BAK and NOXA Are Critical Determinants of Mitochondrial Apoptosis Induced by Bortezomib in Mesothelioma

Sara Busacca1, Alex D. Chacko2, Astero Klabatsa3, Kenneth Arthur2, Michael Sheaff4, Vignesh K. Gunasekharan5, Julia J. Gorski2, Mohamed El-Tanani2, V. Courtney Broaddus6, Giovanni Gaudino7, Dean A. Fennell1*

1Department of Cancer Studies and Molecular Medicine, University of Leicester, Leicester, United Kingdom, 2Centre for Cancer Research and Cell Biology, Queen’s University of Belfast, Belfast, Northern Ireland, 3Division of Cancer Studies, Department of Research Oncology, King’s College London, London, United Kingdom, 4Department of Cellular Pathology, Barts and the London NHS Trust, London, United Kingdom, 5Department of Microbiology-Immunology, The Feinberg School of Medicine, Northwestern University, Chicago, Illinois, United States of America, 6Lung Biology Centre, San Francisco General Hospital, University of California San Francisco, San Francisco, California, United States of America, 7University of Hawaii Cancer Center, Honolulu, Hawaii, United States of America

Abstract

Based on promising preclinical efficacy associated with the 20S proteasome inhibitor bortezomib in malignant pleural mesothelioma (MPM), two phase II clinical trials have been initiated (EORTC 08052 and ICORG 05–10). However, the potential mechanisms underlying resistance to this targeted drug in MPM are still unknown. Functional genetic analyses were conducted to determine the key mitochondrial apoptotic regulators required for bortezomib sensitivity and to establish how their dysregulation may confer resistance. The multidomain proapoptotic protein BAK, but not its orthologue BAX, was found to be essential for bortezomib-induced apoptosis in MPM cell lines. Immunohistochemistry was performed on tissues from the ICORG-05 phase II trial and a TMA of archived mesotheliomas. Loss of BAK was found in 39% of specimens and loss of both BAX/BAK in 37% of samples. However, MPM tissues from patients who failed to respond to bortezomib and MPM cell lines selected for resistance to bortezomib conserved BAK expression. In contrast, c-Myc dependent transactivation of NOXA was abrogated in the resistant cell lines. In summary, the block of mitochondrial apoptosis is a limiting factor for achieving efficacy of bortezomib in MPM, and the observed loss of BAK expression or NOXA transactivation may be relevant mechanisms of resistance in the clinic.

Citation: Busacca S, Chacko AD, Klabatsa A, Arthur K, Sheaff M, et al. (2013) BAK and NOXA Are Critical Determinants of Mitochondrial Apoptosis Induced by Bortezomib in Mesothelioma. PLoS ONE 8(6): e65489. doi:10.1371/journal.pone.0065489

Editor: Andrei L. Gartel, University of Illinois at Chicago, United States of America

Received November 1, 2012; Accepted April 25, 2013; Published June 7, 2013

Copyright: © 2013 Busacca et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: SW was supported by an NCI/NIH grant (CA188413). DAF was supported by a Cancer Research UK Clinician Scientist Fellowship. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: df132@le.ac.uk

Introduction

Malignant pleural mesothelioma (MPM) is an aggressive cancer caused by exposure to asbestos. It is increasing in incidence worldwide however there is a paucity of effective therapy [1]. Pemetrexed or raltitrexed when combined with cisplatin have been shown to lead to modest improvements in overall survival [2,3]. However, patients universally relapse; following which, there is no agreed standard of care. MPM is a highly drug resistant solid tumour, and this is correlated with apoptosis resistance [4]. There is a pressing need for new, more effective therapies, particularly where there is an unmet clinical need after first-line chemotherapy.

The proteasome inhibitor bortezomib has shown promising activity in preclinical models both in vitro and in vivo [5,6], which has led to initiation of clinical trials evaluating the effect of bortezomib alone [7] or in combination with cisplatin or oxaliplatin (www.clinicaltrials.gov). However our data from Phase II study of bortezomib activity as monotherapy in an unselected population of MPM patients showed only minimal (5%) response rate, implicating inherent resistance [7]. In contrast to hematopoietic malignancies, the poor response of solid tumours to bortezomib treatment appears to be due to the existence of both primary and acquired resistance [8]. Several mechanisms of resistance have been proposed, including mutations in the proteasome subunits and alteration in their expression levels [9–14], increases in the efficiency of alternative mechanisms of protein degradation such as the lysosomal system, the ER-associated protein degradation (ERAD) [15], and aggresome formation [16]. Bortezomib is an activator of the mitochondrial apoptosis pathway, and as such, defects in this signalling pathway could confer resistance [17].

