, were purified using the $CD34^+$ selection kit. Cell type was confirmed by microscopy and flow cytometry.

RNA extraction and real-time PCR

Total RNA was extracted from 5×10^5 cells using the Nucleic acid PrepStation from Applied Biosystems, according to the manufacturer's instructions. Reverse transcription was carried out as described previously (12), using the specific primers:

Gene ID	Forward	Reverse
GAPDH	5′-ACCAGCCTCA AGATCATCAGC-3′	5′-TGCTAAGCAGTTG GTGGTGC-3′
HO-1	5′-ATGGCCTCCC TGTACCACATC-3′	5′-TGTTGCGCTCAAT CTCCTCCT-3′
GCLC	5′- GGCGATGAG GTGGAATACAT-3′	5′- GTCCTTTCCCCCT TCTCTTG-3′
GCLM	5′-GCGAGGAGCT TCATGATTGT-3′	5′- CTGGAAACTCCCT GACCAAA-3′
NQO1	5′-GCCGCAGACCT TGTGATATT-3′	5′-TTTCAGAATGGCAG GGACTC-3′
GSR	5′-ACTTGCCCATC GACTTTTTG-3′	5′-GGTGGCTGAAGAC CACAGTT-3′
Ferritin	5′- CTGGAGCTCTA CGCCTCCTA-3′	5′-TGGTTCTGCAGCT TCATCAG-3′

Chromatin immunoprecipitation assays

THP-1 cells were treated as described (12) before chromatin was immunoprecipitated with IgG, anti-Nrf2, or anti-Bach1 (Abcam) antisera. Association of Nrf2 and Bach1 was measured by PCR using primers spanning the ARE-site located at -4100 5′-TTTGCTGAGTCACCAGTGC-3′ (forward), 5′-TAAAGCTGCCCTTTCACCTC-3′ (reverse) and was conducted in triplicate on immunoprecipitated and input DNA.

Western immunoblotting, binding assay, and flow cytometry

SDS-PAGE and Western analyses were done as described (17). Cytosolic and nuclear extracts were prepared as described (18). Flow cytometry for measuring apoptosis was carried out on an Accuri-C6 flow cytometer. A dichlorofluorescein (DCF) assay was used to determine cellular ROS generation in AML cells (19, 20).

Transfections

Cells (1×10^6/well) were transfected by Amaxa Nucleofector, with equivalent molar concentrations of siRNAs (30 nmol/L final), and then incubated for 24 hours before treatments (12).

Removal of nonviable cells from test samples

A total of 5×10^6 cells mixed with 25 mL of Dead-Cert nanoparticles (30 minutes) had bound dead cells removed by magnetic separation. Viable cells were then ready for RNA/protein extraction.

Proliferation/death assays

Cells were treated with different doses of bortezomib then viable numbers measured with MTS one-solution assay (Promega; 1 hour) before reading absorbance at 490 nmol/L.

Determination of intracellular glutathione levels

To detect intracellular glutathione levels, 1×10^4 HL60 and THP-1 cells were seeded in 96-well cell culture dishes and

allowed to grow for 24 hours. Cells were then incubated with bortezomib for up to 24 hours. Intracellular glutathione levels were quantified using the bioluminescent GSH-Glo glutathione assay (Promega).

Statistical analyses

Student's t test was conducted. $P < 0.05$ was considered statistically significant (*). Results represent mean ± SEMs of 3 independent experiments. For Western blotting experiments, data are representative of 3 independent experiments.

Results

AML cell sensitivity to proteasome inhibition

To understand the mechanism by which AML cells resist apoptosis in response to the proteasome inhibitor bortezomib (21–23), we analyzed the response of AML patient samples (Table 1), AML cell lines and nonmalignant control cells to increasing concentrations of bortezomib. Figure 1A shows the IC_{50} values comparing AML cells and nonmalignant control cells. We observe significant cell death in all samples in response to bortezomib; however, IC_{50} values for AML samples range between 5 and 71 nmol/L with control nonmalignant $CD34^+$ cells having IC_{50}s between 11 and 16 nmol/L and primary monocytes have IC_{50}s of 4–6 nmol/L. The mean IC_{50}s for bortezomib was significantly lower in noncancerous cell types versus AML (8.8 ± 2.0 vs. 26.3 ± 3.9 nmol/L, means ± SEM, $n = 6$ and 19, respectively; $P = 0.02$). Figure 1B and Supplementary Figure 1 examines the time response (0–72 hours) of AML and control cells to 25 nmol/L of bortezomib. This figure shows that bortezomib does not induce apoptosis in all AML cells with a large percentage of AML cells surviving even at 72 hours treatment (30%–35%), unlike nonmalignant cells (<5%). Control cells also undergo apoptosis earlier than AML cells.

Bortezomib induces cytoprotective and detoxification gene expression in AML cells

A potential role for antioxidant behavior (24, 25) of bortezomib was investigated. Under normal physiologic conditions, Keap1 mediates the ubiquitin-26S proteasome–mediated degradation of Nrf2 (10, 26) and regulates redox-sensitive activation of Nrf2 (26, 27). As the generation of ROS is now considered to be the early critical event for initiation of bortezomib-induced apoptosis in some cancer cells (28, 29), the role of bortezomib in regulating Nrf2-mediated gene induction was examined. Figure 2 shows that bortezomib significantly induces a number of Nrf2-regulated genes, namely, the antioxidants ferritin, GSR, and GCLM, the phase I drug oxidation enzyme NQO1, and also the cytoprotective enzyme HO-1. Interestingly, bortezomib did not induce cytoprotection or detoxification genes in nonmalignant control $CD34^+$ cells. Given the extent to which bortezomib induces AML cytoprotective genes, any number of genes could protect AML cells from bortezomib-induced apoptosis.

