High basal nuclear levels of Nrf2 in acute myeloid leukemia reduces sensitivity to proteasome inhibitors

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

1School of Pharmacy, University of East Anglia, Norwich NR4 7TJ, United Kingdom

2Department of Haematology, Norfolk and Norwich University Hospitals NHS Trust, Colney Lane, Norwich, NR4 7UY United Kingdom

Corresponding Author:
Professor David J MacEwan
School of Pharmacy
University of East Anglia
Norwich
NR4 7TJ
United Kingdom

e-mail: d.macewan@uea.ac.uk
Tel: +44 (0)1603 592005
Fax: +44 (0)1603 592003

Running title: Nrf2 protects AML from proteasome inhibitor treatment

Key words: leukemia, AML, apoptosis, transcription factor, repressor, antioxidant

Footnotes: This work was funded by the Big C Appeal, Leukaemia & Lymphoma Research and the Association for International Cancer Research. Manuscript Information: 28 text pages, 6 figures and 1 table. Words 4,999.
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 non-cancerous 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 which protect AML cells from bortezomib-induced apoptosis. By contrast, non-malignant 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 co-treatment with either an Nrf2-inhibitory or Bach1-inhibitory treatment, rationalizing a targeted therapy against AML.
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 15–70% 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 anti-cancer 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 two 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 physiological conditions, the inhibitor of Nrf2, 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 up-regulation 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). The present 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 (Salisbury, UK) 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 (ImmunoSolv, UK). 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 PBMCs 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, Auburn, CA). For all experiments at least three different donors were used to obtain the results presented. AML samples <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 x 10^5 cells using the Nucleic acid PrepStation from Applied Biosystems, according to the manufacturer’s instructions. Reverse transcription was performed as described previously (12) using the specific primers:

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>5'-ACCAGCCTCAAGATCATCAGC-3'</td>
<td>5'-TGCTAAGCAGTTGGTGTTG-3'</td>
</tr>
<tr>
<td>HO-1</td>
<td>5'-ATGGCCCTCCTGTACCACATC-3'</td>
<td>5'-TGTTGCGCTCAATCTCCTC-3'</td>
</tr>
<tr>
<td>GCLC</td>
<td>5'-GGCGATGAGGTGGAATACAT-3'</td>
<td>5'-GTCCTTTCCCCTTCTCTG-3'</td>
</tr>
<tr>
<td>GCLM</td>
<td>5'-GCGAGAGCTTCATGATG-3'</td>
<td>5'-CTGGAAACTCCCTGACAAA-3'</td>
</tr>
</tbody>
</table>
NQO1 5’-GCCGCAGACCTTGATATT-3’ 5’-TTTCAGAATGGCAGGGACTC-3’
GSR 5’-ACTTGCCCATCGACTTTTTG-3’ 5’-GGTGCTGAAGACCACAGTT-3’
Ferritin 5’-CTGGAGCTCTACGCCTCCTA-3’ 5’-TGGTTCTGCAGCTTCATCAG-3’

ChIP 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 performed in triplicate on immunoprecipitated and input DNA.

Western immunoblotting, binding assay and flow cytometry
SDS-PAGE and Western analyses were performed as described (17). Cytosolic and nuclear extracts were prepared as described (18). Flow cytometry for measuring apoptosis was performed 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 x 10^6/well) were transfected by Amaxa Nucleofector, with equivalent molar concentrations of siRNAs (30 nM final) then incubated 24 h before treatments (12).

Removal of non-viable cells from test samples.
5 x 10^6 cells mixed with 25 µL of Dead-Cert nanoparticles (30 min) had bound dead cells removed by a magnet. 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 h) before reading absorbance at 490 nM.
Determination of intracellular glutathione levels

To detect intracellular glutathione levels, 1x10^4 HL60 and THP-1 cells were seeded in 96-well cell culture dishes and allowed to grow for 24 h. Cells were then incubated with bortezomib for up to 24 h. Intracellular glutathione levels were quantified using the bioluminescent GSH-Glo™ glutathione assay (Promega).