Here, we show that specific components of the mitochondrial signalling pathway are lost or dysregulated in MPM, and can directly cause bortezomib resistance.
Materials and Methods

Reagents and Antibodies

Bortezomib was courtesy of Millenium; MG132 was purchased from Sigma-Aldrich, (St. Louis, MO). Antibodies against c-Myc, BAX and BAK were from Cell Signaling (Danvers, MA, USA), anti-PARP from Alexis (Nottingham, UK), anti-NOXA from Calbiochem (Gibbstown, NY), GAPDH and β-Tubulin from Abcam (Cambridge, UK). Secondary antibodies were: goat anti-rabbit HRP (DAKO, Glostrup, Denmark), donkey anti-mouse HRP (GE Healthcare).

Cell Lines

REN [18] (kindly provided by Dr. S.M. Albelda, University of Pennsylvania, Philadelphia, USA), were grown in Nutrient mixture F12 Ham (Invitrogen, Carlsbad, CA), L-Glutamine, 10% (FBS) Foetal Bovine Serum (PAA) and penicillin/streptomycin (Gibco). JU77 [19], and Wild type (WT) and BAX/BAK double knockout (DKO) mouse embryonic fibroblasts (MEFs) [20] (kind gift from Dr. Scott Oakes, University of California, San Francisco, USA) were grown in RPMI Medium 1640, L-Glutamine and 10% FBS. Bortezomib resistant cells (RENBZR, JU77BZR) were generated by increasing exposure to bortezomib. Generation of c-Myc shRNA expressing stable clones employed retroviral transduction using 4 \times 10^5 Phoenix Ampho cells [21] (kindly provided by P. Mullan, Queen’s University of Belfast, Northern Ireland). Cells were then subjected to puromycin (Calbiochem) (4 μg/mL) selection.

Measurement of Cell Viability and Apoptosis

Cell viability was assessed by a Vialight Plus kit (Lonza, Basel, Switzerland). For the caspase-3 luminescence assay, cells were

Figure 1. In MEF cells, BAX and BAK expression regulates bortezomib activity. A) WT MEF and BAX/BAK DKO MEF cells were treated with bortezomib 10 nM for 24 h. PARP cleavage was measured by western blot. B) Caspase3 activity was assessed by luminescence assay. Data were normalized to untreated control (WT; p < 0.0001; DKO n.s.). C) BAX and BAK were transiently overexpressed in DKO cells and 24 h post transfection cells were treated with bortezomib 10 nM for a further 24 hours. BAX and BAK expression were then analysed by western blot. D) Caspase 3 activation after bortezomib treatment was also analysed by luminescence assay. Data were normalized to untreated control (EV: n.s.; GSTBAX: p < 0.0001 GSTBAK: p < 0.0001).

doi:10.1371/journal.pone.0065489.g001
analysed by using a Caspase-Glo 3/7 Assay (Promega, Southampton, Hampshire).

Protein Extraction and Immunoblotting

Cells were lysed in RIPA buffer containing protease inhibitors (Roche, Burgess Hill, UK). Cell lysates were separated on SDS-PAGE denaturing gels, transferred to nitrocellulose membranes, and blocked in 5% milk-PBS-0.1% tween. Membranes were probed with primary antibodies diluted in 5% milk-PBS-0.1% tween at 4°C overnight. Signal detection was performed with the ECL-plus chemiluminescent system (GE Healthcare, Amersham, UK).

siRNA Transfections

Non-silencing control (NT), BAX, BAK, and NOXA targeting siRNAs were obtained from Qiagen. siRNA (50 nM for BAX and BAK, 20 nM for NOXA) transfections were performed using the RNAiMAX transfection reagent (Invitrogen) according to manufacturer’s instructions.

BAX and BAK Overexpression

BAX/BAK DKO cells were transiently transfected with GST-tagged BAX and BAK (pEGFP-C3 vector), using X-tremeGENE transfection reagent (Roche) according to manufacturer’s instructions.

Tumour Samples

BAX and BAK protein expression was assessed by immunohistochemistry (IHC) on two sets of samples. 16 tissues were from ICORG-05 Study [7]. The second set comprised a TMA of 100 archived mesotheliomas; however partial of full loss of 30% TMA cores has been observed during the staining. Appropriate ethical approval was obtained from the local research ethics committees to carry out this work (Ireland: SJH/AMNCH (The St James’s Hospital & Adelaide & Meath Hospital incorporating the National Children’s Hospital) Research Ethics Committee; Netherlands: Ethics Committee of NKI-AVL (Nederlands Kanker Instituut - Antoni van Leeuwenhoek Ziekenhuis); Belgium: Ethics Committee of the University Hospital Ghent; United Kingdom: Belfast Central Research, OREC (Office of research ethics committees) UK).

Immunohistochemistry and Scoring

Immunohistochemistry for the samples from the ICORG-05 study was performed within the Tissue Core Technology Unit at the Centre for Cancer Research and Cell Biology and sections were then scanned in the Queen’s University of Belfast Bioimaging Unit. BAK primary antibody was used at a 1:800 dilution; BAX antibody was used at a 1:50 dilution. IHC scoring of tissue slides from the Phase II trial was carried out through the PathXL™ TMA Toolbox [i-Path Diagnostics Ltd, Belfast, UK]. The TMA was stained and scored in the Pathology Core Facility, Department of pathology, Bart’s and the London NHS Trust.

