

# Bevacizumab attenuates major signaling cascades and eIF4E translation initiation factor in multiple myeloma cells

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Multiple myeloma (MM), a malignancy of plasma cells, remains fatal despite introduction of novel therapies, partially due to humoral factors, including vascular endothelial growth factor (VEGF), in their microenvironment. The aim of this study was to explore the efficacy of anti-VEGF treatment with bevacizumab directly on MM cells. Particular attention was directed to the affect of VEGF inhibition on protein translation initiation. Experiments were conducted on MM cells (lines, bone marrow (BM) samples) cultured on plastic. Inhibition of VEGF was achieved with the clinically employed anti-VEGF antibody, bevacizumab, as a platform and its consequences on viability, proliferation, and survival was assessed. VEGF downstream signals of established importance to MM cell biology were assayed as well, with particular emphasis on translation initiation factor eIF4E. We showed that blocking VEGF is deleterious to the MM cells and causes cytostasis. This was evidenced in MM cell lines, as well as in primary BM samples (BM MM). A common bevacizumab-induced attenuation of critical signaling effectors was determined: VEGFR1, mTOR, c-Myc, Akt, STAT3, (cell lines) and eIF4E translation initiation factor (lines and BM). ERK1/2 displayed a variegated response to bevacizumab (lines). Utilizing a constitutively Akt-expressing MM model, we showed that the effect of bevacizumab on viability and eIF4E status is Akt-dependent. Of note, the effect of bevacizumab was achieved with high concentrations (2 mg/ml), but was shown to be specific. These findings demonstrate that bevacizumab has a direct influence on major pathways critically activated in MM that is independent from its established effect on angiogenesis. The cytostatic effect of VEGF inhibition on MM cells underscores its potential in combined therapy, and our findings, regarding its influence on translation initiation, suggest that drugs that unbalance cellular proteostasis may be particularly effective.

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Multiple myeloma (MM) is an incurable malignancy of end-stage B-lymphocytes that primarily accumulate in the bone marrow (BM). Characteristically, the malignant plasma cells produce extensive quantities of monoclonal immunoglobulins and cause end organ damage.<sup>1</sup> Current disease treatment includes conventional chemotherapy, autologous stem cell transplantation, targeted therapies with a proteasome inhibitor that exploits the dependency of myeloma cells on protein synthesis homeostasis, or immunomodulators such as lenalidomide or thalidomide that also have anti-angiogenic effect.<sup>1</sup> The anti-angiogenic strategy in MM is justified by increased micro-vessel density in the BM, highly correlated with plasma cell labeling index.<sup>2</sup> Increased

angiogenesis persists even after achievement of complete response following stem cell transplantation, suggesting the utility of combined chemotherapeutic and anti-angiogenic drug regimens.<sup>2</sup> Anti-angiogenic therapy targeting the major signal of vascular endothelial growth factor (VEGF) may be directed at its receptors (VEGFR) allowing distinction between MM cells (VEGFR1) and endothelial cells (VEGFR2), or aimed at VEGF itself.<sup>3,4</sup> The elevated secreted levels of VEGF produced by MM cells and BM stroma cells denotes it as a potential target for directed MM therapy.<sup>5,6</sup> VEGF-A (hereafter VEGF) signaling in MM cells directly stimulates an array of pathways in MM cells, affecting cell migration, proliferation, survival, and drug resistance.<sup>2,7</sup>

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Exogenous VEGF/VEGFR-1 signaling triggers PI3K/PKC $\alpha$ -dependent MM cell migration, MEK/ERK MM-dependent cell proliferation, and Mcl1- and surviving-induced cell survival.<sup>7</sup> It was also established that an assortment of cytokines and growth factors, including VEGF, stimulate the PI3K/Akt/FKHR, PI3K/Akt/mTOR pathways, and promote survival and proliferation of MM primary cells and cell lines.<sup>2</sup> None of these studies addressed the downstream effectors of mTOR, specifically eukaryotic translation initiation factor eIF4E.<sup>5,8</sup> Dereglulation of protein synthesis has lately been linked to various human malignancies with elevated global translation, as well as increased synthesis of proteins involved in proliferation, survival, metastasis, and other malignant characteristics.<sup>8</sup> Many tumors display elevated levels of activated eIF4E, which is the rate-limiting regulator of 5' cap-dependent translation.<sup>8</sup> It was recently reported that elevated translational machinery, including the eIF4E, is evident in distinct subtypes of MM patients as well.<sup>9</sup> Activation of eIF4E enhances translation of genes with highly structured 5'-untranslated region, lending them a relative increase in translation efficiency.<sup>10</sup> Hence, eIF4E activation dictates not only rate of protein synthesis, but also its quality. Overexpression of eIF4E causes malignant transformation and drug resistance (mTOR inhibitor-rapamycin) in several cell models.<sup>11</sup> This can be explained by specific genes translationally regulated by eIF4E and essential for tumorigenesis (MM included), such as c-Myc that participates in cell growth, cyclin D essential for cell cycle progression, Bcl-XL, Bcl-2, Mcl, and survivin implicated in cell death inhibition, VEGF instrumental in angiogenesis, and MMP-9 vital for metastasis.<sup>12,13</sup> Interestingly, several studies have demonstrated that eIF4E knockdown did not affect global protein synthesis, but the levels of specific, highly dependent targets.<sup>14</sup>

In the present study, we examined the efficacy of anti-VEGF treatment directly on MM cells using the platform of the *bona-fide* VEGF inhibitor, bevacizumab. The research concentrated on signaling pathways affected by VEGF inhibition in MM cells, irrespective of its source in an *in-vitro* model of MM cell lines cultured without additional cell types (such as stroma, endothelium), thereby limiting the findings to direct VEGF-MM signaling cascades only. We also tested the effects of VEGF inhibition on the BM samples of MM patients under identical experimental conditions. We explored the effect of VEGF inhibition on the viability, proliferation, and survival of the MM cells, as well as multiple downstream signaling cascades of established importance to MM cell biology, with specific emphasis on protein translation initiation.

## MATERIALS AND METHODS

### Cell Lines

MM cell lines RPMI 8226, U266 (ATCC, Manassas, VA, USA), ARP1, and ARK (Professor Epstein, Little Rock, AR, USA) were cultured in RPMI 1640, as described previously<sup>15</sup> (Biological Industries, Kibbutz Beit-Haemek, Israel).

### Materials

Bevacizumab (Roche, Israel) was used according to company recommendations<sup>16</sup> for 5 days, and dose-response curves in 0.01  $\mu$ g/ml–4 mg/ml concentration as described elsewhere,<sup>17</sup> with an estimated IC<sub>50</sub> of 2.8–5.6 mg/ml. Human IgG antibody (Sigma, St Louis, MO, USA) served as a negative control at equal concentration to bevacizumab as described previously.<sup>18</sup> Bevacizumab and IgG control solutions were saline-based. Autophagosome formation inhibitor 3-methyladenine (3MA; Sigma; dissolved in ddw), inhibitor of class III phosphatidylinositol 3-kinase,<sup>19</sup> and Mek1/2/ERK signaling inhibitor, U0126 (Cell Signaling Technology, Danvers, MA, USA; dissolved in DMSO) were used at final concentrations of 10 mM and 10  $\mu$ M, respectively.

### Study Group

The study group included BM samples obtained from patients diagnosed with MM according to accepted clinical criteria ( $n = 10$ ). Patients included in the study were 52–82 years old, and displayed 15–70% plasma cells of which 74–99% were CD138+. BM leukocytes were separated by Ficoll (Sigma) gradient according to manufacturer's instructions. The study approved by Meir Medical Center Helsinki committee was conducted according to Helsinki guidelines, and all participants signed informed consent forms.

### Cell Viability and Count

Briefly, 20 000 cells per 200  $\mu$ l medium per 96-well cultured with bevacizumab for 5 days were counted (ADVIA 120 Automated Hematology Analyzer; GMI, Minneapolis, MN, USA) and assayed for viability with WST1 Cell Proliferation Reagent (Roche, Basel, Switzerland) according to manufacturer's instructions.<sup>20</sup>

### Cell Survival and Death

Cells were harvested and stained with Annexin V-PE (250  $\mu$ g/ml; BioVision, Mountain View, CA, USA) and 7AAD (0.05  $\mu$ g/ $\mu$ l; eBioscience, San Diego, CA, USA) 5 days post-treatment, and assayed with Coulter flow cytometer (FACS; EPICS-XL, Beckman Coulter, Fullerton, CA, USA) for Annexin V + /7AAD– (apoptotic) and Annexin V + /7AAD+ cells (necrotic). All results are expressed as percent of total cell number.

### Cell Cycle

Harvested cells were exposed to 40  $\mu$ g/ml propidium iodide and 100  $\mu$ g/ml Ribonuclease A (Sigma) in PBS for 30 min at room temperature in the dark, and analyzed by FACS.