Table 1. AML sample information

Number	Age	Gender	WHO diagnosis	Cytogenetics	% Blasts	Previous treatment
AML204	80	Male	AML without maturation	Normal	95	Nil
AML205	66	Female	Therapy-related myeloid neoplasm	Complex	85	1999 DAT, DAT MACE, MiDAC (50)
AML206[a]	61	Male	AML with myelodysplasia-related changes	Complex	80	Nil
AML207	53	Male	Acute promyelocytic leukemia with t(15;17) (q22:q12) PML-RARA	t(15;17)	95[b]	Nil
AML208	77	Male	AML with myelodysplasia -related changes	Complex	95	Nil
AML209	49	Male	AML with maturation	Normal	80	Nil
AML210	39	Male	AML with maturation	Normal	65	Nil
AML211	58	Male	Acute erythroid leukemia	Normal	70	Nil
AML212	64	Male	AML with t(8;21)(q22;q22);RUNX1-RUNX1T1	t(8;21)	85	Nil
AML213	92	Female	AML with myelodysplasia- related changes	Not available	70	Nil
AML216	57	Male	AML with myelodysplasia- related changes	Normal	55	Nil
AML217	82	Female	AML with myelodysplasia -related changes	Deletion 13	85	Hydroxycarbamide
AML301	46	Female	AML with maturation	+4, +8, t(9;22)	70	Nil
AML303	40	Male	Acute promyelocytic leukemia with t(15;17)(q22:q12) PML-RARA	t(15;17)	95[b]	1999 DAT, DAT MACE, MiDAC (50)
AML305	66	Female	AML without maturation	t(2;12)	65	Nil
AML306	78	Male	AML with myelodysplasia- related changes	Not available	85	Nil
AML307	57	Male	AML with minimal differentiation	Not available	95	2009 DA, DA, MACE, MiDAC (50)

NOTE: AML disease characteristics including WHO diagnosis and cytogenetics. Percentage blast denotes % of AML blasts after purification using density gradient and in some instances $CD34^+$ positive selection ([a]isolated through CD34-positive selection. [b]% of blasts and promyelocytes). Previous treatments are as outlined (50).

