Simultaneous targeting of PI3K and mTOR with NVP-BGT226 is highly effective in multiple myeloma

Philipp Baumann, Laura Schneider, Sonja Mandl-Weber, Fuat Oduncu and Ralf Schmidmaier

Multiple myeloma is still uncurable. Myeloma cells become resistant to common drugs and patients eventually die of tumour progression. Therefore, new targets and drugs are urgently needed. NVP-BGT226 is a novel, orally bioavailable small-molecule inhibitor of phosphoinositol-3-kinase and mammalian target of rapamycin. Here, we show that NVP-BGT226 inhibits growth in common myeloma cell lines and primary myeloma cells at nanomolar concentrations in a time-dependent and dose-dependent manner. Western blots for the detection of caspase 3 cleavage and annexin-V–fluorescein isothiocyanate/propidium iodide assays revealed induction of apoptosis in common myeloma cells lines. Induction of apoptosis was accompanied by upregulation of proapoptotic Bim and a moderate upregulation of Mcl-1 and Bad and a downregulation of Bcl-2, Bax and Bcl-Xl. Inhibition of cell growth was mainly due to inhibition of myeloma cell proliferation, as shown by the 5-bromo-2′-deoxyuridine assay. Cell cycle analysis revealed induction of cell cycle arrest in the G1 phase, which was due to downregulation of cyclin D1, cyclin D2, pRb and cdc25a. NVP-BGT226 inhibited phosphorylation of protein kinase B (Akt), P70S6k and 4E-BP-1 in a time-dependent and dose-dependent manner. Furthermore, we show that the stimulatory effect of insulin-like growth factor 1, interleukin-6 and conditioned medium of HS-5 stromal cells on myeloma cell growth is completely abrogated by NVP-BGT226. Overall, inhibition of phosphoinositol-3-kinase/mammalian target of rapamycin by NVP-BGT226 is highly effective, and NVP-BGT226 represents a potential new candidate for targeted therapy in multiple myeloma. *Anti-Cancer Drugs* 23:131–138 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2012, **23**:131–138

Keywords: Akt, mTOR, multiple myeloma, NVP-BGT226, PI3 kinase

Abteilung Hämatologie und Onkologie, Medizinische Klinik, Campus Innenstadt, Klinikum der Universität München, München, Germany

Correspondence to Philipp Baumann, MD, Medizinische Klinik Campus Innenstadt, Klinikum der LMU, Ziemssenstr. 1, 80336 München, Germany
Tel: +49 89 5160 2237; fax: +49 89 5160 4412;
e-mail: Philipp.Baumann@med.uni-muenchen.de

Received 5 May 2011 **Revised form accepted** 31 August 2011

Introduction

Multiple myeloma (MM) remains an incurable disease and is characterized by the accumulation of malignant plasma cells in the bone marrow [1]. The disease is characterized by impaired haematopoesis, renal dysfunction and bone destruction [2]. In the past two decades, management and treatment of MM has changed considerably. The introduction of high-dose chemotherapy with autologous stem cell support has prolonged the median overall survival [3,4]. Furthermore, the understanding of the pathophysiology of the disease has led to the approval of novel drugs such as the proteasome inhibitor bortezomib and immunmodulatory drugs such as thalidomide and lenalidomide. Despite novel agents in the therapy, MM cells eventually become resistant to cytotoxic drugs and patients die of tumour progression [5–8].

The molecular understanding of this disease has changed rapidly. Interactions between the bone marrow stromal cells and MM cells lead to increased adhesion and cytokine release [9–11]. Soluble factors such as insulin-like growth factor 1 (IGF-1), vascular endothelial growth factor and cytokines such as interleukin-6 (IL-6) [12–16] and cellular adhesion to extracellular matrix or bone marrow stromal cells lead to a consecutive upregulation of intracellular signalling pathways and confer to drug resistance, survival and MM cell proliferation. The identification of signalling pathways such as the Jak/Stat-pathway, the Ras/Raf/MAPK-pathway, the PI3K/Akt/mTOR pathway and the role of NFκB has led to the development of specific signalling pathway inhibitors.

Phosphoinositol-3-kinase (PI3K) is a crucial kinase that influences many pathways that regulate cell growth, apoptosis and survival. In particular, class 1a PI3Ks, consisting of a p85 regulatory and a p110 catalytic group, play major roles concerning these cellular events. Once activated, PI3K phosphorylates Akt. Akt in turn activates a large variety of cellular signalling molecules including, glycogen synthase kinase 3, tuberous sclerosis 2 and protein S6 kinase (P70S6k), and acts on 4EBP1, thereby inducing protein transcription. Promotion of cell cycle is warranted by acting on cell cycle inhibitors p27, p21, cMyc and cyclinD1. In addition, activated Akt exerts its antiapototic effects by acting on proapoptotic and antiapoptotic mitochondrial proteins such as Bad [17,18]. Combined inhibition of PI3K and mamma-

0959-4973 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/CAD.0b013e32834c8683

lian target of rapamycin (mTOR) by NVP-BGT226 has been proven to be very effective in terms of induction of apoptosis and inhibition of proliferation [19]. The first-generation drug NVP-BEZ235 has already entered early clinical trials. Here, we present for the first time NVP-BGT226, a second generation dual inhibitor of PI3K/mTOR.