### Immunoblotting

Protein lysates of bevacizumab-treated cells were immunoblotted as described elsewhere.<sup>15</sup> Nuclear and cytosolic proteins were extracted with NucBuster protein extraction kit (Novagen, Madison, WI, USA) according to manufacturer's instructions. Rabbit anti-human antibodies were used for detection of the following: pmTOR(Ser2448)/total mTOR,

peIF4E(Ser209)/total eIF4E, pAkt(Ser473)/total Akt, pSTAT3 (Ser727/Tyr705)/total STAT3, pERK1/2(Thr202/Tyr204)/total ERK1/2, pMNK(Thr 197/202)/total MNK, p4EBP (Ser65)/total 4EBP, Cyclin D, c-Myc (Cell Signaling Technology), HSC-70, total VEGFR1 (Santa Cruz, CA, USA), pVEGFR1(Tyr 1213; R&D Systems, Minneapolis, MN, USA) and LC3/LC3II (Sigma). Peroxidase-conjugated secondary goat anti-rabbit antibody and enhanced chemiluminescence detection (ECL kit, Pierce, Rockford, IL, USA) facilitated the identification of bound antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Products were visualized with LAS3000 Imager (Fujifilm, Greenwood, SC, USA). Integrated optical densities of the immunoreactive protein bands were measured as arbitrary units, employing Multi Gauge software v3 (Fujifilm), and normalized to protein amount.

### Immunocytochemistry

Treated cells were cytopspinned, fixated (4% paraformaldehyde, 100% methanol), blocked (5.5% goat serum), and incubated with primary antibody for total eIF4E (Cell Signaling Technology) over night at 4 °C. Next, the slides were incubated with fluorescent secondary antibody Alexa-Fluor 594 (Invitrogen, Carlsbad, CA, USA), washed, and incubated with the fluorescent DNA-binding compound Hoechst 33342 dye (Sigma). Cells were visualized with a BX41 microscope ( $\times 40$ ); images were taken with DP70 digital camera and DP Controller software (Olympus, Center Valley, PA, USA).

### Stable Transfection of Plasmid DNAs

For stable transfection of Akt1/PKB $\alpha$  in pUSEamp and empty control vector (Millipore, Billerica, MA, USA), RPMI 8226 cells were transfected and selected with 1 mg/ml G418 (Clontech, Mountain View, CA, USA) as described previously.<sup>21</sup>

### MMP9 Gelatinase Activity

Supernatants of bevacizumab-treated cells were assayed for MMP9 activity by zymography as described elsewhere.<sup>22</sup> Band intensity was measured using LAS3000, and calculated with Multi-gauge v3.0 software.

### VEGF Secretion

VEGF levels in cell culture media (originating from serum and MM secretion; three experiments in duplicate) were measured using Human VEGF Quantikine ELISA Kits (R&D Systems) and read at 450 nm, using multiwell spectrophotometer (ELISA reader model Sunrise; Tecan, Salzburg, Austria). Concentrations were interpolated from a standard curve.

### Statistical Analysis

Student's paired *t*-tests were used to analyze differences between cohorts and considered significant when *P*-value was

$\leq 0.05$ . Interaction between treatments was assayed by the attitude formula of drugs  $q = P(A + B)/P(A) + P(B) - P(A) \cdot P(B)$  ( $q < 0.85$ —antagonist;  $q > 1.15$ —synergistic;  $1.15 > q > 0.85$ —additive),<sup>23</sup> assuming that bevacizumab is the first treatment (A), and 3MA or ERK inhibitor treatment is the second (B). At least, three separate experiments were conducted.

## RESULTS

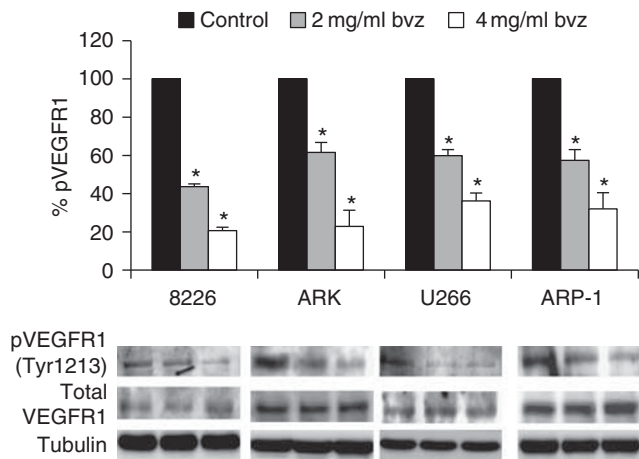
### Research Model

MM cell lines cultured on plastic were treated with different concentrations of the only VEGF inhibitor available, bevacizumab<sup>24</sup> (0.01  $\mu$ g/ml–4 mg/ml) for 0–5 days. Human IgG antibody, used as a negative control, did not affect the viability of the MM cell lines, thereby demonstrating the specificity of bevacizumab (91–94% in 2 mg/ml IgG; 91–98% in 4 mg/ml; compared with saline; non-significant; Supplementary Data, Figure 1). VEGF depletion by bevacizumab was validated by measuring its secreted levels in culture media of treated (2 and 4 mg/ml bevacizumab) and control MM cell lines (Supplementary Data, Figure 2). Importantly, VEGF was undetectable at both bevacizumab concentrations, yet as will be evident in the results presented hereafter, differences in cell response between 2 and 4 mg/ml were determined. Others made similar observations, but failed to offer a conclusive explanation, yet suggested that the detection method may not be sensitive enough.<sup>17</sup>

The common clinical application of bevacizumab is targeted at endothelial cells involved in angiogenesis. This is not the case in our study, and the response was evident at higher concentrations. Several publications describe responses to bevacizumab at similar concentrations, including a study in MM.<sup>25</sup> Moreover, in use of various antibody therapies, higher doses have been employed.<sup>26</sup> These control experiments are in accordance with previous studies conducted in other cell models.<sup>27</sup> Multiple publications demonstrate that bevacizumab has specific anti-VEGF activity<sup>27</sup> and lacks biological activity via the Fc receptors. Further studies should address the disparity between the dose response to bevacizumab and the remaining VEGF levels.

### VEGF Inhibition of VEGFR1 Phosphorylation in MM Cell Lines

Previous studies depict VEGFR1 as the major mediator of VEGF signaling in MM cells.<sup>3,4</sup> Therefore, we assessed the baseline expression of VEGFR1 in MM cell lines, and indeed, VEGFR1 was expressed in all the MM cell lines (Figure 1). Next, we studied the effect of VEGF inhibition on VEGFR1 levels and phosphorylation rate. No changes in levels of total VEGFR1 were determined, yet a distinct decrease in phosphorylated VEGFR1 was observed in all bevacizumab-treated MM cell lines (40–54%  $\downarrow$  in 2 mg/ml; 65–80%  $\downarrow$  in 4 mg/ml;  $P < 0.05$ ; Figure 1). The dose-dependent distinction between phosphorylated VEGFR1 levels suggests that despite undetectable levels of VEGF, there is a



**Figure 1** Inhibition of vascular endothelial growth factor (VEGF) attenuated VEGF receptor 1 (VEGFR1) phosphorylation in multiple myeloma (MM) cell lines; bevacizumab (bvz) (2 and 4 mg/ml)-treated MM lines were lysed and immunoblotted for pVEGFR1 (Tyr1213). Results are expressed as averages of relative expression of control cells ( $n=3$ ; mean  $\pm$  s.e.; y axis; left) and representative immunoblots are displayed (right). Tubulin served as loading control. Asterisks depict statistical significance ( $*P<0.05$ ).