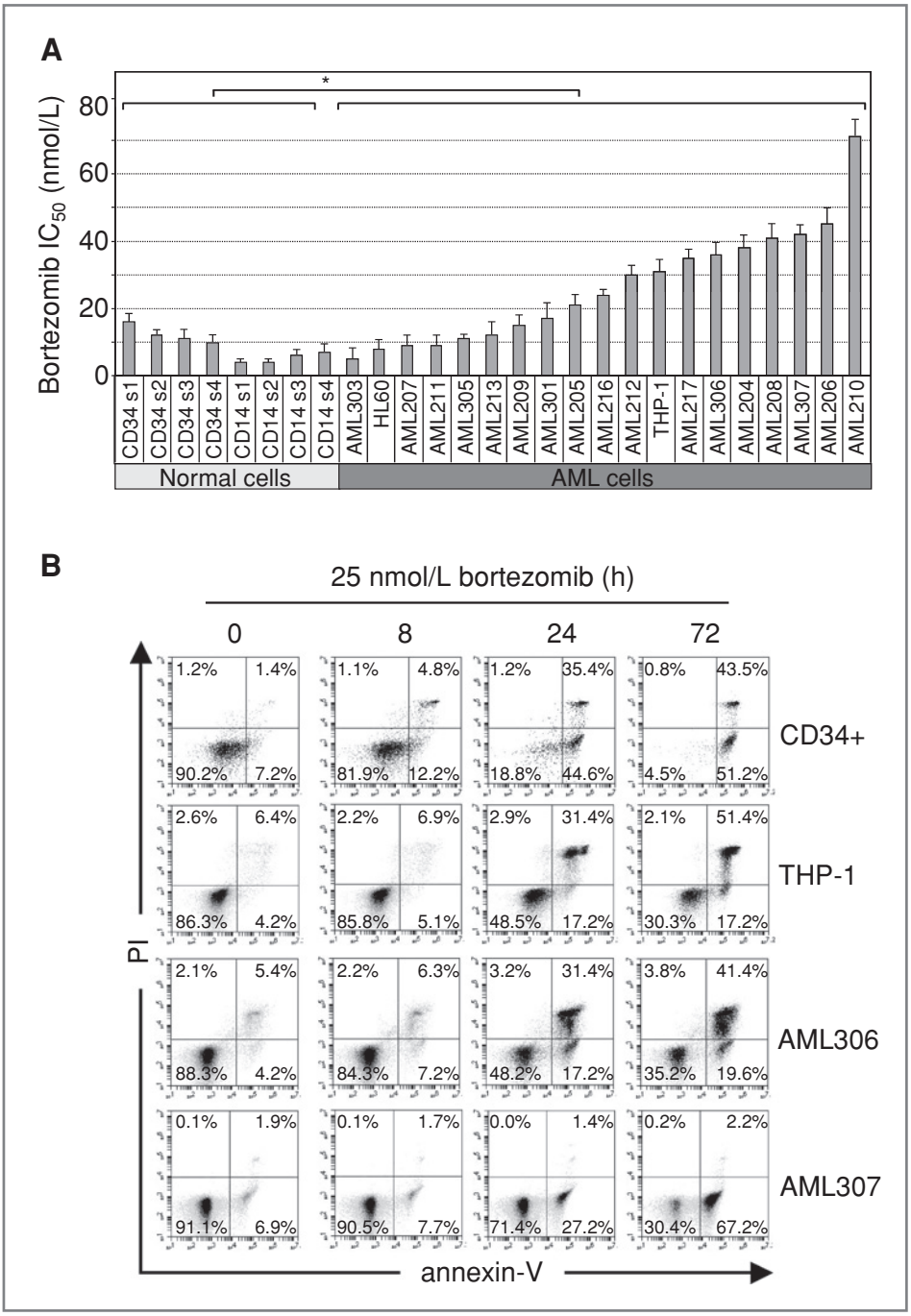

Figure 1. Reduced sensitivity of AML cells to bortezomib-induced apoptosis. A, AML and control cells treated with increasing doses of bortezomib (1–100 nmol/L) for 24 hours and then assessed by MTS. Values indicate means ± SEM, $n = 3$. IC_{50} concentrations determined for each sample. B, cells were treated with 25 nmol/L bortezomib for the indicated times. % cells positive for apoptosis was determined by Annexin V–FITC (fluorescein isothiocyanate)/PI (propidium iodide) FACS (fluorescence activated cell sorting) analysis.

Bortezomib induces stabilization and subsequent activation of Nrf2 in AML

As bortezomib induced the expression of cytoprotective and detoxification genes regulated by Nrf2 in AML cells, subcellular localization of Nrf2 was examined in response to bortezomib. Indications of Nrf2 activation include protein accumulation and nuclear localization in activated cells, therefore we examined Nrf2 protein expression levels in both whole cell extracts and cytosolic versus nuclear extracts in response to bortezomib in AML cells. Figure 3A shows that bortezomib induces Nrf2 protein expression in whole cell extracts from THP-1 cells and that levels remain stable up to 72 hours. Moreover, examination of cytosolic and nuclear fractions showed Nrf2 localized in the nucleus in response to bortezomib (Fig. 3A). To determine if Nrf2 was responsible for activation of HO-1 in response to bortezomib in AML cells, we used siRNA to silence Nrf2 expression. Supplementary Figure 2 shows that THP-1 transfected cells with siRNA exhibit Nrf2 knockdown. Figure 3B shows bortezomib treatment of Nrf2-silenced AML cells

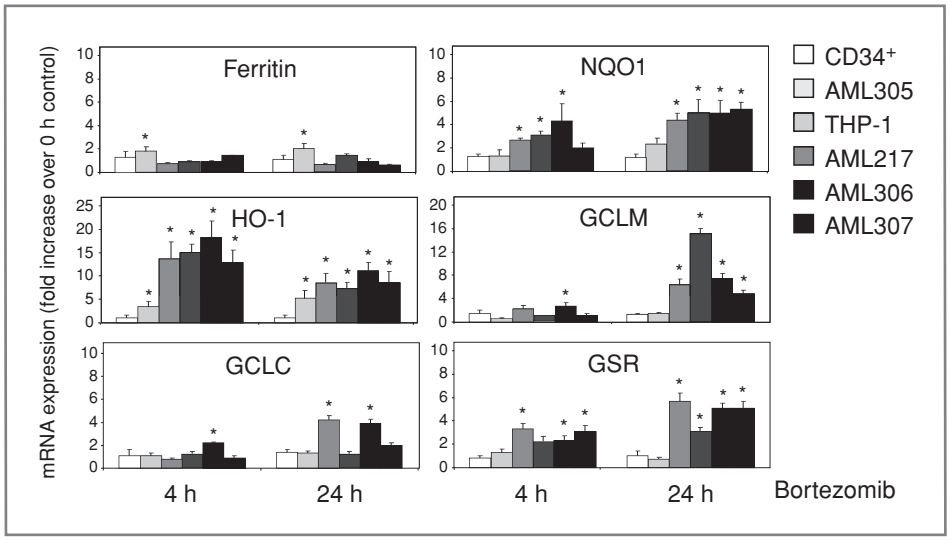

Figure 2. Cytoprotective gene induction by bortezomib in AML and nonmalignant control cells. Cells were treated with 25 nmol/L bortezomib. Extracted mRNA was measured using real-time PCR. mRNA expression was normalized to GAPDH.

inhibits HO-1 expression compared with control siRNA-treated AML cells.

In our previous study we also observed some nuclear expression of Nrf2 in unactivated THP-1 cells (27). To address the importance of this finding we wanted to determine the extent to which primary AML cells and control cells had nuclear expression of Nrf2. Figure 3C (also Supplementary Fig. 3) shows AML samples with higher bortezomib-resistance (Fig. 1A) possess nuclear expression of Nrf2, which is not true of control cells or bortezomib-sensitive AML cells. Finally, we examined if silencing Nrf2 or HO-1 could induce apoptosis in AML cells that are resistant to bortezomib. When we knocked down Nrf2 in AML cell line THP-1, AML306, and human monocytes (Fig. 3D) we showed that bortezomib then induced a significantly greater apoptotic response in THP-1 and primary AML blasts. Knockdown of Nrf2 allowed bortezomib to kill off almost all AML cells. Thus, Nrf2 knockdown reveals the full apoptotic potential of bortezomib in AML. Unlike Nrf2, silencing of HO-1 did not allow full bortezomib-induced AML death to be revealed (Supplementary Fig. 4) with additional NF-κB inhibition possibly also needed (12) in regard to bortezomib-induced AML death. These findings show that basal nuclear Nrf2 is responsible for protecting AML cells from bortezomib-induced apoptosis and the mechanism by which Nrf2 is protecting AML cells is via combined upregulation of cytoprotective and detoxifying genes, and not directly mediated through HO-1.