The staining results were semi-quantitatively assessed by two individual examiners. Tumours were graded for expression of BAX and BAK as follows: 0 = no cells stained, 1 = <25% cells positive (light staining), 2 = 25–75% cells positive (moderate staining) 3 = >75% cells positive (strong staining). In case of discrepancy between the two examiners, a result was obtained by consensus while reviewing the slides using a double-headed microscope. Survival data were available for 30 out of 70 patients; therefore the analysis has been carried out on this unselected
population only. The survival analysis was performed by using Kaplan-Meier estimation and significance was measured by the log-rank test.

The adopted statistics software was SPSS17.0 (Chicago, IL, USA).

Mitochondria Isolation
Cells were washed in Mitochondrial Isolation Buffer (200 mM Mannitol, 70 mM Sucrose, 1 mM EGTA, 10 mM HEPES, 0.5 mg/ml BSA, pH7.4). Mitochondria were then isolated by dounce homogenization followed by centrifugation at 800×g for 10 minutes at 4°C to remove debris and heavy membranes, then by centrifugation at 10,000×g for 10 minutes at 4°C. The mitochondrial-free cytosolic fraction was used for Western blot analysis [22].

Real Time Quantitative RT-PCR
Total RNA was extracted using an RNeasy Plus mini kit (Qiagen Valencia, CA, USA) according to manufacturer's instructions. Quality control was performed by Phalanx Biotech Group (Palo Alto, CA, USA). Reverse transcription was performed with M-MLV Reverse transcriptase (Invitrogen). Real-Time PCR was carried out using Power SYBR® Green PCR Master Mix (Applied Biosystem).

c-Myc Reporter Assay
c-Myc reporter assay was performed using Cignal Reporter Assay Kits (SABiosciences, Frederick, MD). Transfections were carried out by using the Lipofoctamine2000 transfection reagent (Invitrogen). Luciferase activity was measured using the dual-luciferase reporter assay system (Promega).

---

Figure 3. Immunohistochemical analysis of BAX and BAK expression in malignant mesothelioma patients. A) Representative image of normal tissue control and positive tissues stained for BAX and BAK by immunohistochemistry. B) Pie-charts representing the frequency of BAX, BAK and BAX-BAK negativity in previously treated 30 mesothelioma patients. C) Kaplan Meier curves correlating BAX, BAK, and double BAX/BAK expression respectively with survival in the total of 30 previously-treated patients.
doi:10.1371/journal.pone.0065489.g003
Chromatin Immunoprecipitation

Chromatin immunoprecipitation assays were performed as previously described [23]. The antibodies used for immunoprecipitation were: TBP antibody, HA-probe (Y11), anti-c-Myc (N262), from Santa Cruz and rabbit IgG from Dako. PCR amplification was performed on the purified DNA using the primers for MYC-BS III [24].

Statistical Analysis

Dose-response curves were fitted using non-linear regression (GraphPad Prism version 4.0, GraphPad Software, Inc. LaJolla, CA, USA).

One or two-way analysis of variance was used to evaluate statistical significance and a Bonferroni post-test was performed. A p value less than 0.05 was considered significant.

Results

BAK is an Essential Regulator of Bortezomib-induced Apoptosis in MPM Cells

Bortezomib induced apoptosis in wild type mouse embryonic fibroblasts (WT MEF), but this effect was dramatically reduced in cells with homozygous deletion of BAX and BAK (BAX/BAK DKO MEF), as evidenced by PARP cleavage (Figure 1A, middle panels). Apoptosis induction measured by caspase 3 activation (4.13 and 1.13 fold increase in WT and DKO respectively) (Figure 1B). Apoptosis induction measured by caspase 3 activation, was restored by knocking-in BAK or BAX (EV: 0.77, GST-BAX: 3.48 and GST-BAK: 3.04 fold increase) (Figure 1C, D), suggesting that both BAX and BAK mediate bortezomib-induced apoptosis.

We then tested for the individual and combined contribution of BAX and BAK by using RNA interference to silence BAX or BAK or both in MPM cell lines (REN and JU77). Here in contrast to our findings using MEFs, we found that silencing of BAX alone did not reduced sensitivity to bortezomib. However, BAK proved to be important; BAX and BAK silencing significantly reduced bortezomib-induced caspase 3 activity in both REN (siNT: 3.25, siBAX: 3.14, siBAX: 0.83 and siBAX/BAK: 1.01 fold increase) (Figure 2A) and JU77 (siNT: 4.8, siBAX: 5.13, siBAX: 0.9 and siBAX/BAK: 0.76 fold increase) (Figure 2B). Similarly, also PARP cleavage was reduced only by the expression of BAK, but not of BAX in both REN (Figure 2C) and JU77 (Figure 2D).