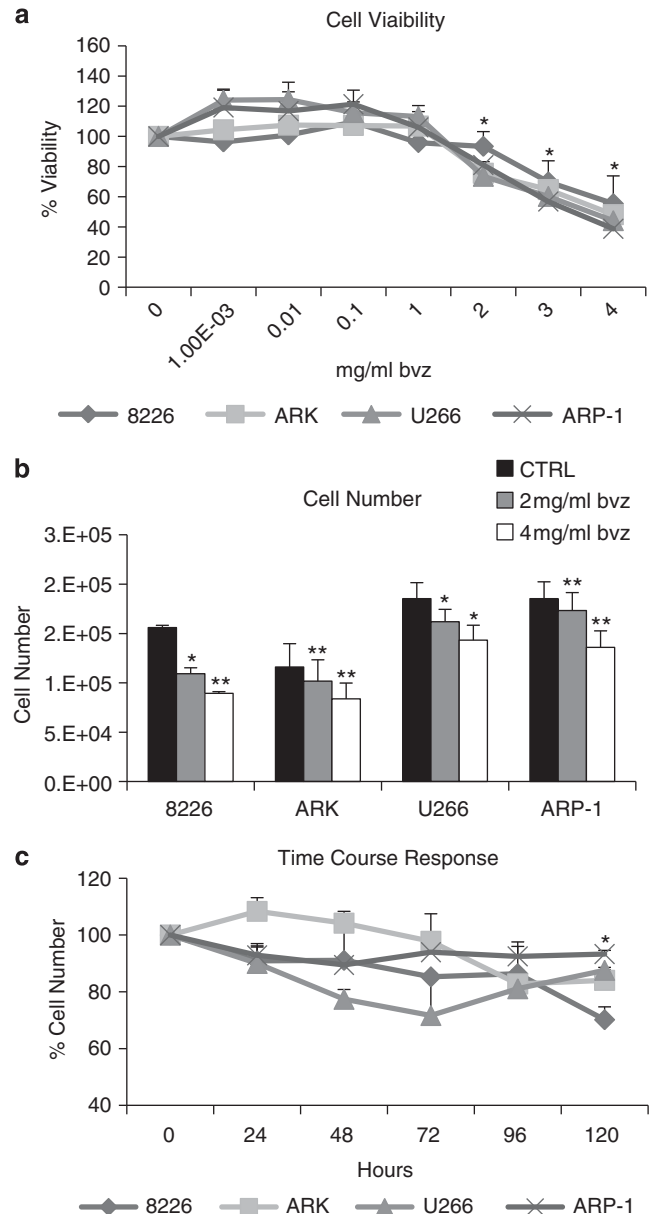
difference in cellular kinetics between 2 and 4 mg/ml of bevacizumab.

### Inhibition of VEGF has a Deleterious Effect on MM Cell Lines

RPMI 8226, ARK, U266, and ARP-1 were treated with bevacizumab (0.01  $\mu$ g/ml–4 mg/ml), and assayed for viability. VEGF inhibition reduced cell viability in a dose-dependent manner in all four cell lines (10–30%  $\downarrow$  in 2 mg/ml; 45–60%  $\downarrow$  in 4 mg/ml;  $P<0.05$ ; Figure 2a). Significant decreases in total cell numbers were also determined in all bevacizumab-treated cell lines (5–30%  $\downarrow$  in 2 mg/ml; 20–40%  $\downarrow$  in 4 mg/ml;  $P<0.05$ ; Figure 2b). The decrease in cell numbers of the MM cell lines was time-dependent, and the best response to 2 mg bevacizumab was evident after 5 days of treatment (20–40%  $\downarrow$ ;  $P<0.05$ ; Figure 2c). Experiments conducted hereafter were designed to study the mechanisms underlying the deleterious effect of the drug on the MM cell lines. These experiments depict an effect on cell phenotype and major signaling cascades critical to MM.

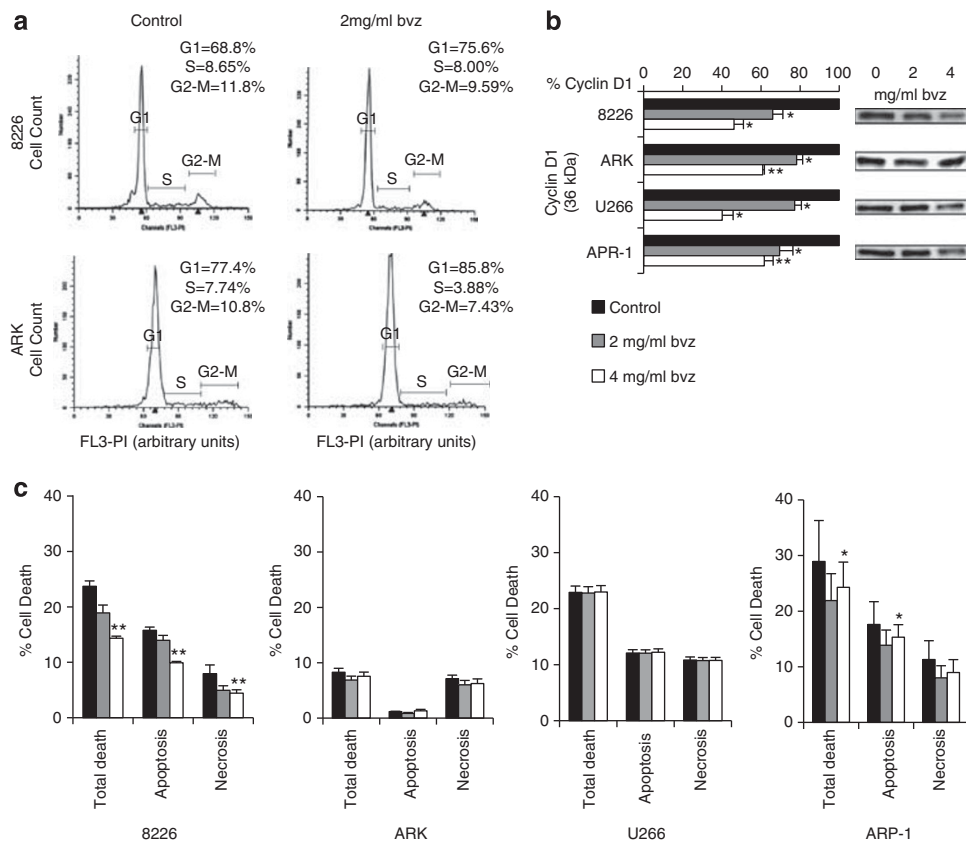
### VEGF Inhibition Induced Cell Cycle Arrest and Reduced Death in RPMI 8226 and ARK Cell Lines

Reduced cell counts and viability could be explained by decreased proliferation and/or increased death; therefore, we assessed changes in cell cycle and cell death. RPMI 8226 and ARK cell lines treated with bevacizumab (2 mg/ml) displayed a slight arrest in G1 cell cycle phase, compared with control (5–6%  $\uparrow$  in G1; 2–5%  $\downarrow$  in S;  $P<0.05$ ; Figure 3a, Table in Supplementary Data Figure 3). Furthermore, reduced levels of cyclin D1 observed by immunoblotting corroborated these results (20–35%  $\downarrow$  in 2 mg/ml; 40–65%  $\downarrow$  in 4 mg/ml;  $P<0.05$ ; Figure 3b).



**Figure 2** Inhibition of vascular endothelial growth factor (VEGF) with bevacizumab has a deleterious effect on multiple myeloma (MM) cell lines; MM cell lines were treated with different concentrations of bevacizumab (bvz; 0.01  $\mu$ g/ml–4 mg/ml) for 5 days, and assayed for: (a) viability detected with WST-1 cell proliferation assay. Results are expressed as averages of relative expression of control cells ( $n=5-12$ ; mean  $\pm$  s.e.; y axis); (b) cell count detected with ADVIA 120 Automated Hematology Analyzer. Results are expressed as absolute values of cell count ( $n=3-8$ ; mean  $\pm$  s.e.; y axis); (c) MM cell lines were treated with 2 mg/ml bvz for 24–120 h (x axis). Time-course response was determined by detecting cell count with ADVIA 120 Automated Hematology Analyzer. Results are expressed as averages of relative expression of control cells ( $n=3$ ; mean  $\pm$  s.e.; y axis). Asterisks depict statistical significance ( $*P<0.05$ ;  $**P<0.01$ ).

Analysis of cell death in bevacizumab-treated MM cell lines demonstrated slightly decreased death rates in RPMI 8226 (4–5%  $\downarrow$  in 2 mg/ml; 9%  $\downarrow$  in 4 mg/ml;  $P<0.05$ ), yet, no



**Figure 3** Inhibition of vascular endothelial growth factor (VEGF) with bevacizumab affect cell cycle and cell death in multiple myeloma (MM) cell lines; MM cell lines were treated with different concentrations of bevacizumab (bvz; 2 and 4 mg/ml) for 5 days, and assayed for (a) cell cycle (representing images; 2 mg/ml); (b) lysed and immunoblot for cyclin D1 (2 and 4 mg/ml); results are expressed as averages of relative expression of control cells ( $n = 3$ ; mean  $\pm$  s.e.; y axis; left panel), and representative images of western blot analysis (right panel) are shown; (c) Annexin + /7AAD- (apoptotic) and Annexin + /7AAD+ (necrotic) cells (x axis) were assayed in bvz (2 mg/ml)-treated cell lines (bottom) by FACS. Results are expressed as averages of relative expression (mean  $\pm$  s.e., y axis) of control cells ( $n = 5-12$ ). Asterisks depict statistical significance (\* $P < 0.05$ ; \*\* $P < 0.01$ ).

significant change in death of ARK cells (Figure 3c). Declines in cell numbers accompanied by G1 cell cycle arrest are in accordance with reduced proliferation and afford a reasonable explanation for decreased viability observed in these cell lines upon VEGF inhibition. The decrease in cell death underscores the protective role of the G1-cell cycle arrest in our research model and timeframe. In total, it can be conjectured that VEGF inhibition near RPMI 8226 and ARK caused the cells to acquire a new homeostasis of proliferation and death that resulted in a more limited expansion of their populations (summarized in top section of Table 1).