Methods

Cells

NCI-H929, U266, RPMI-8226 and OPM2 MM cell lines and the human stromal cell line HS-5 were obtained from the American Type Culture Collection (Rockville, MD, USA), grown in RPMI 1640 medium (Boehringer, Ingelheim, Germany) containing 10% heat-inactivated foetal calf serum (Boehringer) in a humidified atmosphere (37.0°C; 5% CO_2), and seeded at a concentration of 2×10^5 cells/ml. Cells have been regularly tested for mycoplasma and were free of this contamination.

Purification of primary MM cells

After informed consent was obtained from patients, mononuclear cells from bone marrow aspirates were isolated using CD138 MACS beads (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions. In brief, mononuclear cells were washed twice with chilled buffer (containing Dulbecco's PBS, 0.5% foetal calf serum and 2 mmol/l EDTA), and were resuspended in buffer. CD138 MACS beads were added to the cells and incubated for 15 min at 4°C. After that, cells were washed again, resuspended in 1 ml buffer and subjected to separation columns. After unlabelled cells passed through the column, the separation columns were removed from the separator, and the labelled cells were flushed out of the separation columns. The separation procedure was repeated twice. After separation, the purity of the CD138 cells was examined by flow cytometry using a CD138 PE-labelled antibody (BD Sciences, Heidelberg, Germany). A purity of more than 90% CD138-positive cells was accepted for further experiments. The ethics committee of the University of Munich approved the study.

Reagents

Propidium iodide (PI) was purchased from Calbiochem (Schwalbach, Germany) and WST-1 from Roche (Penzberg, Germany). NVP-BGT226 was kindly provided by Novartis Pharma AG (Basel, Switzerland). Polyclonal primary antibodies against p-Akt1/2 (Thr308 and Ser473), p-p85α (Tyr508), Akt1/2, p85α, Bax, Bcl-2, Bim, Mcl-1, FasL and actin were obtained from Santa Cruz (Heidelberg, Germany), and p-P70S6k (Thr421/Ser424), p-mTOR (Ser2448), p-4E-EBP1 (Thr70), P70S6k, cyclinD1, pRb (Ser807/811), cyclinE, cdk2/4/6, cdc25a, cleaved caspase 3, caspase 8, cleaved caspase 8, caspase 9, cleaved caspase 9 and m-TOR from Cell Signalling (Frankfurt/Main, Germany). Secondary antibodies raised against goat, mouse or rabbit epitopes were purchased from GE Healthcare (Uppsala, Sweden). Bortezomib was purchased from Millenium Pharmaceuticals (Cambridge, MA, USA), IL-6 from Sigma-Aldrich (Taufkrichen, Germany) and IGF-1 and CD40L from R&D Systems (Wiesbaden, Germany). Annexin-V–fluorescein isothiocyanate (FITC) was purchased from Becton Dickinson (Heidelberg, Germany).

Cell growth assay

For quantification of metabolically active cells in suspension, a WST-1 growth inhibition assay protocol was used as recommended by the manufacturer (Roche). After an incubation period, the WST-1 agent was added to the myeloma cell culture. After another 2 h, absorbance at 450 nm was measured using a microplate reader (reference wavelength: 680 nm).

Analysis of apoptosis and cell death

Cells were stained with PI and annexin-V-FITC. In brief, after two treatments with washing buffer (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 and 1 l H_2O, pH 7.2), cells were resuspended in 400 µl of Dulbecco's PBS. One hundred microlitres of this cell suspension were incubated with 10 µl of 50 µg/ml PI and 5 µl of annexin-V-FITC for 15 min at room temperature in the dark. Cells were analysed by flow cytometry (Coulter EPICS XL-MCL; System II, Krefeld, Germany).