Statistical analyses

Student's t test was performed. 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 analysed the response of AML patient samples (Table 1), AML cell lines and non-malignant control cells to increasing concentrations of bortezomib. Figure 1A shows the IC\textsubscript{50} values comparing AML cells and non-malignant control cells. We observe significant cell death in all samples in response to bortezomib, however IC\textsubscript{50} values for AML samples range between 5-71 nM with control non-malignant CD34+ cells having IC\textsubscript{50}s between 11-16 nM and primary monocytes have IC\textsubscript{50}s of 4-6 nM. The mean IC\textsubscript{50}s for bortezomib was significantly lower in non-cancerous cell types versus AML (8.8 ± 2.0 versus 26.3 ± 3.9 nM, means ± SEM, n = 6 and 19 respectively; P = 0.02). Figure 1B and Supplementary Figure 1 examines the time response (0-72 h) of AML and control cells to 25 nM 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 h treatment (30-35%), unlike non-malignant 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 behaviour (24,25) of bortezomib was investigated. Under normal physiological 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 non-malignant
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.

**Bortezomib induces stabilization and subsequent activation of Nrf2 in AML**

Since bortezomib induced the expression of cytoprotective and detoxification genes regulated by Nrf2 in AML cells, subcellular localisation of Nrf2 was examined in response to bortezomib. Indications of Nrf2 activation include protein accumulation and nuclear localisation in activated cells, therefore we examined Nrf2 protein expression levels in both whole cell extracts and cytosolic verses 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 h. Moreover, examination of cytosolic and nuclear fractions showed Nrf2 localised in the nucleus in response to bortezomib (Figure 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 knock-down. Figure 3B shows bortezomib treatment of Nrf2-silenced AML cells inhibits HO-1 expression compared to 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 (and Supplementary Figure 3) shows AML samples with higher bortezomib-resistance (Figure 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 (Figure 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 knock-down 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 Figure 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 analysed 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 nM bortezomib over 24 h. 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. ChIP analysis revealed recruitment of Bach1 was markedly decreased at the ARE from 1 h bortezomib treatment (Figure 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 two AML samples, one with low normal nuclear Nrf2 (AML303) and one 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 Figure 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 suggests 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.

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

Since 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 (Figure 5A). ROS activation kinetics correlate with nuclear accumulation of Nrf2 following bortezomib (Figure 3B). Moreover, we observed a secondary decrease in ROS levels in bortezomib-resistant AML cells (AML217, AML306, THP-1) apparent 2-8 h after treatment, that was not observed in more bortezomib-sensitive cells (CD34+, AML303, AML205)(Figure 5A). Comparing these data, there are two 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 we get the same initial increase in ROS but no decrease as is seen with the control siRNA (Nrf2 knock-down 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. Since 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 h 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 binds and inhibits chymotryptic-like proteolytic activity of the proteasome, localized in the β5 subunit of the 20S core, resulting in accumulation of poly-ubiquitinated 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 this study we demonstrated that normal myeloid cells (including CD34+ HSC and primary monocytes) have lower IC₅₀s to bortezomib than the majority of AML samples tested. We showed in AML cells that having IC₅₀s greater than control cells (ie 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 non-malignant 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 et al showed bortezomib preferentially induced CD20 in normal B cells compared to 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 pathological 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 defence 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 pro-oxidant 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 to 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 Figure 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 to more sensitive AML samples (AML303, HL60).

Our understanding of the sequence of events leads us to conclude that AML cells which have basal nuclear expression of Nrf2 are more likely to be resistant to bortezomib-induced death and possibly other SACT. Interestingly, Nrf2 can modulate expression of alpha and beta subunits of the 26S proteasome, however, we demonstrated no obvious change in proteasomal subunits expression patterns between bortezomib-resistant and -sensitive AML cells (Supplementary Figure 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
demonstrated 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 defence 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 defence 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, since Nrf2 regulates many genes including HO-1 to provide reduced sensitivity towards proteasomal inhibition, targeting Nrf2 may be more effective than targeting any of these genes alone. In conclusion, our data demonstrate that inhibition of Nrf2
sensitizes cells to proteasome inhibition, suggesting that Nrf2 inhibitors may be used concomitantly to increase the efficacy of anticancer therapy.