**Inhibition of VEGF Caused Enhanced Autophagy in U266 and ARP-1**

Bevacizumab-treated ARP-1 and U266 did not show significant changes in cell cycle (data not shown) and a differential response in cell death. ARP-1 displayed a decreased death rate similar to RPMI 8226 (7%  $\downarrow$  in 2 mg/ml; 6%  $\downarrow$  in 4 mg/ml;  $P < 0.05$ ; Figure 3c), yet similar to ARK, no change was observed in U266. Previously, we showed that MM cell lines depend on autophagy for survival;<sup>15</sup> thus, we speculated that

the cells might have induced an autophagic response to the VEGF inhibition promoting survival, but at a metabolic cost evidenced in the viability assay. By immunoblotting, we determined increased LC3-II (autophagy-induced lipidated form of LC3-I)<sup>28</sup> in the VEGF-inhibited cells (2.7- and 1.5-fold change in 2 mg/ml-treated U266 and ARP-1, respectively,  $P < 0.05$ ), indicating elevated autophagy. No changes in LC3-II levels were observed in VEGF-inhibited RPMI 8226 and ARK (Figure 4a). To validate the survival-promoting role of autophagy in VEGF-inhibited ARP-1 cells, we blocked the formation of autophagosomes (3MA) and assessed the effect on cell viability and proliferation. Combined 3MA and bevacizumab (2 mg/ml) caused a synergistic negative effect on ARP-1 cells viability (65%  $\downarrow$  compared with untreated control, and 60%  $\downarrow$  compared with bevacizumab-treated cells;  $q = 2.44$  (see Materials and Methods),  $P < 0.01$ ; Figure 4b), and an additive negative effect on total cell number (38%  $\downarrow$  compared with control;  $q = 0.92$ ,  $P < 0.05$ ; Figure 4c). The effect of 3MA on bevacizumab-induced cell death (2 mg/ml) demonstrated an increase in apoptotic cell numbers (12%  $\uparrow$  compared with bevacizumab-treated cells,  $P < 0.05$ ;

Figure 4d). Taken together, our results suggest that VEGF inhibition stimulated autophagic pathways in ARP-1 and U266, and that these increased levels of autophagy are

essential for cell survival under conditions of VEGF inhibition with bevacizumab. Indeed, when autophagy was blocked, the cells displayed higher death rates altogether and by apoptotic death mode (Table 1).

**Table 1 Summary of bevacizumab-induced changes in MM cell lines**

	RPMI 8226	ARK	U266	ARP-1
Cell fate	Cytostasis G1 arrest	Cytostasis G1 arrest	Cytostasis Autophagy	Cytostasis Autophagy
Mitogenic signals	↓mTOR ↓Akt ↓STAT3 ↓ERK	↓mTOR ↓Akt ↓STAT3 ↑ERK	↓mTOR ↓Akt ↓STAT3 ↓ERK	↓mTOR ↓Akt ↓STAT3 ↑ERK
eIF4E targets <sup>a</sup>	↓c-Myc ↓cyclin D1 ↓MMP9	↓c-Myc ↓cyclin D1 ↓MMP9	↓c-Myc ↓cyclin D1 ↓MMP9	↓c-Myc ↓cyclin D1 ↓MMP9
Translation initiation	↓eIF4E ↓4EBP ↓Mnk	↓eIF4E ↓4EBP ↓Mnk	↓eIF4E ↓4EBP ↓Mnk	↓eIF4E ↓4EBP ↓Mnk

Abbreviation: MM, multiple myeloma.

<sup>a</sup>Total proteins (contrary to ratios of phosphorylated/total presented in other table categories).

Grey shading highlights dissimilar findings.

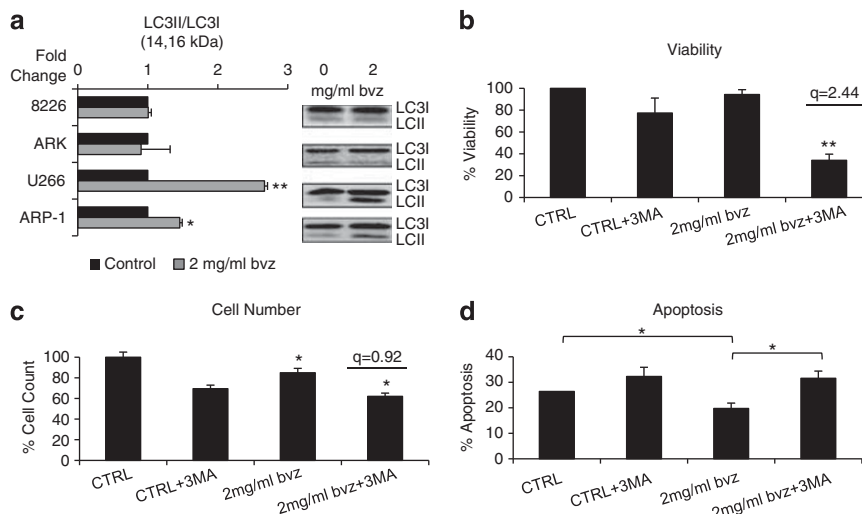
### Inhibition of VEGF Affected Multiple Signal Cascades in MM Cell Lines

Having established a general deleterious effect of VEGF inhibition on the MM cell lines, we examined molecular targets implicated in various functions (mTOR, c-Myc, Akt, STAT3, ERK, and MMP9; Table 1; Figure 5). Lack of significant changes in expression of the housekeeping HSC-70, previously used in MM cell lines,<sup>29</sup> served as loading control for all following immunoblot analyses (Supplementary Data, Figure 4).

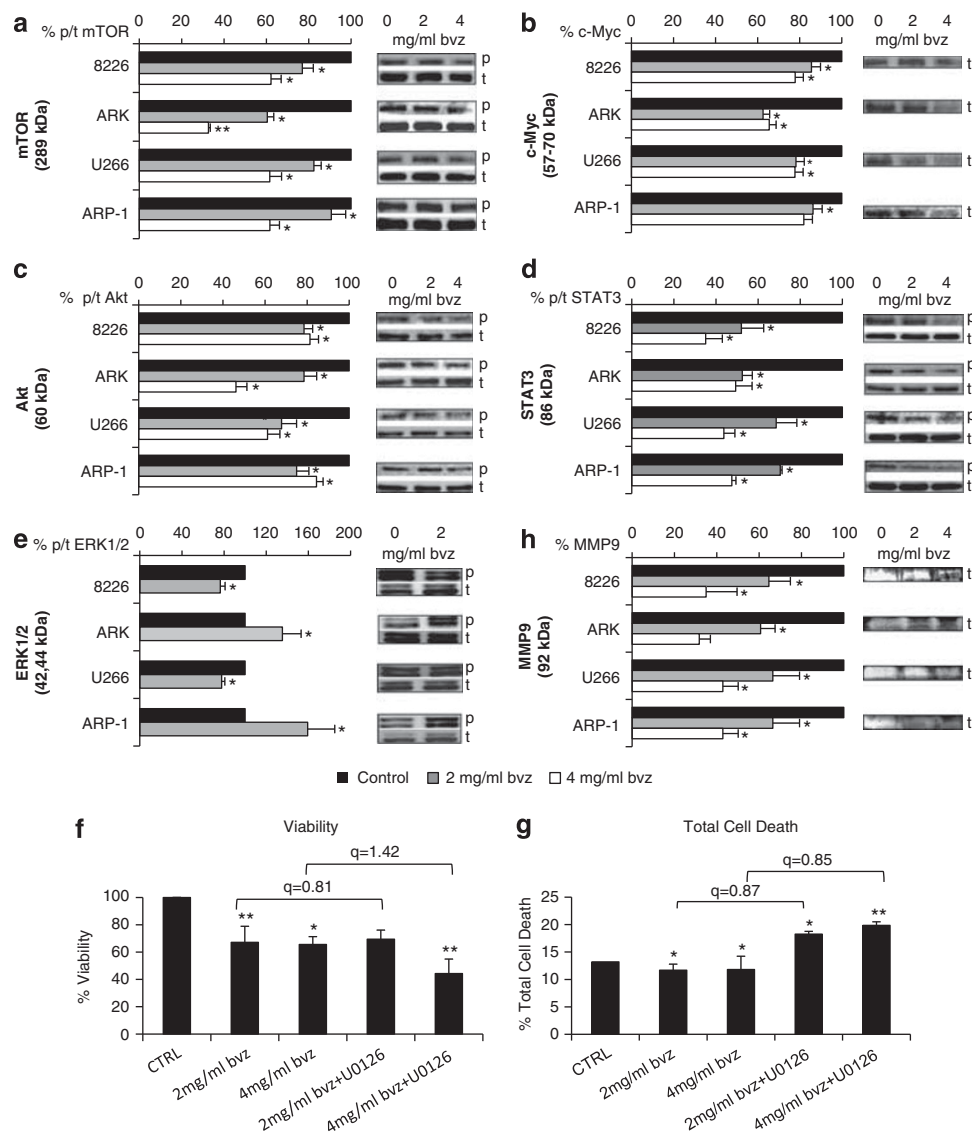
Assessment of phosphorylated (active) and total mTOR ratios in the bevacizumab-treated MM cell lines demonstrated significantly decreased levels of phosphorylated mTOR (14-38% ↓ in 2 mg/ml; 18-45% ↓ in 4 mg/ml;  $P < 0.05$ ; Figure 5a). Moreover, in U266 and ARP-1, decreased phosphorylated-mTOR levels are also in accordance with the increase in autophagy, as phosphorylated mTOR is an established key inhibitor of autophagy.