Table 1. Scoring of samples from ICORG-05 Phase II Trial [7].

| Staining intensity | Sample | BAX | BAK | Sample | BAX | BAK |
|--------------------|--------|-----|-----|--------|-----|-----|
| a                  | 1      | 1   | i   | 2      | 2   |     |
| b                  | 0      | 1   | j   | 0      | 2   |     |
| c                  | 1      | 2   | k   | 0      | 1   |     |
| d                  | 0      | 1   | l   | 1      | 1   |     |
| e                  | 0      | 1   | m   | 0      | 2   |     |
| f                  | 1      | 1   | n   | 1      | 1   |     |
| g                  | 0      | 1   | o   | 2      | 3   |     |
| h                  | 0      | 3   | p   | 1      | 1   |     |

0 = No cells stained 1 = <25% cells stained 2 = 26–75% cells stained 3 = >75% cells stained.

doi:10.1371/journal.pone.0065489.t001

BAK is Lost in Primary Mesothelioma

We used an immunohistochemistry-based approach to measure the expression of BAX and BAK in two different cohorts of MPM patients (Figure 3A). In the first cohort, out of 69 cases assessed for BAK expression, 43 were positive (62.3%) and 26 were negative (37.7%). The staining was associated mainly with moderate or strong intensity in both cases. The expression of BAX could be assessed in 70 cases overall the TMA of which 44 cases were positive (62.9%) and 26 were negative (37.1%). The loss of both BAX and BAK expression was also analysed in 70 cases of which 44 were positive for both proteins (62.9%) and 26 were negative for both proteins (37.1%). Thirty of these patients were previously treated and survival data were available; of these patients the 80% presented positive staining for BAX, 70% were positive for BAK and 70% were positive for both BAX and BAK (Figure 3B). Survival analysis revealed that in BAX positive patients the overall median survival was 18 versus 6 months in negative patients (HR = 0.518, 95% CI = 0.36–0.75). In the case of BAX, its expression was associated with a survival of 16 months, compared to 5 months of BAX negative patients (HR = 0.42, 95% CI = 0.29–0.62). The overall median survival was 18 months in BAX/BAK positive samples and 3 months in BAX/BAK negative samples (HR = 0.197, 95% CI = 0.08–0.49) (Figure 3 C).

A second cohort consisted of 16 specimens obtained from MPM patients treated with bortezomib in a phase II trial [7]. BAX expression was absent in 50% of the tumours, whereas BAK was expressed in all the samples (100%). Notably, only one patient (sample i) of this set had stable disease [7] and the corresponding tissue was positive for both BAX and BAK staining (Table 1).

Neither BAX or BAK Expression are Altered in Mesothelioma Cell Lines Selected for Bortezomib Resistance

Both REN and JU77 cell lines were exposed to increasing concentrations of bortezomib leading to selection of two isogenic resistant cell lines (REN RZR, JU77 BZR). REN RZR and JU77 BZR cells exhibited 6-fold and 7-fold resistance compared to parental cells (IC50 12 nM and 50 nM respectively) (Figure 4A, left panels). Resistance was accompanied by a significant reduction in caspase 3 activity (REN: 1.83, REN RZR, 0.8, JU77 BZR, 2.9, JU77 BZR: 1.1 fold increased) (Figure 4A, middle panels) and reduced PARP cleavage in both resistant cell lines compared to parental cells (Figure 4A, right panels). Basal expression of both BAX and BAK was not significantly different in resistant cells compared to parental cell lines after 6 h (Figure 4B). Finally, cytochrome C release upon bortezomib treatment was also investigated; cytosolic levels of Cytochrome C in a cytoplasmic mitochondria-free fraction were detected in parental cell lines only, but not in the two resistant cell lines (Figure 4C).

The BH3-only Protein NOXA is Critical for Bortezomib-induced Apoptosis

Upregulation of NOXA has been implicated as a regulator of bortezomib induced apoptosis specifically in tumour cells [17]. After NOXA was silenced using RNA interference, bortezomib induced caspase 3 activity was significantly inhibited in REN (Figure 5A) and JU77 transfected cells (Figure 5B). These data were confirmed by western blot showing a significant decrease in PARP cleavage induced by bortezomib in both REN (Figure 5C) and JU77 transfected cells (Figure 5D).
Bortezomib Resistant Cells Fail to Activate Transcription of NOXA

The upregulation of NOXA protein expression following bortezomib was significantly reduced in resistant cells compared to parental cells (Figure 6A). Analysis of NOXA mRNA level revealed significant transcriptional upregulation of NOXA after bortezomib treatment in parental cells. This was not reflected in the resistant cell lines where bortezomib induced very little or no upregulation of NOXA (REN: 4.42; RENBZR: 0.82; JU77: 3.19; JU77BZR: 1.24 fold increase, respectively) (Figure 6B).