Bortezomib inhibits Bach1/ARE in AML paving the way for Nrf2 activation

To further understand the significance of constitutive nuclear Nrf2 activity in AML cells, we analyzed the expression of the Nrf2 transcriptional repressor Bach1. Figure 4A shows that Bach1 nuclear expression is virtually unchanged between control and AML samples, suggesting that Bach1 is repressing the nuclear Nrf2 seen in bortezomib-resistant AML samples from binding to the ARE and switching on ARE-regulated genes. To determine if bortezomib can induce Bach1 nuclear export in to the cytoplasm we examined cytosolic and nuclear expression of Bach1 in response to 25 nmol/L bortezomib over 24 hours. Figure 4B shows that nuclear Bach1 levels decreased at the same time that cytosolic Bach1 levels increased in response to bortezomib. We next examined the *in vivo* relevance of Nrf2 and Bach1 in regulating HO-1 expression in AML cells in response to bortezomib. We evaluated the effect of bortezomib on recruitment of Bach1 and Nrf2 to the HO-1 ARE promoter site. Figure 4C shows real-time PCR (gel panel) and PCR analysis (bar graph) of the levels of Bach1 and Nrf2 at the ARE. Chromatin immunoprecipitation (ChIP) analysis revealed recruitment of Bach1 was markedly decreased at the ARE from 1 hour bortezomib treatment (Fig. 4C). In contrast Nrf2 binding to ARE over the same time, was markedly greater with bortezomib treatment. Finally, to determine if Bach1 is repressing cytoprotective gene induction in AML cells that have nuclear expression of Nrf2, we used siRNA to knockdown Bach1 expression in 2 AML samples, 1 with low normal nuclear Nrf2 (AML303) and 1 with high nuclear Nrf2 (AML307). Supplementary Figure 2 shows that we can knockdown Bach1 protein effectively in AML cells. Figure 4D shows that Bach1-knockdown induced expression of cytoprotective genes HO-1, NQO1, GCLM, and GSR in AML307 (bortezomib-resistant) but not AML303 (bortezomib-sensitive). Furthermore, we also examined HL60 and THP-1 cell response to Bach1-knockdown and as a positive control Keap1 knockdown (Supplementary Fig. 5). Again this showed that Bach1-knockdown induced expression of HO-1, NQO1, GCLM, and GSR in THP-1 (bortezomib-resistant) but not bortezomib-sensitive HL60s. Keap1-knockdown showed that these genes could be induced in HL60 as well as THP-1 cells. Taken together these results suggest that under normal basal conditions in AML Bach1 prevents nuclear Nrf2 binding to the ARE to induce cytoprotective genes. However, upon bortezomib treatment, Bach1 quickly detaches from the ARE, is exported from the nucleus, and the high basal Nrf2 causes immediate upregulation of cytoprotective genes, thus protecting AML from bortezomib-induced apoptosis.