Analysis of c-Myc protein levels in bevacizumab-treated MM cell lines established notable dose-dependent reductions in all four lines (10-40% ↓ in 2 mg/ml; 40-70% ↓ in 4 mg/ml;  $P < 0.05$ ; Figure 5b).

The effect of VEGF inhibition on Akt was examined in the MM cell lines, and as expected, a decrease in active



**Figure 4** Bevacizumab treated U266 and ARP-1 cell lines displayed enhanced autophagic response. (a) Protein lysates of multiple myeloma (MM) cell lines (y axis) treated with 2 mg/ml bevacizumab (bvz) were immunoblotted for autophagic marker LC3-II. Representative images (right panel) and averages (left panel) of relative LC3II/LC3I expression levels (x axis) compared with control are shown. HSC-70 served as loading control for these analyses (Supplementary Data, Figure 4). ARP-1 cells treated with 2 mg/ml bvz, 3-methyladenine (3MA; 10 mM) or combination of both (x axis) were assessed for (b) viability detected with WST-1 cell proliferation assay; (c) cell count detected with ADVIA 120 Automated Hematology Analyzer; (d) apoptosis (Annexin + /7AAD-) assayed by FACS; in comparison to solvent-treated controls. All results are expressed as mean percent  $\pm$  s.e. ( $n \geq 3$ ; y axis). Asterisks depict statistical significance ( $*P < 0.05$ ;  $**P < 0.01$ ) and treatment interaction is indicated in  $q$ -value.



**Figure 5** Bevacizumab affected multiple signal cascades in multiple myeloma (MM) cell lines; Bevacizumab (bvz; 2 and 4 mg/ml)-treated MM lines were lysed and immunoblotted for several vascular endothelial growth factor (VEGF) downstream signals: (a) pmTOR(Ser2448)/total mTOR; (b) c-Myc; (c) pAkt(Ser473)/total Akt; (d) pSTAT3(Tyr705)/total STAT3; (e) pERK1/2(Thr202/Tyr204)/total ERK. (h) MMP9 activity assayed by zymogram. Results are expressed as averages of relative expression of control cells ( $n \geq 3$ ; mean  $\pm$  s.e.; y axis) and representative immunoblots/zymogram are displayed (right). (f, g) depict the effect of combined bevacizumab and ERK-inhibitor (U0126) on ARP-1 cells. ARP-1 cells treated with solvent, 2 and 4 mg/ml bvz and/or U0126 ( $10 \mu\text{M}$ ; x axis) were assessed for (f) viability and (g) total cell death. Results are expressed as mean percent  $\pm$  s.e. ( $n = 3$ ; y axis). HSC-70 served as loading control for these analyses (Supplementary Data, Figure 4). Asterisks depict statistical significance ( $*P < 0.05$ ;  $**P < 0.01$ ) and treatment interaction is indicated by  $q$ -value.

phosphorylated Akt was observed (20–30%↓ in 2 mg/ml; 15–65%↓ in 4 mg/ml;  $P < 0.05$ ; Figure 5c).

We also explored the effect of VEGF inhibition on established Akt downstream targets, FoxO1/3a proteins, yet determined no significant changes in total and phosphorylated levels of these proteins in all four MM cell lines (data not shown, elaborated upon in Discussion).

Dimerization and activation of STAT3 depends on Tyr705 phosphorylation.<sup>30</sup> In our study, we observed an unvarying decrease in STAT3 Tyr705 phosphorylation in all four MM

cell lines (30–50%↓ in 2 mg/ml; 50–65%↓ in 4 mg/ml;  $P < 0.05$ ; Figure 5d). Assessment of phosphorylated ERK yielded heterogeneous responses to bevacizumab; RPMI 8226 and U266 showed decreased phosphorylated ERK levels (23%↓ in 2 mg/ml;  $P < 0.05$ ), yet ARP-1 and ARK displayed significantly elevated levels of phosphorylated ERK (35–60%↑ in 2 mg/ml;  $P < 0.05$ ; Figure 5e). It is well established that ERK sustains MM survival.<sup>31</sup> To assess whether this is the case in the cell lines with elevated ERK levels, we treated ARP-1 and U266 with MEK1/2/ERK



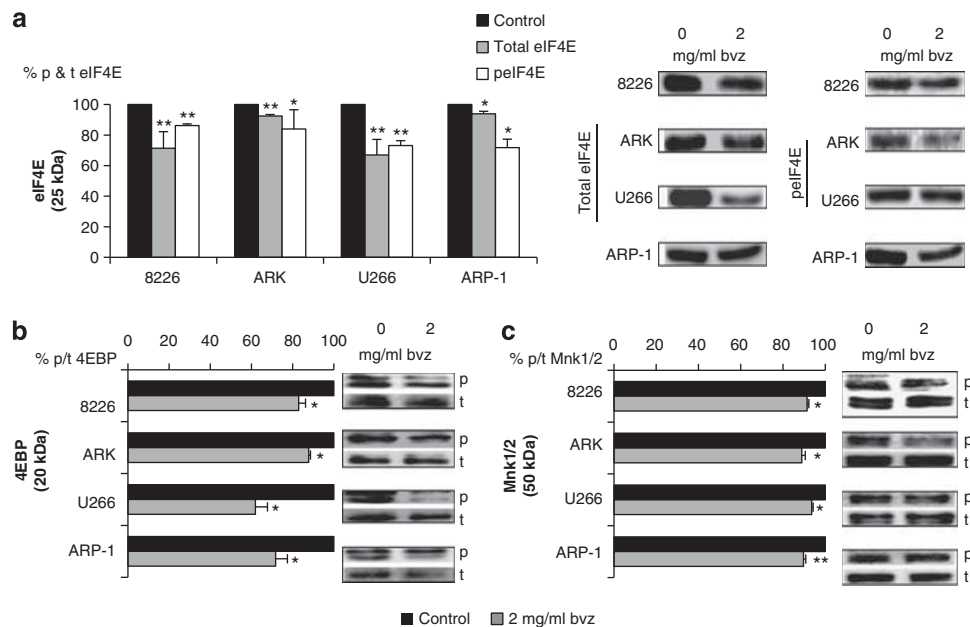
signaling inhibitor (U0126) and assessed viability and survival. The recorded decrease of phosphorylated ERK in U266 made this cell line an appropriate negative control. Reduced phosphorylation of MEK1/2/ERK in U0126-treated ARP-1 cells served as control to its function (40%↓ in ARP-1,  $P < 0.05$ , Supplementary Data, Figure 5). In concordance with our postulate, an interaction between the responses of the ARP-1 cells to ERK and VEGF inhibitions was demonstrated. A synergistic decrease (see Materials and Methods) in ARP-1 viability was evidenced with co-administered U0126 and 4 mg/ml bevacizumab (55%↓,  $P < 0.01$ ,  $q = 1.42$ ; Figure 5f). Indeed, the decreased viability was stratified by the observed elevation in total cell death of co-treated cells, which was antagonistic to the effect of bevacizumab alone (6–8%↑,  $P < 0.05$ ,  $q \cong 0.85$ ; Figure 5g). The death demonstrated in co-treated ARP-1 was of apoptotic mode. In contrast to ARP-1, U266 displayed an additive effect of the two treatments (bevacizumab and U0126) on viability and cell death ( $q = 1$  in both parameters with all bevacizumab concentrations; data not shown), thereby indicating there is no inter-dependence between the cells' responses to bevacizumab and ERK activity. These findings support the necessity of phosphorylated ERK for cell survival upon VEGF inhibition. VEGF signaling is also known to affect motility and invasion of cells.<sup>32</sup> In this study, the activity of MMP9 was explored in MM cell lines treated with bevacizumab. A dose-dependent decrease in MMP9 activity was observed in all four cell lines (40–45%↓ in 2 mg/ml; 60–70%↓ in 4 mg/ml;  $P < 0.05$ ; Figure 5h).