C-Myc is a transcriptional activator of NOXA [24]. To evaluate the role of this transcription factor in MPM cells, REN clones stably expressing shRNA targeting c-Myc (RENsh/Myc), or scrambled control shRNA (REN sh/scr), were generated. In RENsh/Myc cells following bortezomib treatment, the upregulation

Figure 4. Generation and characterization of mesothelioma bortezomib-resistant cell lines. A) REN and JU77 selected for resistance after exposure to increasing doses of bortezomib were tested for cell viability after 24 h treatment with bortezomib at concentrations ranging from 0.5 nM to 50 nM and compared to parental cells. REN/RENBZR and JU77/JU77BZR cells were treated for 24 h with bortezomib 5 nM and 10 nM, respectively. PARP cleavage induced by bortezomib was analysed by western blot and caspase3 activity was measured by luminescence assay. Data were normalized to untreated control (REN: <0.0001; RENBZR: n.s.; JU77: p=0.0002; JU77BZR: n.s.). B) Expression of BAX and BAK was investigated in parental and resistant cells pre- and after 6 h treatment with bortezomib 5 nM and 10 nM in REN/RENBZR and JU77/JU77BZR respectively. C) Cytochrome C release was assessed after 24 h treatment with bortezomib (5 nM and 10 nM in REN/RENBZR and JU77/JU77BZR, respectively). Mitochondrial-free cytosolic fraction has been used for western blot analysis.
doi:10.1371/journal.pone.0065489.g004
of NOXA protein was completely abolished following bortezomib in RENsh/Myc cells (Figure 6C).

In bortezomib resistant MPM cells, c-Myc protein expression was lower at baseline compared to parental cells and was unaffected by exposure to bortezomib (Figure 6A). Consistently, the basal transcriptional activity of c-Myc in resistant cells evaluated by a luciferase reporter assay was significantly reduced compared to parental cells. Following bortezomib treatment c-Myc activity was significantly increased in REN but not in RENBZR cells (REN: 2.08; RENBZR: 1.7 fold increase, respectively) (Figure 6D).

Chromatin immunoprecipitation revealed the interaction of c-Myc with the promoter for NOXA in REN parental cells, which increased after treatment with bortezomib. However, no interaction was observed in RENBZR cells, even after treatment with bortezomib (Figure 6E).

Discussion

Bortezomib exhibits significant preclinical activity in several solid tumour cell lines and animal models including MPM [5,6]. However, this efficacy has not been successfully translated into the clinic due to either primary or acquired resistance [7,25–27]. Here we demonstrate that reconstitution of BAX or BAK in mouse BAX/BAK DKO fibroblasts is sufficient to restore sensitivity to bortezomib. In MPM cells the silencing of BAK dramatically reduced response to bortezomib, however downregulation of BAX alone was not sufficient to impact the response to bortezomib. Thus, although BAX and BAK appear to be functionally redundant in MEFs, this does not appear to be the case in MPM cells, where BAX was not able to reconstitute loss of BAK. This might be due to the known differential regulation by prosurvival proteins, such as Mcl-1 and Bcl-xL [28–30]. Both Bcl-xL and Mcl-1 are highly expressed in MPM and may restrain BAK constitutively [31]. Moreover, BAX and BAK expression data in our resistant cell lines show that these proteins are not altered in a context of "acquired resistance" after prolonged exposure to the drug. This suggests that the selective pressure may not be enough to lead to loss of expression of proteins that have essential housekeeping roles, such as mitochondrial fusion and fission. However, in a clinical setting we observed de novo lack of BAX and BAK protein expression that can correlate with primary resistance.

The expression of BAX and BAK has been previously investigated in mesothelioma samples and 24% loss of BAK and 42% loss of BAX expression were found, but no correlation with histology was reported [31]. Conversely, another group showed 100% expression of BAX in the MPM specimens analysed [32]. In our population of unselected MPM samples loss of both BAX and BAK was observed and it was correlated with clinical outcome implicating a prognostic significance of defective mitochondrial apoptosis. However, Cox regression analysis was only possible for histology, age and sex and no association between those and BAX/BAK expression could be seen. This is due to the low number of BAX/BAK positive cases with survival data not allowing statistical analysis. Therefore, the prognostic value of BAX/BAK levels associated with other common prognostic...
factors requires further analysis in a larger cohort with available clinical data.

Apoptosis block is a hallmark of cancer and may contribute to aggressive tumour progression in this sub-population of patients with MPM, as well as potentially conferring drug resistance [33]. Bortezomib upregulates the BH3-only protein and Mcl-1 inhibitor NOXA, at both the protein and mRNA level after 6 hours from exposure [22,34,35]; The Mcl-1 anti-apoptotic protein inhibits apoptosis by sequestering BAK from activating the mitochondrial outer membrane permeabilization [30]. It has been demonstrated that NOXA can displace BAK from Mcl-1 and can also promote Mcl-1 degradation through the proteasome system [30]. Here we show that NOXA is downregulated in both resistant cell lines; this may explain why NOXA or BAK silencing
recapitulate the resistant phenotype. Moreover, treatment with bortezomib failed to induce NOXA upregulation at both protein and mRNA level in resistant cells.