**Acknowledgments**

This study was supported by research funding in the form of grant support from the Big C Appeal, Association for International Cancer Research (AICR) and Leukaemia and Lymphoma Research Foundation. We thank Professor Richard Ball (N&NUH Human Tissue Bank) for sample storage and Dr Shalal Sadullah (James Paget University Hospital, Norfolk) for collection of some of the AML samples.

**Disclosure of Conflicts of Interest:** The authors declare no competing financial interests.
References


10. Bloom DA, Jaiswal AK. Phosphorylation of Nrf2 at Ser(40) by protein kinase C in response to antioxidants leads to the release of Nrf2 from INrf2, but is not


35. Rushworth SA, Chen XL, Mackman N, Ogborne RM, O'Connell MA. Lipopolysaccharide-induced heme oxygenase-1 expression in human


46. Reichard JF, Sartor MA, Puga A. Bach1 is a specific repressor of HMOX1 that is inactivated by arsenite. J Biol Chem. 2008;283:22363-70.


<table>
<thead>
<tr>
<th>Number</th>
<th>Age</th>
<th>Gender</th>
<th>WHO diagnosis</th>
<th>Cytogenetics</th>
<th>% Blasts</th>
<th>Previous treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML204</td>
<td>80</td>
<td>male</td>
<td>AML without maturation</td>
<td>normal</td>
<td>95</td>
<td>nil</td>
</tr>
<tr>
<td>AML205</td>
<td>66</td>
<td>female</td>
<td>therapy related myeloid neoplasm</td>
<td>complex</td>
<td>85</td>
<td>1999 DAT, DAT MACE, MiDAC (ref 50)</td>
</tr>
<tr>
<td>AML206</td>
<td>61</td>
<td>male</td>
<td>AML with myelodysplasia related changes</td>
<td>complex</td>
<td>80</td>
<td>nil</td>
</tr>
<tr>
<td>AML207</td>
<td>53</td>
<td>male</td>
<td>acute promyelocytic leukaemia with t(15;17)(q22;q12) PML-RARA</td>
<td>t(15;17)</td>
<td>95**</td>
<td>nil</td>
</tr>
<tr>
<td>AML208</td>
<td>77</td>
<td>male</td>
<td>AML with myelodysplasia related changes</td>
<td>complex</td>
<td>95</td>
<td>nil</td>
</tr>
<tr>
<td>AML209</td>
<td>49</td>
<td>male</td>
<td>AML with maturation</td>
<td>normal</td>
<td>80</td>
<td>nil</td>
</tr>
<tr>
<td>AML210</td>
<td>39</td>
<td>male</td>
<td>AML with maturation</td>
<td>normal</td>
<td>65</td>
<td>nil</td>
</tr>
<tr>
<td>AML211</td>
<td>58</td>
<td>male</td>
<td>Acute erythroid leukameia</td>
<td>normal</td>
<td>70</td>
<td>nil</td>
</tr>
<tr>
<td>AML212</td>
<td>64</td>
<td>male</td>
<td>AML with t(8;21)(q22;22);RUNX1-RUNX1T1</td>
<td>t(8;21)</td>
<td>85</td>
<td>nil</td>
</tr>
<tr>
<td>AML213</td>
<td>92</td>
<td>female</td>
<td>AML with myelodysplasia related changes</td>
<td>not available</td>
<td>70</td>
<td>nil</td>
</tr>
<tr>
<td>AML216</td>
<td>57</td>
<td>male</td>
<td>AML with myelodysplasia related changes</td>
<td>normal</td>
<td>55</td>
<td>nil</td>
</tr>
<tr>
<td>AML217</td>
<td>82</td>
<td>female</td>
<td>AML with myelodysplasia related changes</td>
<td>deletion 13</td>
<td>85</td>
<td>Hydroxycarbamide</td>
</tr>
<tr>
<td>AML301</td>
<td>46</td>
<td>female</td>
<td>AML with maturation</td>
<td>+4, +8, t(9;22)</td>
<td>70</td>
<td>nil</td>
</tr>
<tr>
<td>AML303</td>
<td>40</td>
<td>male</td>
<td>Acute promyelocytic leukaemia with t(15;17)(q22;q12) PML-RARA</td>
<td>t(15;17)</td>
<td>95**</td>
<td>1999 DAT, DAT MACE, MiDAC (ref 50)</td>
</tr>
<tr>
<td>AML305</td>
<td>66</td>
<td>female</td>
<td>AML without maturation</td>
<td>t(2;12)</td>
<td>65</td>
<td>nil</td>
</tr>
<tr>
<td>AML306</td>
<td>78</td>
<td>male</td>
<td>AML with myelodysplasia related changes</td>
<td>not available</td>
<td>85</td>
<td>nil</td>
</tr>
<tr>
<td>AML307</td>
<td>57</td>
<td>male</td>
<td>AML with minimal differentiation</td>
<td>not available</td>
<td>95</td>
<td>2009 DA, DA, MACE, MiDAC (ref 50)</td>
</tr>
</tbody>
</table>
Table legend