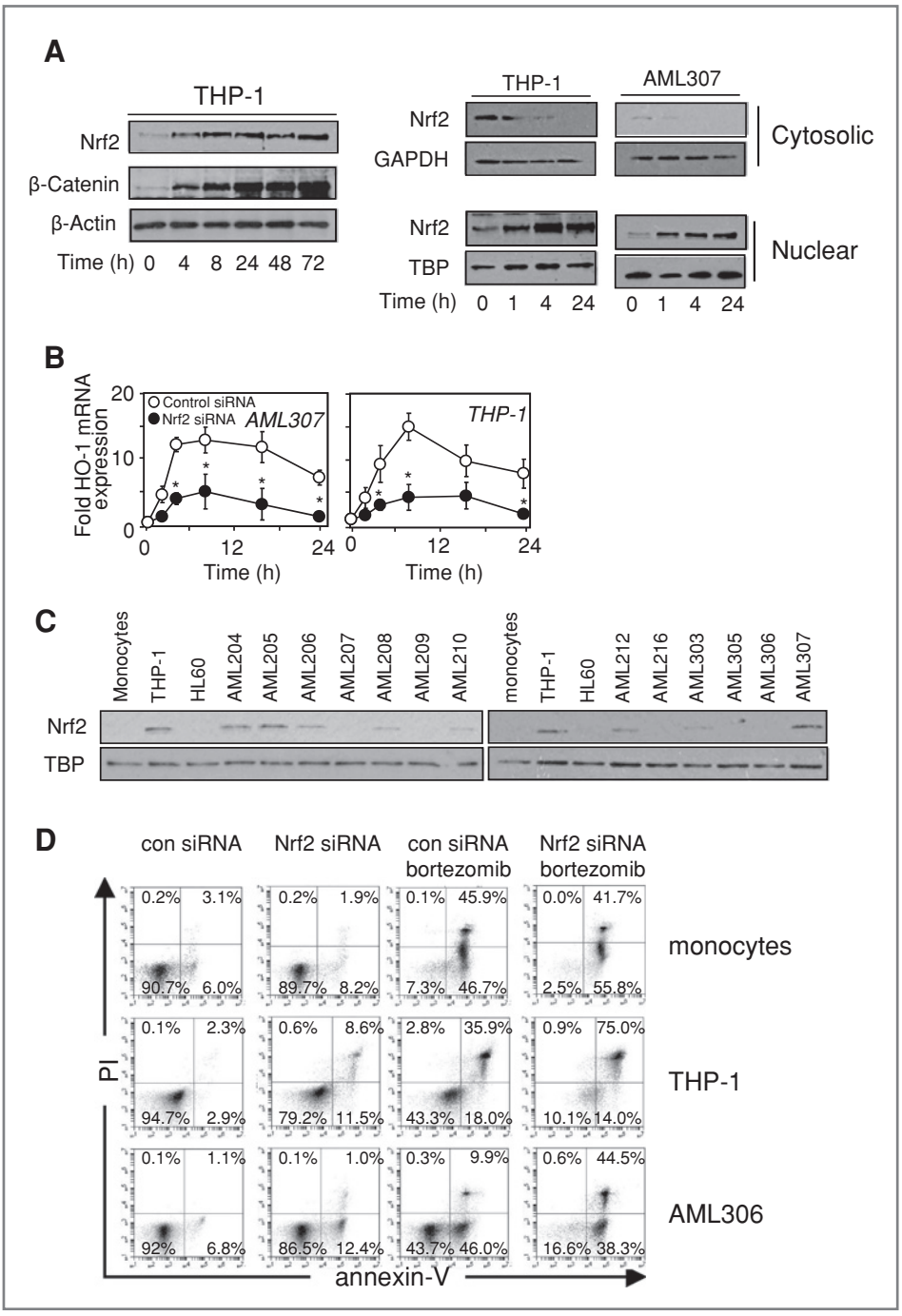

Figure 3. Bortezomib induces the activation of Nrf2 in AML. A, THP-1 cells treated with 25 nmol/L bortezomib for the indicated times. Whole cell protein extracts and cytosolic and nuclear extracts were probed for Nrf2 protein levels and reprobed for β-actin, GAPDH, or TBP to confirm equal loading. B, AML cells were transfected with 30 nmol/L siRNA and incubated for 24 hours before treatment with 25 nmol/L bortezomib for the indicated times. RNA was extracted and HO-1 mRNA measured using real-time PCR. mRNA expression was normalized to GAPDH. C, nuclear extracts were prepared from numerous AML samples and analyzed for Nrf2 protein. D, AML cells and control monocytes were transfected with 30 nmol/L of siRNA and incubated for 24 hours before 25 nmol/L bortezomib treatment for 24 hours before FACS analysis.

Bortezomib induces ROS to activate Nrf2 and Bach1, protecting AML from apoptosis

As Keap1 regulates redox-sensitive activation of Nrf2 (26), and ROS are considered critical in bortezomib-induced effects in some cancer cell lines (28, 29), the role of ROS was examined here in AML. In response to bortezomib treatment, ROS generation occurred in a time-dependent manner (Fig. 5A). ROS activation kinetics correlate with nuclear accumulation of Nrf2 following bortezomib (Fig. 3B). Moreover, we observed a secondary decrease in ROS levels in bortezomib-resistant AML cells (AML217, AML306, THP-1), apparent 2–8 hours after treatment, that was not observed in more bortezomib-sensitive cells ($CD34^+$, AML303, AML205; Fig. 5A). Comparing these data, there are 2 clear groupings: (i) those cells sensitive to bortezomib and without a secondary reduction in ROS levels; and (ii) cells that are insensitive to bortezomib with a substantial secondary reduction in ROS levels. Next we examined the role of Nrf2 in regulating ROS levels in AML cells. Figure 5B shows that if we inhibit Nrf2 expression and activation using an Nrf2 siRNA., in AML306 and THP-1 cells