Cell proliferation assay using 5-bromo-2′-deoxyuridine (BrdU)

Cell proliferation was determined using the BrdU Labelling and Detection Kit III from Roche (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions. In brief, cells were seeded in 96-well plates (Corning Costar, Corning, New York, USA) at a concentration of 1.5×10^4 cells/well in RPMI medium supplemented with 10% foetal bovine serum with or without the substance that was to be tested. After 36 h, BrdU-labelling solution was added (final concentration: 10 µmol/l), and cells were cultured for another 12 h in a humidified atmosphere (37°C/5%CO_2). Then, the plates were centrifuged (10 min, 300*g*), and the supernatants were discarded. The plates were dried at 60°C for 2 h. After fixation with ethanol/HCl for 30 min at −20°C, the DNA was partially digested by nuclease treatment for 30 min at 37°C. The cells were washed three times with medium and incubated with anti-BrdU-POD labelling solution for 30 min at 37°C. The anti-POD solution was removed and the cells were washed three times with washing buffer. The ABTS substrate solution was added, and absorbance was measured in a microplate reader at 405 nm with a reference wavelength of 490 nm.

Cell cycle analysis

Cellular DNA content was determined by flow cytometry. MM cells were harvested and washed twice with ice-cold

PBS. After that, cells were resuspended in 1 ml of 70% ethanol and fixed for 2 h at −20°C. Fixed cells were washed once with ice-cold PBS and resuspended in 400 µl of PBS, and 40 µl of 50 µg/ml PI were added to the cells. Cells were acquired by flow cytometry.

Western blot analysis

Cells were lysed with lysis buffer, and protein concentrations were determined using the biophotometer. Protein was separated by SDS-PAGE. After electrophoresis, protein was transferred to nylon membranes (Millipore, Massachusetts, USA), blocked in TBS-T and 5% non-fat dry milk for 1 h, and subsequently washed and incubated with TBS-T and the primary antibodies for 12 h. After washing with TBS-T, membranes were incubated with a peroxidase-conjugated secondary antibody for 1 h. Signals were detected by chemoluminescence using the ECL detection system (GE Healthcare). β-Actin served as the internal control for equal loading.

Statistics

Mean values with SDs from representative experiments are shown in the figures. Data were confirmed by at least two independent experiments. The Wilcoxon test was used to compare different groups. P less than 0.05 was considered statistically significant.

Results

The dual PI3K/mTOR inhibitor NVP-BGT226 inhibits cell growth and induces apoptosis in MM cells *in vitro*

First of all, we tested the cytotoxic effect of NVP-BGT226 on a variety of myeloma cells. For this, cell viability was determined time dependently by WST-1 assay. NCI-H929, RPMI-8226, U266 and OPM-2 myeloma cells were incubated with increasing concentrations of NVP-BGT226. Cell growth was determined after 48 and 96 h. As shown in Fig. 1a, MM cell growth was completely inhibited in a time-dependent and dose-dependent manner. Effective concentrations were found in the nanomolar range in all four cell lines with an IC_{50} in the range between 20 and 100 nmol/l.

Further experiments should elucidate whether NVP-BGT226-induced abrogation of cell growth is due to apoptosis and/or inhibition of proliferation. First, apoptosis was assessed. Cells of all four cell lines were incubated with increasing concentrations of NVP-BGT226 for 48 h. After the incubation period, cells were harvested and further processed for flow cytometry analysis. As shown in Fig. 1b and c, significant induction of apoptosis occurred in three of four cell lines. OPM-2, NCI-H929 and RPMI-8226 myeloma cells showed marked annexin-V positivity after incubation with NVP-BGT226, the IC_{50} was in the range between 100 and 250 nmol/l. Interestingly, although strong and almost complete inhibition of growth could be observed in the WST-1 viability assay, U266 cells stained less with annexin-V-FITC and PI compared with the other cell lines.