Table 1. **AML sample information.** AML disease characteristics including WHO diagnosis and cytogenetics. Percent blast denotes % of AML blasts after purification using density gradient and in some instances CD34+ positive selection (* denotes isolated through CD34 positive selection. ** denotes % of blasts and promyelocytes). Previous treatments are as outlined (50).
Figure legends

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

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

Figure 3. Bortezomib induces the activation of Nrf2 in AML. (A) THP-1 cells treated with 25 nM bortezomib for the indicated times. Whole cell protein extracts as well as 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 nM siRNA and incubated for 24 h before treatment with 25 nM 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 analysed for Nrf2 protein. (D) AML cells and control monocytes were transfected with 30 nM of siRNA and incubated for 24 h before 25 nM bortezomib treatment for 24 h, before FACS analysis.

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 nM 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 nM bortezomib for various times before ChIP using antibodies against Bach1, Nrf2 and normal rabbit IgG as a control. Data presented as percent 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 nM bortezomib for various times. (D) Cells were transfected with 30 nM of siRNA and incubated for 24-48 h. HO-1, NQO1, GCLM and GSR mRNA was measured using real-time PCR, with expression normalized to GAPDH.

Figure 5. Bortezomib-induced ROS activates Nrf2 and Bach1 to protect AML cells from apoptosis. (A) AML cells and non-malignant CD34+ control cells were treated with bortezomib followed by incubation with H2DCFDA 10 μM, 15 min. Cellular ROS was assessed by flow cytometry. (B) AML cells were transfected with 30 nM of siRNA and incubated for 24 h, then treated 25 nM bortezomib followed by ROS measurement. (C). Cells treated with bortezomib for 1 h with or without 10 mM NAC pretreatment for 30 min. Cytosolic and Nuclear Bach1 and Nrf2 protein was determined then reprobed with either GAPDH or TBP to confirm similar loading. (D) Cells treated with bortezomib for 4 h with or without 10 mM NAC pretreatment for 30 min. HO-1 and NQO1 mRNA was measured using real-time PCR, with expression normalized to GAPDH.

Figure 6. Overview of the mechanism by which human AML leukemia cells are resistant to bortezomib treatment.
A Rushworth et al, Figure 1

B

25 nM bortezomib (h)

annexin-V

CD34+

THP-1

AML306

AML307

normal cells
AML cells

bortezomib IC50 (nM)

0

20

40

60

80

0

25 nM bortezomib (h)

82 4 7 2

B

1.2% 1.4% 1.1% 4.8% 1.2% 35.4% 0.8% 43.5%

90.2% 7.2% 81.9% 12.2% 18.8% 44.6% 4.5% 51.2%

2.6% 6.4% 2.9% 31.4% 2.2% 6.9% 2.1% 51.4%

AML306

AML307
A

B

C

D

Rushworth et al, Figure 5
Rushworth et al, Figure 6

**Normal cells**
- bortezomib
- ROS
- Death

**AML cells**
- bortezomib
- ROS
- Nrf2
- Bach1
- cytoprotection

*chemo-resistance*