NOXA is commonly described as a p53 target gene as it contains p53 response elements on its promoter and it has also been reported as a key mediator of p53-driven apoptosis [36]. However, it has been shown that in different cellular systems the up-regulation of NOXA at both protein and mRNA level induced by bortezomib can also occur through p53-independent mechanisms [22,34,37,38], such as myc transcriptional regulation [24]. Indeed, it was demonstrated that c-Myc can bind the Noxa promoter and regulate its transcription [24,39]. As shown in Figure 6, Noxa overexpression occurs in REN cells upon treatment with bortezomib. The transcriptional mechanism involved in REN cells must be p53-independent because REN cells are known to contain a rearranged p53 gene and lack expression of p53 protein [40]. We thus suggest the c-Myc is the only driver of Noxa expression in this context.

The induction of NOXA and the subsequent activation of the mitochondrial apoptosis pathway by bortezomib has been reported not to correlate linearly with c-Myc protein or mRNA expression [24], but being dependent on c-Myc activity in binding the Noxa promoter. However, no data are available to date on c-Myc dysregulation in bortezomib resistance cells. Here we show that resistant cells express a lower level of c-Myc protein, compared to parental cells and that c-Myc is dysfunctional in the resistant setting. No binding at the Noxa promoter was detected in resistant cells and as expected it was not induced by bortezomib treatment. Our data support the thesis of c-Myc stabilisation induced by bortezomib (as shown by western blot), consequent increase in binding to the Noxa promoter (ChIP data) and final activation (increased reporter activity).

These events may occur in cooperation with proteasome function in regulating histone acetylases [41], chromatin modulating proteins, basal transcriptional factors and DNA methyltransferase [42] which play an important role in c-Myc transcription regulation [43–45].

Alternative mechanisms of resistance to bortezomib have been proposed, in fact, increasing evidence in haematological tumours support the importance of the expression levels of proteasome subunits and their composition. Mutation of PSMB5 has been shown to be a cause of bortezomib resistance [10,11,13,46], however G322A or C326T mutations were not observed in our cells implicating an alternative gene alteration, to account for lack of inhibition. Moreover, in our system expression levels of the β1 subunit before or after treatment were not altered. Conversely the β2 subunit was decreased in resistant cells, supporting data which showed a correlation between resistance and expression levels of proteasome subunits [47]. Finally, the β5 subunit level was increased in RENBZR cells compared to parental cells (data not shown).

These findings suggest that disruption of c-Myc-dependent NOXA-mediated death signalling and BAK could play a potential role in resistance to bortezomib in the clinical setting, highlighting the putative role of BAK and NOXA as valid prognostic markers for bortezomib. However, our data from 16 patients enrolled in the Phase II clinical trial showed that NOXA was expressed in the tissues from all the MPM patients examined, including the one showing stable disease [7]. The number of samples included in this analysis was too small to allow a statistically reliable analysis; actually, we know that a portion of patients will be BAX and/or BAK negative as observed in a larger cohort (70 samples). Nevertheless these patients will still show resistance even were NOXA expression is observed. It is also possible that other resistance mechanisms are involved in the clinical setting as suggested from data available from the sanger database (http://www.cancerrxgene.org/) regarding genomics of proteasome inhibitors, such as bortezomib and MG132, sensitivity/resistance.

In summary, bortezomib requires functional BAK and NOXA to induce apoptosis in MPM cells. The loss of BAK expression occurring in a subset of patients with MPM may contribute to resistance to this drug in the clinical setting. However, dysregulation of NOXA transactivation may be an alternative mechanism as evidenced in MPM cells selected for resistance to bortezomib.

**Author Contributions**

Conceived and designed the experiments: AF MET VCB GG. Performed the experiments: SB AK KA JJG. Analyzed the data: AK MS. Contributed reagents/materials/analysis tools: AC VKG. Wrote the paper: SB DAF.