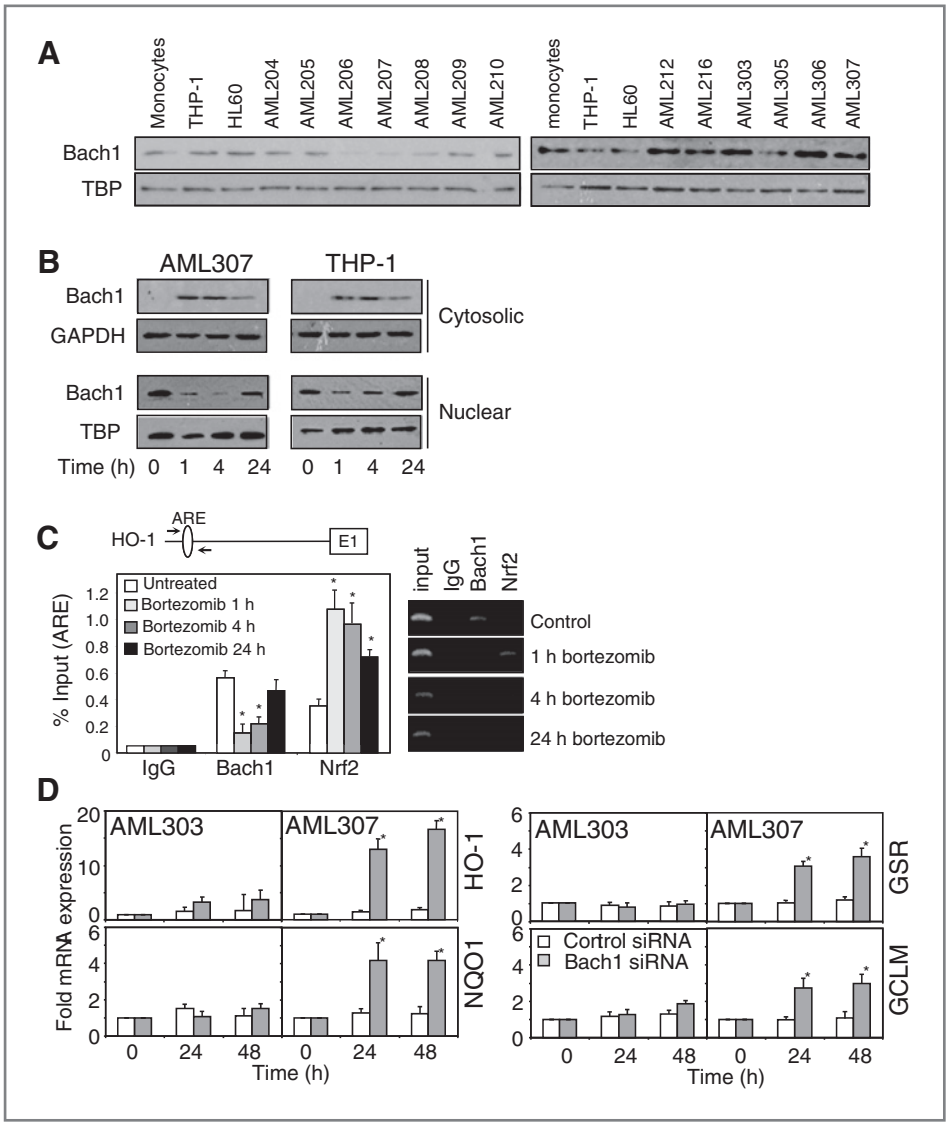

Figure 4. Bortezomib inhibits Bach1/ARE in AML. A, nuclear extracts were prepared and Bach1 protein levels determined. Blots were reprobed with TBP to confirm similar loading. B, AML cells were treated with 25 nmol/L bortezomib for the indicated time. Cytosolic and nuclear extracts were probed for Bach1 protein. C, ChIP analysis of the HO-1 promoter ARE site. The bar graph shows the real-time PCR analysis in triplicate of THP-1 cells which were untreated or treated with 25 nmol/L bortezomib for various times before ChIP, using antibodies against bach1, Nrf2, and normal rabbit IgG as a control. Data presented as percentage of input. Values are means ± SD ($n = 4$). The gel panels show PCR analysis of ChIP for the same site using the same antibodies in THP-1 cells in response to 25 nmol/L bortezomib for various times. D, cells were transfected with 30 nmol/L of siRNA and incubated for 24–48 hours. HO-1, NQO1, GCLM, and GSR mRNA were measured using real-time PCR, with expression normalized to GAPDH.

we get the same initial increase in ROS but no decrease as is seen with the control siRNA (Nrf2 knockdown AML303 cells showed no change in secondary ROS). This suggests that activation of the high basal Nrf2 and its subsequent cytoprotective gene induction reduces the amount of cellular ROS created by bortezomib, and thus protecting AML cells against apoptosis. Finally we determined if ROS were responsible for regulation of Nrf2 and Bach1 in response to bortezomib. Figure 5C shows that the ROS quencher *N*-acetyl cysteine (NAC) inhibited the bortezomib increase of nuclear Nrf2 in resistant AML cells, but with sensitive AML or human monocytes, bortezomib did not induce a Nrf2 response in these cells. NAC also inhibited bortezomib-induced export of nuclear Bach1 in resistant cells. No effect was observed in bortezomib-sensitive or human monocytes, as subcellular redistribution of Nrf2 or Bach1 was not seen in response to bortezomib. Figure 5D shows that NAC inhibited bortezomib-induced Nrf2 gene induction in resistant cells but not sensitive AML cells or human monocytes, where no Nrf2 induction was observed. As in thyroid cancer cells, increased levels of intracellular glutathione in response to bortezomib are known to have greater resistance to apoptosis (30), together with data from this study which shows that bortezomib induces glutathione rate-limiting enzymes in resistant cells, we examined intracellular glutathione response to bortezomib in THP-1 (bortezomib-resistant) and HL60 (bortezomib-sensitive). Supplementary Figure 6 shows that intracellular glutathione reduced in HL60 and increased in THP-1 cells in response to bortezomib over a 24-hour time period.

Discussion

An understanding of the pivotal role of the ubiquitin-proteosome system in normal cell physiology as well as in malignant disease propelled development of proteasome inhibitors for therapeutic applications. Bortezomib reversibly