### VEGF Inhibition Affected Translation Initiation Signals in MM Cell Lines

Findings of this study demonstrated a bevacizumab-induced decline in signals that promote eIF4E translation initiation (Akt, mTOR), as well as a depletion of eIF4E-dependent targets (c-Myc, cyclin D1, MMP9),<sup>12</sup> suggesting that VEGF inhibition affected eIF4E activity. In keeping with this hypothesis, our results indicate significantly reduced levels of phosphorylated and total eIF4E in MM cell lines treated with 2 mg/ml bevacizumab (7–33%↓ in total eIF4E; 14–30%↓ in pEIF4E;  $P < 0.05$ ; Figure 6a). Regulation of eIF4E activity occurs at multiple levels and is yet unresolved.<sup>33–35</sup> eIF4E function in translation is controlled at its expression level and/or by its association (or lack of) with additional translational components. eIF4E binding may also modulate the nature of its activity, directly or via its cellular localization. It was also shown that eIF4E association to certain factors (4EBP, HSP27) may afford protection from proteosomal degradation.<sup>35</sup>

To discern the regulation of eIF4E in our research model, we examined 4EBP1 and determined a significant decrease in Ser65-phosphorylated 4EBP1 in all four MM cell lines treated with bevacizumab (12–40%↓ in 2 mg/ml;  $P < 0.05$ ; Figure 6b). These results are in accordance with the diminished active mTOR we have determined. According to published literature, the phosphorylated 4EBP1 releases its binding of eIF4E, making it available for phosphorylation by Mnk1 or ubiquitination, followed by proteosomal degradation.<sup>36,37</sup>

Examination of eIF4E kinase, Mnk1/2, demonstrated moderate decreases in phosphorylated Mnk1/2 in all four



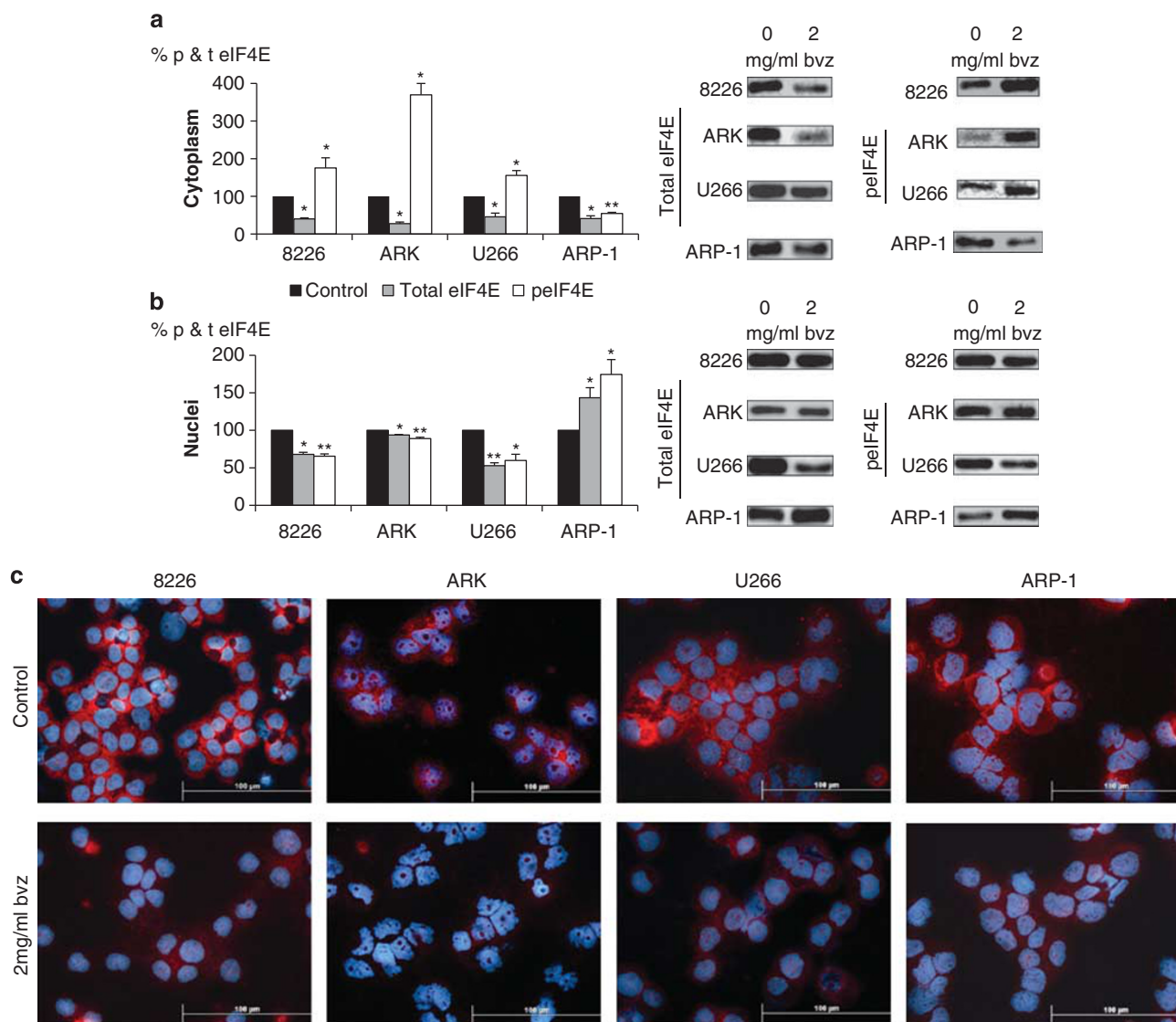
**Figure 6** Bevacizumab attenuated eIF4E translation initiation factor and its regulators in multiple myeloma (MM) cell lines: Whole-cell protein lysates of bevacizumab (bvz, 2 mg/ml)-treated MM cell lines were immunoblotted for: (a) pEIF4E(Ser209)/total eIF4E; (b) p4EBP1(Ser65)/total 4EBP1; (c) pMnk1/2(Thr 197/202)/total Mnk1/2; averages of relative protein expression levels compared with respective controls (left) and representative images of immunoblots (right) are shown. HSC-70 served as loading control for these analyses (Supplementary Data, Figure 4). Results are expressed as percent (mean  $\pm$  s.e.) of control cells ( $n = 3$ ). Asterisks depict statistical significance (\* $P < 0.05$ ; \*\* $P < 0.01$ ).



MM cell lines (~10%↓ in 2 mg/ml;  $P < 0.05$ ; Figure 6c). Hence, the depleted levels of total eIF4E we have observed (with no decrease in mRNA, data not shown) most probably indicate elevated degradation of unbound and unphosphorylated eIF4E.

Next, we evaluated the effect of VEGF inhibition on cellular distribution of total and phosphorylated eIF4E in protein extracts from the nuclei and cytoplasmic fractions of bevacizumab-treated and -untreated MM cell lines. Significant changes in total and phosphorylated eIF4E are evident in all MM cell lines ( $P < 0.05$ ; Figure 7). Cytoplasmic levels of

total eIF4E were decreased in all MM cell lines, thereby making less of the translation factor available for translation initiation *de facto* (55–70%↓ in 2 mg/ml,  $P < 0.05$ ; Figure 7a, gray bars). Moreover, in RPMI 8226, ARK, and U266, decreases in nuclear levels of total eIF4E were also evident (7–50%↓ in 2 mg/ml,  $P < 0.05$ ; Figure 7b, gray bars). The decreased levels in cytoplasmic and nuclear compartments in these three cell lines are in accordance with the decreased levels demonstrated in total cell lysates (Figure 4a). Of the total eIF4E localized to the cytoplasm, a larger fraction was phosphorylated because of VEGF inhibition (50–270%↑



**Figure 7** Bevacizumab affect cellular distribution of total and phosphorylated eIF4E in multiple myeloma (MM) cell lines: Fractionated cell lysates (NucBuster) of bevacizumab (bvz, 2 mg/ml)-treated MM cell lines were immunoblotted for peIF4E(Ser209)/total eIF4E; in cytoplasm (a) and nuclei (b). Averages of relative protein expression levels compared with respective controls (left) and representative images of immunoblots (right) are shown. HSC-70 served as loading control for these analyses (Supplementary Data, Figure 4). Results are expressed as percent (mean ± s.e.) of control cells ( $n = 3$ ). Asterisks depict statistical significance ( $*P < 0.05$ ;  $**P < 0.01$ ). (c) 2 mg/ml bvz-treated MM lines were cytospinned, incubated with primary antibody for total eIF4E and fluorescent secondary antibody (red, Alexa-Fluor 594), followed with fluorescent DNA-binding compound Hoechst 33342 dye (blue). Representing images of control cells (top panel) and treated cells (bottom panel; scale bar—100  $\mu$ M).

in 2 mg/ml,  $P < 0.05$ ; Figure 7a, white bars). An exception is ARP-1 that demonstrated increased nuclear levels of total and phosphorylated eIF4E, and decreased levels of cytoplasmic phosphorylated eIF4E (2 mg/ml; nuclear: 75% ↑ in peIF4E, 44% ↑ in total eIF4E; cytoplasmic: 45% ↓ in peIF4E,  $P < 0.05$ ; Figure 7a,b). Taken together, the decrease in total cell lysates indicates that a larger portion of the remaining eIF4E was localized to the nuclei where it cannot be involved in translation initiation. These data are consistent with previous reports that described increased amount of eIF4E localizing to the nucleus after heat stress.<sup>38</sup> It has also been reported that mTOR inhibition with rapamycin increases nuclear retention of eIF4E.<sup>38</sup> Interestingly, no changes were evident in steady state levels of total cellular protein, an observation that complies with previous studies that showed eIF4E knockdown did not attenuate global protein synthesis.<sup>14</sup> Altogether, our findings demonstrate that VEGF inhibition attenuates eIF4E availability for translation initiation of specific targets by depletion in the cytoplasm and nuclear retention.