**References**

1. Fennell DA, Gaudino G, O’Byrne KJ, Mutti L, van Meerbeeck J (2008) Advances in the systemic therapy of malignant pleural mesothelioma. Nat Clin Pract Oncol 5: 136–147.
2. van Meerbeeck JP, Gaspar R, Manegold C, Van Klaveren RJ, Van Marck EA, et al. (2005) Randomized phase III study of cisplatin with or without rituximab in patients with malignant pleural mesothelioma: an intergroup study of the European Organisation for Research and Treatment of Cancer Lung Cancer Group and the National Cancer Institute of Canada. J Clin Oncol 23: 6081–6089.
3. Vogelzang NJ, Rusthoven JJ, Symanowski J, Denham C, Kazak E, et al. (2003) Phase III study of pemetrexed in combination with cisplatin versus cisplatin alone in patients with malignant pleural mesothelioma. J Clin Oncol 21: 2636–2644.
4. Fennell DA, Rudd RM (2004) Defective core-antigen signalling in diffuse malignant pleural mesothelioma: opportunities for effective drug development. Lancet Oncol 5: 354–362.
5. Gordon GJ, Mani M, Maslik G, Mukhopadhyay L, Yeap BY, et al. (2008) Preclinical studies of the proteasome inhibitor bortezomib in malignant pleural mesothelioma. Cancer Chemother Pharmacol 61: 349–358.
6. Sarto-Rebischon A, Gasparri F, Galvani A, Nicu I, Darnowski JW, et al. (2007) Bortezomib inhibits nuclear factor-kappaB dependent survival and has potency in vivo activity in mesothelioma. Clin Cancer Res 13: 5942–5951.
7. Fennell DA, McDowell C, Buacasa S, Webb G, Moulton B, et al. (2012) Phase II clinical trial of first or second-line treatment with bortezomib in patients with malignant pleural mesothelioma. J Thorac Oncol 7: 1466–1470.
8. Wolf J, Richardson PG, Schuster M, LeBlanc A, Walters IB, et al. (2008) Utility of bortezomib retreatment in relapsed or refractory multiple myeloma patients: a multicenter case series. Clin Adv Hematol Oncol 6: 735–740.
9. Fuchs D, Berges C, Opelz G, Daniel V, Naujokat C (2008) Increased expression and altered subunit composition of proteasomes induced by continuous proteasome inhibition establish apoptotic resistance and hyperproliferation of Burkitt lymphoma cells. J Cell Biochem 103: 270–283.
10. Lu S, Chen Z, Yang J, Chen L, Gong S, et al. (2008) Overexpression of the PSMB5 gene contributes to bortezomib resistance in T-lymphoblastic lymphoma/leukemia cells derived from Jurkat line. Exp Hematol 36: 1278–1284.
11. Lu S, Yang J, Chen Z, Gong S, Zhou H, et al. (2009) Different mutants of PSMB5 confer varying bortezomib resistance in T lymphoblastic lymphoma/leukemia cells derived from the Jurkat cell line. Exp Hematol 37: 831–837.
12. Lu S, Yang J, Song X, Gong S, Zhou H, et al. (2000) Point mutation of the proteasome beta5 subunit gene is an important mechanism of bortezomib resistance in bortezomib-selected variants of Jurkat T cell lymphoblastic lymphoma/leukemia line. J Pharmacol Exp Ther 326: 423–431.
13. Oserlemans R, Franke NE, Assaf YG, Ciba J, van Zantwijck J, et al. (2008) Molecular basis of bortezomib resistance: proteasome subunit beta3 (PSMB5) gene mutation and overexpression of PSMB5 protein. Blood 112: 2489–2499.
14. Voortman J, Checchina A, Giaccone G (2007) The proteasomal and apoptotic phenotype determine bortezomib sensitivity of non-small cell lung cancer cells. Mol Cancer 6: 73.
15. Wang Q, Mora-Jensen H, Weniger MA, Perez-Galan P, Woldorf C, et al. (2009) ERAD inhibitors integrate ER stress with an epigenetic mechanism to activate BH3-only protein NOXA in cancer cells. Proc Natl Acad Sci U S A 106: 2200–2205.
16. Raymútsi T, Milián M, Singleton DC, Harris AL (2009) Role of ATF4 in regulation of autophagy and resistance to drugs and hypoxia. Cell Cycle 8: 3838–3847.
17. Fennell DA, Chacko A, Mutti L (2008) BCL-2 family regulation by the 20S proteasome inhibitor bortezomib. Oncogene 27: 1189–1197.

18. Smythe WR, Kaiser LR, Hwang HC, Amin KM, Pilewski JM, et al. (1994) Successful adenovirus-mediated gene transfer in an in vivo model of human malignant mesothelioma. Ann Thorac Surg 57: 1395–1402.

19. Manning LS, Whitaker D, Murch AR, Garlepp MJ, Davis MR, et al. (1991) Establishment and characterization of five human malignant mesothelioma cell lines derived from pleural effusions. Int J Cancer 47: 285–290.

20. Scorrano L, Oakes SA, Opferman JT, Cheng EH, Sorcinelli MD, et al. (2003) BAX and BAK regulation of endoplasmic reticulum Ca2+: a control point for apoptosis. Science 300: 135–139.

21. Swift S, Lorens J, Achatz JF, Nolan GP (2001) Rapid production of retroviruses for efficient gene delivery to mammalian cells using 293T cell-based systems. Curr Protoc Immunol Chapter 10: Unit 10 17C.

22. Qin JZ, Zifra J, Sturni J, Bodner B, Bonish BK, et al. (2005) Proteasome inhibitors trigger NOXA-mediated apoptosis in melanoma and myeloma cells. Cancer Res 65: 6292–6293.

23. Hosey AM, Gorski JJ, Murray MM, Quinn JE, Chung WY, et al. (2007) Molecular basis for estrogen receptor alpha deficiency in BRCA1-linked breast cancer. J Natl Cancer Inst 99: 1603–1604.

24. Nikiforov MA, Riblett M, Tang WH, Gratchouk V, Zhuang D, et al. (2007) Tumor cell-selective regulation of NOXA by c-MYC in response to proteasome inhibition. Proc Natl Acad Sci U S A 104: 19488–19493.

25. Agajanian C, Soignet S, Dzou DS, Penn CA, Adams J, et al. (2002) A phase I trial of the novel proteasome inhibitor PS-341 in advanced solid tumor malignancies. Clin Cancer Res 8: 2503–2511.