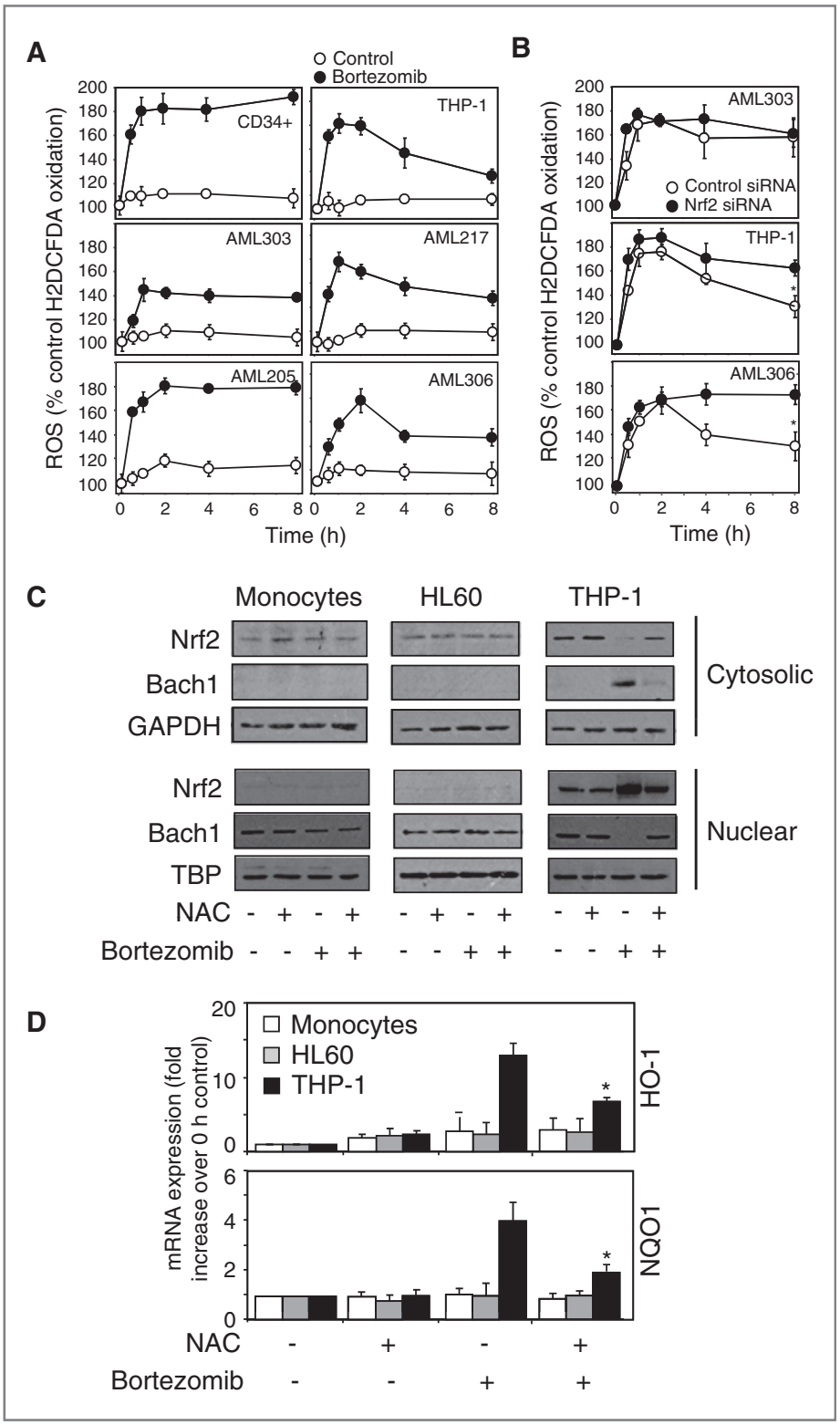

Figure 5. Bortezomib-induced ROS activates Nrf2 and Bach1 to protect AML cells from apoptosis. A, AML cells and nonmalignant $CD34^+$ control cells were treated with bortezomib followed by incubation with H2DCFDA 10 μmol/L, 15 minutes. Cellular ROS was assessed by flow cytometry. B, AML cells were transfected with 30 nmol/L of siRNA and incubated for 24 hours and then treated with 25 nmol/L bortezomib, followed by ROS measurement. C, cells treated with bortezomib for 1 hour with or without 10 mmol/L NAC pretreatment for 30 minutes. Cytosolic and nuclear Bach1 and Nrf2 protein was determined and then reprobed with either GAPDH or TBP to confirm similar loading. D, cells treated with bortezomib for 4 hours with or without 10 mmol/L NAC pretreatment for 30 minutes. HO-1 and NQO1 mRNA were measured using real-time PCR, with expression normalized to GAPDH.

binds and inhibits chymotryptic-like proteolytic activity of the proteasome, localized in the β5 subunit of the 20S core, resulting in accumulation of polyubiquitinated proteins and disturbed protein homeostasis. This may trigger apoptosis, with relative selectivity for malignant cells while leaving normal cells comparatively untouched. However, our study and others suggests bortezomib is not as selective in targeting malignant cells as first thought, especially for treating AML. In

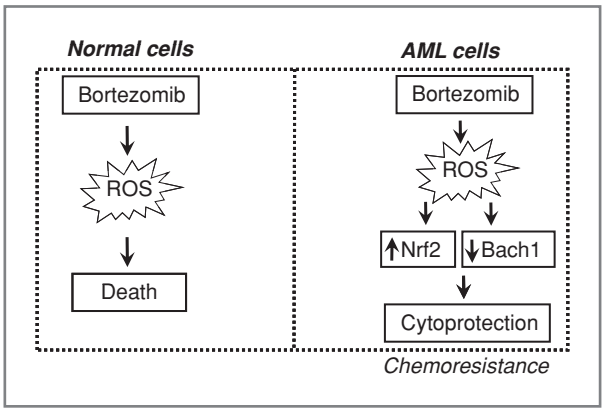

Figure 6. Overview of the mechanism by which human AML leukemia cells are resistant to bortezomib treatment.

this study we showed that normal myeloid cells (including $CD34^+$ HSC and primary monocytes) have lower IC_{50}s to bortezomib than the majority of AML samples tested. We showed in AML cells that having IC_{50}s greater than control cells (i.e. bortezomib-resistant) that cytoprotective transcription factor Nrf2 is not only induced by bortezomib, but is already in their nucleus and therefore primed for activation. Moreover, we observe that bortezomib induces nuclear export of Nrf2 repressor Bach1, through a ROS-dependent mechanism. Altogether, these signals trigger rapid induction of cytoprotective and detoxification genes, including HO-1, NQO1, GCLM, and GRS, that combine to act to protect AML cells from bortezomib-induced apoptosis.

Interestingly, other proteasomal inhibitors like MG132 preferentially induce apoptosis in AML cells over nonmalignant control cells (31). However, peptidyl aldehydes such as MG132 are less specific than bortezomib as they inhibit serine/cysteine proteases in addition to the proteasome, with poorer stability and bioavailability (32), and therefore are less likely to become clinically useful. Bacterial product lactacystin is more proteasome-specific, however irreversibly blocks several proteasomal proteolytic activities, whereas bortezomib reversibly inhibits more the chymotrypsin-like activities. These unique features of bortezomib make it easier to administer in an *in vivo* setting and result in its decreased toxicity. However, as we and others have discovered, using better targeted proteasome inhibitors like bortezomib leads to no preferential apoptosis in AML cells over control cells (14, 15, 22). We show here that bortezomib preferentially induced apoptosis in control cells over AML cells, making it less likely to be used to treat AML. Other studies have also shown unexpected results when using bortezomib, for instance Bil and colleagues showed bortezomib preferentially induced CD20 in normal B cells compared with a decrease in malignant B cells, and this differential CD20 expression led to a significant increase in rituximab-mediated complement-dependent cytotoxicity in normal B cells (33). With increasing interest in inhibiting the ubiquitin-proteosome system, better targeted proteasome inhibitors are being clinically developed. We need to understand the specific pathways protecting AML cells and other cancer cells from such drugs.