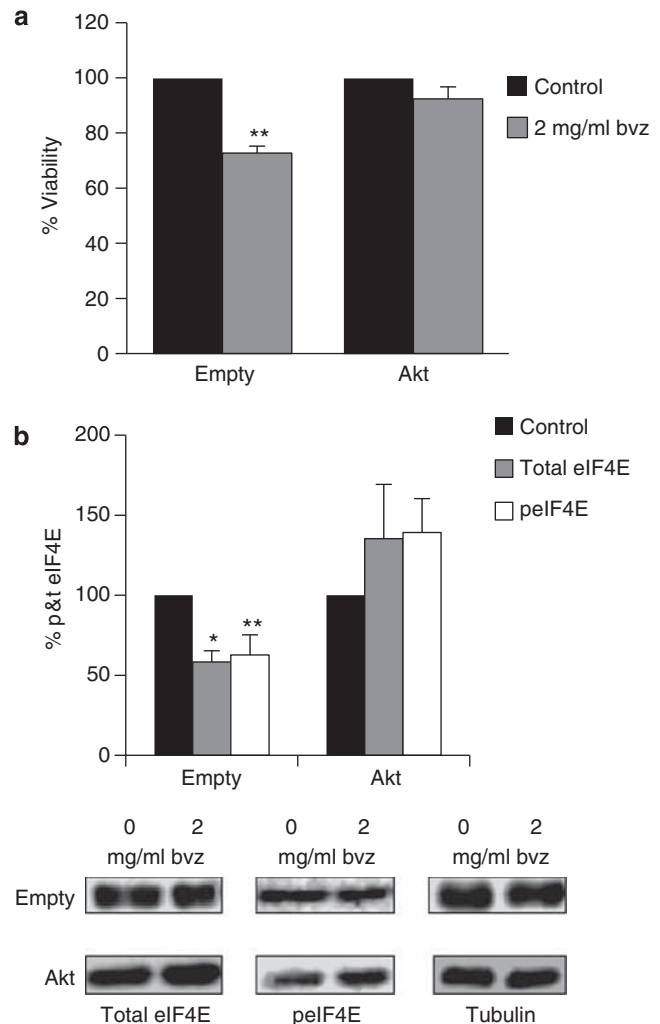
### The Effect of VEGF Inhibition on eIF4E and Cell Viability is Akt-Dependent

Our results so far demonstrated an adverse affect of VEGF inhibition on the phenotype of MM cell lines and an attenuation of multiple signals. We wished to connect both observations. As it is well established that Akt is an upstream regulator of most signals including eIF4E (mTOR/4EBP and Ras/Mnk1/2),<sup>39</sup> we used a constitutively expressing Akt MM cell line as a model (8226-AKT). Control (empty vector) and 8226-AKT cells were treated with bevacizumab (2 and 4 mg/ml) for 5 days and assessed for cell viability, and phosphorylated eIF4E/total eIF4E levels. 8226-AKT demonstrated a minor and non-significant decrease in viability upon bevacizumab treatment (2 mg/ml) compared with control that displayed significantly reduced viability rates (8226-AKT: 7.5% ↓, NS; empty vector: 30% ↓,  $P < 0.001$ ; Figure 8a).

Analysis of phosphorylated and total eIF4E in this model demonstrated a significant decrease of both in the control cells (37% ↓ in peIF4E, 42% ↓ in total eIF4E; 2 mg/ml bevacizumab (bvz),  $P < 0.05$ ). In 8226-AKT cells, no significant changes in phosphorylated eIF4E or total eIF4E were observed (Figure 8b). In fact, an opposite trend of elevated total and phosphorylated eIF4E in Akt expressing cells is indicated (NS). Taken together, these results demonstrate that the effect of VEGF inhibition on MM cell viability and eIF4E status is Akt-dependent.

### VEGF Inhibition has a Deleterious Effect on MM BM Samples

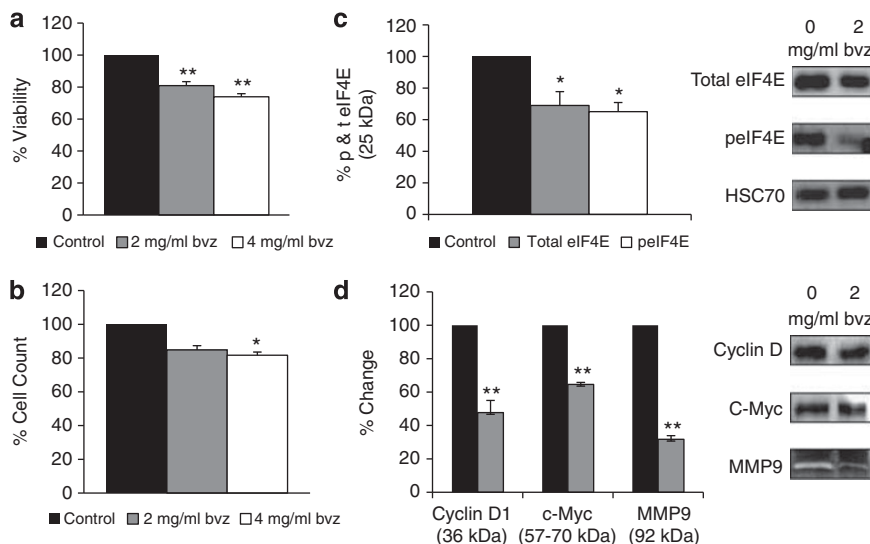
On the basis of the promising observations we have made so far, we decided to test the efficacy of VEGF inhibition on primary mononuclear BM samples (enriched with MM cells) extracted from consenting myeloma patients, using identical experimental conditions to those used for the cell lines.



**Figure 8** Bevacizumab effect on eIF4E and cell viability is Akt-dependent; constitutively expressing Akt and control RPMI 8226 cells were treated with bevacizumab (bvz, 2 mg/ml) and assayed for (a) viability, (b) immunoblotted for peIF4E/total eIF4E; averages of relative protein expression levels compared with respective control (top panel) and representative images of immunoblots are shown (bottom panel). Tubulin served as loading control. Results are expressed as percent (mean  $\pm$  s.e.) of control cells ( $n = 3$ ). Asterisks depict statistical significance (\* $P < 0.05$ ; \*\* $P < 0.01$ ).

Decreased cell viability was determined in all bevacizumab-treated MM BM samples (20% ↓ in 2 mg/ml; 26% ↓ in 4 mg/ml;  $P < 0.01$ ) in a dose-dependent manner ( $P < 0.05$ ; Figure 9a). Decreases in total cell numbers were also determined in all MM BM-treated samples (15% ↓ in 2 mg/ml, NS; 20% ↓ in 4 mg/ml;  $P < 0.05$ ; Figure 9b). No significant changes in cell death were observed.

Next, we examined the effect of bevacizumab on eIF4E levels in the MM BM cells, and determined a significant decrease in both phosphorylated eIF4E and total eIF4E levels compared with untreated MM BM cells (35% ↓ in peIF4E, 30% ↓ in total eIF4E; 2 mg/ml bvz,  $P < 0.05$ ,  $n = 7-8$ ) (Figure 9c).



**Figure 9** Bevacizumab has a deleterious effect on multiple myeloma (MM) bone marrow (BM) samples; BM Samples of MM patients ( $n \geq 6$ ) were treated with bevacizumab (bvz) (2 and 4 mg/ml) for 5 days, and assayed for (a) viability detected with WST-1 cell proliferation assay; (b) cell count detected with ADVIA 120 Automated Hematology Analyzer. Results are expressed as averages of relative expression compared with untreated control BM cells (mean  $\pm$  s.e., y axis). Protein lysates of 2 mg/ml bvz (5 days)-treated MM BM cells were immunoblotted for (c) pEIF4E(Ser209)/total eIF4E; (d) cyclin-1, c-Myc, and (e) MMP9 assayed by activity zymogram assay. Averages of relative protein expression levels compared with respective controls (left) and representative images of immunoblots/ zymogram (right) are shown. HSC-70 served as loading control for these analyses. Results are expressed as percent (mean  $\pm$  s.e.) of control cells ( $n \geq 3$ ). Asterisks depict statistical significance (\* $P < 0.05$ ; \*\* $P < 0.01$ ).