26. Fanucchi MP, Fossella FV, Belt R, Natale R, Fidias P, et al. (2006) Randomized phase II study of bortezomib alone and bortezomib in combination with docetaxel in previously treated advanced non-small-cell lung cancer. J Clin Oncol 24: 5025–5033.

27. Scaglotti GV, Germonpre P, Bousquet L, Vansteenkiste J, Gervais R, et al. (2010) A randomized phase II study of bortezomib and penetrated, in combination or alone, in patients with previously treated advanced non-small-cell lung cancer. Lung Cancer 68: 420–426.

28. Du X, Youde RJ, FlitGerald DJ, Patast A (2010) Pseudomonas exotoxin A-based systems. Curr Protoc Immunol Chapter 10: Unit 10 17C.

29. Johnson TR, Stone K, Nikrad M, Yeh T, Zong WX, et al. (2003) The novel ATM-related protein TRRAP is an essential cofactor for the c-Myc transcription through recruitment of DNA methyltransferase corepressor. EMBO J 24: 336–346.

30. Willis SN, Chen L, Dewson G, Wei A, Naik E, et al. (2005) Bortezomib-induced apoptosis in mature T-cell lymphoma cells partially depends on upregulation of Noxa and functional repression of Mcl-1. Cancer Sci.

31. O'Kane SL, Pound RJ, Campbell A, Chaudhuri N, Lind MJ, et al. (2006) The composition of proteasome subunits. Cancer 112: 659–670.

32. Soito Y, Kinnula V, Kaarteenaho-Wiik R, Kurttula E, Linnainma K, et al. (1999) Bortezomib-induced apoptosis and expression of apoptosis regulating proteins bcl-2, mcl-1, bcl-X, and bax in malignant mesothelioma. Clin Cancer Res 5: 3508–3515.

33. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144: 664–674.

34. Fernandez Y, Verhaegen M, Miller TP, Ruhl JL, Steinier P, et al. (2005) Differential regulation of noxa in normal melanocytes and melanoma cells by proteasome inhibition: therapeutic implications. Cancer Res 65: 8294–8304.

35. Ri M, Iida S, Ishida T, Ito A, Yano H, et al. (2008) Bortezomib-induced apoptosis in mature T-cell lymphoma cells partially depends on upregulation of Noxa and functional repression of Mcl-1. Cancer Sci.

36. Oda E, Okiki R, Murasawa H, Nemoto J, Shihue T, et al. (2000) Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. Science 288: 1053–1058.

37. Combaret V, Boyault S, Lacouo I, Brejon S, Rousseau R, et al. (2008) Effect of bortezomib on human neuroblastoma: analysis of molecular mechanisms involved in cytotoxicity. Mol Cancer 7: 50.

38. Pandit B, Pastel AI (2011) Proteasome inhibitors induce p53-independent apoptosis in human cancer cells. Am J Pathol 178: 355–360.

39. Liu W, Sweitz WM, Medisetti R, Das GM (2011) Estrogen-mediated upregulation of Noxa is associated with cell cycle progression in estrogen receptor-positive breast cancer cells. PLoS One 6: e29466.

40. Pietruska JR, Kane AB (2007) SV40 oncoproteins enhance asbestos-induced DNA double-strand breaks and abrogate senescence in murine mesothelial cells. Cancer Res 67: 3637–3643.

41. Ehebauer M, Hayward P, Arias AM (2006) Notch, a universal arbiter of cell fate decisions. Science 314: 1414–1415.

42. Xiong H, Chen ZF, Liang QC, Du W, Chen HM, et al. (2009) Inhibition of DNA methyltransferase inhibits cell cycle arrest and apoptosis in human colorectal cancer cells via inhibition of JAK2/STAT3/STAT5 signalling. J Cell Mol Med 13: 3668–3679.

43. Brenner C, Dephus R, Dideot C, Loriot A, Vire E, et al. (2005) Mys represses transcription through recruitment of DNA methyltransferase corepressor. EMBO J 24: 336–346.

44. Fernandez PC, Frank SR, Wang L, Schroeder M, Liu S, et al. (2003) Genomic targets of the human c-Myc protein. Genes Dev 17: 1113–1129.

45. McMahon SB, Van Buskirk HA, Dugan KA, Copeland ND, Cole MD (1998) The novel viral protein TRAP is an essential cofactor for the c-Myc and E2F oncoproteins. Cell 94: 363–374.

46. Ri M, Iida S, Nakashima T, Miyazaki H, Mori F, et al. (2010) Bortezomib-resistant myeloma cell lines: a role for mutated PSMB5 in preventing the accumulation of unfolded proteins and fatal ER stress. Leukemia.

47. Busse A, Kraus M, Na IK, Rietz A, Scheibenbogen C, et al. (2008) Sensitivity of prostate cancer cells derived from pleural effusions. Int J Cancer 47: 285–290.

BAK and NOXA Regulate Bortezomib-Induced Apoptosis