The role of Nrf2 has been intensively studied showing that Nrf2 activation protects against many human diseases or pathologic states, such as cancer, neurodegenerative diseases, aging, cardiovascular disease, inflammation, pulmonary fibrosis, and acute pulmonary injury (7, 34–39). Using dietary or synthetic compounds to boost Nrf2-mediated cellular defense responses to prevent disease has been intensively studied (40–44). Many Nrf2 activators have been identified and their efficacy in cancer prevention has been verified both in animal models and in human clinical trials (40, 45). Here, we provide a different perspective, one where the activation of Nrf2 protects cancer cells from undergoing apoptosis in response to a SACT, bortezomib. We show that many AML samples have nuclear Nrf2, and therefore are primed for the activation of the ARE. The transcriptional repressor Bach1 prevents Nrf2 activation but with the addition of bortezomib (which induces a prooxidant state) this induces Bach1 to disassociate from the ARE, allowing Nrf2 to bind the ARE and induce transcription of Nrf2-regulated genes. The experiment described in Figure 4D shows that siRNA knockdown of Bach1 induces expression of HO-1, NQO1, GCLM, and GSR in AML307 but not AML303. We believe the reason for this to be related to the presence of nuclear Nrf2 in AML307 (but not AML303) thus allowing activation at the ARE. The role of Bach1 compared with the more expected Keap1 (46, 47) to repress ARE gene transcription is less clear, as some studies show conflicting data regarding specific genes repressed by Bach1. For example, knockdown of Bach1 induced HO-1 but not NQO1, with the data for GCLM, GCLC, and GSR being less clear. Here we show that knockdown of Bach1 or Keap1 are both as equally influential over the expression of these antioxidant genes in AML cells (Supplementary Fig. 5). Bach1 can compete at the ARE with Nrf2 in the NQO1 promoter. Taken together, silencing of only Bach1 would have no effect on NQO1 expression, but observing any change requires additional Nrf2 activation, such as we observe here in resistant AML samples (AML307, THP-1) compared with more sensitive AML samples (AML303, HL60).

Our understanding of the sequence of events leads us to conclude that AML cells that have basal nuclear expression of Nrf2 are more likely to be resistant to bortezomib-induced death and possibly other SACT (Fig. 6). Interestingly, Nrf2 can modulate expression of alpha and beta subunits of the 26S proteasome, however, we showed no obvious change in proteasomal subunits expression patterns between bortezomib-resistant and -sensitive AML cells (Supplementary Fig. 7). Moreover, based on these observations, the identification of small molecules that potently and specifically inhibit the Nrf2-dependent response or prevent ROS-dependent Bach1 activation would be extremely important in increasing the efficacy of new and existing proteasomal inhibitors for therapeutic applications, particularly in AML.

One of the main questions to be raised by this study is why is Nrf2 constitutively active in some but not all AML cells and not in control cells? This is not the first time that Nrf2 has been shown to be constitutively active in human cancer cells (45, 48). In human lung cancer tissues and lung carcinoma lines, mutation in the inhibitor of Nrf2, Keap1, inactivated its

repressor function causing Nrf2 activation as showed by elevated Nrf2 protein levels, Nrf2 nuclear localization, and increased mRNA expression of Nrf2 target genes. Moreover, Nrf2 was found to be increased in 91.5% of tumors from patients with head and neck squamous cell carcinoma, plus abnormally elevated Nrf2 protein levels were observed in pancreatic cancer tissues and cell lines, resulting in their increased drug resistance (49). Therefore, it is highly likely that cancer cells acquire growth advantage during the course of transformation by either eliminating any Keap1-mediated negative control of Nrf2, or by increasing Nrf2 protein levels to the point which Keap1 is saturated, subsequently leading to better activation of Nrf2-dependent defense responses. Further work to determine why Nrf2 is abnormally high in AML is underway, as understanding this mechanism may pave the way to developing combinational therapies for the treatment of AML.

Our results clearly indicate that the Nrf2-dependent defense response helps survival of AML cells during treatment with bortezomib. It is also clear that Nrf2-dependent protection accounts for drug sensitivity of AML cells to bortezomib. Drug sensitivity to SACT is the major obstacle to the successful treatment of many cancers including neuroblastoma, breast cancers, and lung cancers. In the course of SACT, a strong initial response is frequently followed by the appearance of drug-resistant variants. Moreover, many genes reported to play roles in drug sensitivity seemingly have functional links with Nrf2. For instance, Nrf2 is a key regulator of phase II-detoxifying enzyme NQO1. In addition, Nrf2 regulates many of the key enzymes important in maintaining cellular redox homeostasis, such as GCLC and GCLM, which are the rate-limiting enzymes controlling glutathione biosynthesis. Moreover, GSR facilitates the reduction of glutathione disulfide to the sulfhydryl form of glutathione. Therefore, as Nrf2 regulates many genes including HO-1 to provide reduced sensitivity toward proteasomal inhibition, targeting Nrf2 may be more effective than targeting any of these genes alone. In conclusion, our data show that inhibition of Nrf2 sensitizes cells to proteasome inhibition, suggesting that Nrf2 inhibitors may be used concomitantly to increase the efficacy of anticancer therapy.

Disclosure of Potential Conflicts of Interest

The authors declare no competing financial interests.

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