Having established that the expression of eIF4E was decreased, we assayed the expression of eIF4E-dependent targets. Significant attenuation of cyclin D (50%  $\downarrow$  in 2 mg/ml bvz,  $P < 0.01$ ,  $n = 6$ ), c-Myc (35%  $\downarrow$  in 2 mg/ml bvz,  $P < 0.01$ ,  $n = 5$ ), and MMP9 (70%  $\downarrow$  in 2 mg/ml,  $n = 3$ ) (Figure 9d) was observed in all MM BM samples, demonstrating that eIF4E activity was indeed compromised.

## DISCUSSION

MM therapy is hampered by the complex interaction of the cells with the BM microenvironment and the difficulties in defining genes that are integral to the malignant phenotype. This MM–BM interaction contributes to the multistep progression of myeloma, development of drug resistance, and eventual relapse of patients.<sup>40</sup> In addressing disease treatment, two major concepts are well established; it is imperative to treat the cells in the context of their supportive microenvironment; and only combined application of drugs targeting multiple signaling cascades in MM is expected to overcome cell heterogeneity and evolving resistance.<sup>1</sup> Angiogenesis is recognized as instrumental to MM progression, and VEGF is an established survival and growth-promoting agent in the myeloma cells.<sup>40</sup> Currently, five clinical trials testing the clinical benefit of the anti-angiogenic activity of bevacizumab in MM patients are underway (<http://clinicaltrials.gov>). Unfortunately, single-agent anti-angiogenic drug trials have been disappointing so far, underscoring the need to formulate rational drug combinations.<sup>40</sup> A recent publication described results of a limited size, phase II randomized trial of bevacizumab *versus* bevacizumab and thalidomide. The

results of this study indicated that there was no benefit to the addition of bevacizumab. It was concluded that future trials targeting VEGF/VEGFR should include patients with elevated VEGF expression and drug combination with agents of proven efficacy. Educated and effective combined drug application is contingent on the elucidation of their respective mechanisms of action. The clinical studies of bevacizumab in myeloma are designed to explore the anti-angiogenic effects of the drug and not the direct effects of blocking the VEGF signaling in the malignant cells themselves. This study was designed to assess the direct effect of VEGF inhibition, using the effective VEGF inhibitor, bevacizumab, on MM cell lines *in vitro* and identify protective mechanisms activated by the cells. Indeed, this strategy yielded novel findings that may be exploited for better design of combined therapy in the future. Our findings demonstrate that blocking VEGF in the vicinity of the MM cell lines is deleterious to the cells. The effect manifested in reduced viability and proliferation, attenuation of VEGFR1 phosphorylation, and major signaling cascades of established importance in MM, particularly a decrease in translation initiation. Assayed MM cell lines displayed slightly decreased cell death due to an arrest in cell cycle or elevated rates of protective autophagy. The common anti-proliferative effects of VEGF inhibition on the MM cell lines, but lack of induced cell death, comply with the accepted definition of cytostatic agents.<sup>41</sup> Previous studies with anti-VEGFR drugs also demonstrated cytostasis in a panel of tumors.<sup>41</sup> Indeed, it is recognized that targeted therapy is most often directed at proliferative signals, such as VEGF, and therefore, their

inhibition culminates in reduced proliferation, ie, cytostasis.<sup>41</sup> Moreover, the cytostatic affect (*vs* cytotoxic) of a given drug is often contingent on the cellular context, with the propensity of the cell to die determining its fate.<sup>41</sup> MM cells are relatively resistant to apoptosis, affording insight into their survival upon VEGF inhibition.<sup>42</sup> Conversely, it is accepted that overcoming drug resistance in MM necessitates inhibition of growth and survival pathways combined with damage inducing drugs.<sup>42</sup>

Analysis of specific VEGF downstream targets demonstrated a common inhibitory effect of bevacizumab on multiple critical signaling effectors: mTOR, c-Myc, Akt, and STAT3, all established growth-promoting mediators.<sup>43,44</sup> Particularly important is the co-inhibition of Akt and mTOR we identified upon VEGF inhibition in the vicinity of the MM cell lines. A common phenomenon described in myeloma depicts activation of Akt by mTOR inhibitors and *vice versa*.<sup>21</sup> This reciprocal response is counterproductive, because both signals promote cell survival, growth, and proliferation. Furthermore, malignancies with constitutively activated Akt characteristic of specific MM subtypes appear to be mTOR-dependent.<sup>21,45</sup> Co-reduction in the activity of mTOR and Akt was also demonstrated on several occasions previously.<sup>21,45</sup>

Another intriguing finding regards the lack of response of *bona fide* Akt targets, the FoxO transcription factors, to VEGF inhibition. This finding is in accordance with a recently published study, which showed that an mTOR inhibitor (PP242) decreased phosphorylation of Akt/mTOR/4EBP/eIF4E, yet had no effect on the Akt/FoxO1/3a signaling arm.<sup>46</sup>

We also showed reduced levels of established eIF4E-dependent proteins of known importance to MM pathogenesis, the proliferation promoting c-Myc and cyclin D1, and the metastasis promoting MMP9. This is the first study to address eIF4E in MM cell cultures and our findings demonstrate for the first time to the best of our knowledge, a direct link between VEGF signaling and translation initiation in MM. Moreover, the unanimous decrease in active eIF4E, its regulators, and targets upon VEGF inhibition stratifies the importance of microenvironmental cues to protein synthesis in MM. The obvious necessity to synthesize proteins for cell growth and proliferation marks its regulation as a means for manipulating cancer. Indeed, as the importance of protein synthesis mode in malignancy and MM is increasingly acknowledged, it will be vital to assimilate the acquired data on its regulation and translate it into disease therapy. Studies currently conducted in our laboratory are designed to accomplish this aim.

Contrary to the uniform attenuation of most signals assessed in our study, we observed an inconsistent response of ERK phosphorylation in the VEGF-inhibited MM cell lines. Differential regulation of ERK in MM cell lines was described previously and associated with disparity in cellular responses to external stimuli.<sup>47</sup> It is well accepted that both STAT3 and ERK sustain MM survival, and that inhibition of

either signal alone is insufficient to induce cell death.<sup>31</sup> Our results can be reconciled with this consensus, as the response of the cells to bevacizumab was cytostatic and not cytotoxic. A joint regulator of STAT3 and ERK is the stress-induced heat-shock protein 90 (HSP90), already established as a survival-promoting agent in MM.<sup>31</sup> It is possible that VEGF inhibition induced a stress response in the form of HSP90/STAT3 and/or HSP90/ERK, thereby contributing to cells' endurance of the damage.

Use of a MM cell line that expresses Akt in a constitutive manner served to demonstrate irrevocably that VEGF's affect on cell viability, and eIF4E activation are Akt-dependent. It was also evident that the bevacizumab-resistant cells maintained high levels of phosphorylated eIF4e, possibly indicating its involvement in cell phenotype. Further studies of eIF4E significance to MM pathogenesis and drug resistance are currently underway in our laboratory. In a model of eIF4E knockdown, we have observed a significant anti-myeloma effect and a diminution of eIF4E targets, very similar to effects of VEGF inhibition (unpublished data).

A major obstacle to development of cancer treatments is the disparity between results demonstrated in research models and those achieved in actual patients. Our results depict the specific molecular consequences of VEGF depletion from the surrounding area of the MM cells. Furthermore, the affect of bevacizumab directly on MM BM samples was similar to its affect on MM cell lines. Thus, it can be assumed that any affect bevacizumab may have in MM patients will be a combination of its anti-angiogenic activity and diminution of critical signals in the myeloma cells. Although the clinical benefits of bevacizumab application in MM are yet to be explored and are not advocated by the current study, we do present a novel, though preliminary mechanistic, outline of the effect of VEGF inhibition on MM cells. We suggest that these results justify further research into the potential of VEGF inhibition in treatment of MM not only based on its anti-angiogenic activity, but also due to its direct influence on major pathways critically activated in this malignancy. Future studies should address the combined application of bevacizumab with chemotherapy that targets cells in their G1 phase, such as dexamethasone commonly used for myeloma treatment.<sup>48</sup> Moreover, the co-administration of VEGF inhibitors and drugs that block autophagy or, on the contrary, critically elevate ER-golgi stress may also be beneficial (bortezomib<sup>15</sup> for instance).

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

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## DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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