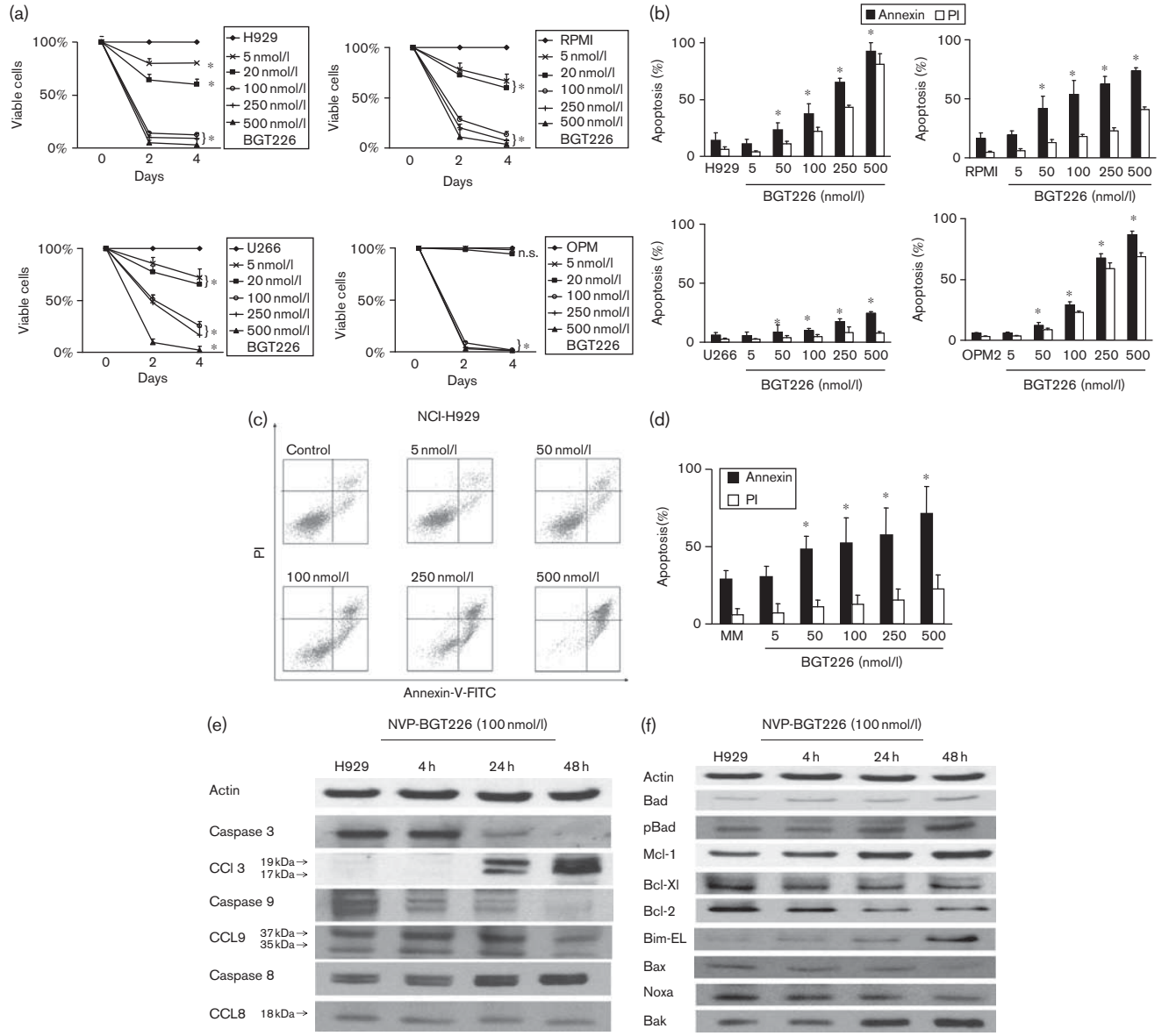
Furthermore, we tested primary myeloma cells obtained from bone marrow aspirates from seven patients. Primary myeloma cells were incubated with 5–500 nmol/l NVP-BGT226 for 48 h, and cell growth was determined by the WST-1 assay (Fig. 1d). Even in slowly proliferating and usually poorly sensitive primary MM cells, cell growth was significantly inhibited by NVP-BGT226, comparable to myeloma cell lines.

In addition, we wanted to confirm the induction of apoptosis by western blotting. NCI-H929 myeloma cells were incubated with 100 nmol/l NVP-BGT226 for 4, 24 and 48 h. Cells were analysed for expression of caspase 3 and cleaved caspase 3. We observed strong cleavage of caspase 3 in NVP-BGT226-treated myeloma cells. Additional analysis of caspase 8, cleaved caspase 8, caspase 9 and cleaved caspase 9 revealed a decreased expression of caspase 9 and a moderate increase in cleaved caspase 9 upon NVP-BGT226 incubation, indicating that apoptosis is induced by means of the intrinsic pathway of apoptosis (Fig. 1e). To further investigate the mechanism of induction of apoptosis, we repeated the same experiments and blotted proapoptotic and antiapoptotic mitochondrial proteins of the Bcl-2 family. Here, we noticed a strong upregulation of proapoptotic bim and bak and a moderate upregulation of Mcl-1, Bad and phospho-Bad. Furthermore, we detected a downregulation of antiapoptotic Bcl-2, Bax, Noxa and Bcl-Xl (Fig. 1f). We conclude that NVP-BGT226 strongly inhibits cell growth of myeloma cells and induces apoptosis at nanomolar concentrations accompanied by the upregulation of proapoptotic and downregulation of antiapoptotic mitochondrial proteins.

NVP-BGT226 inhibits myeloma cell proliferation and induces a G1 cell cycle arrest

Because myeloma cell growth was inhibited by NVP-BGT226, we next asked whether NVP-BGT226 also inhibits myeloma cell proliferation, and performed the BrdU assay. We incubated the four cell lines with increasing doses of NVP-BGT226 for 48 h. BrdU was added 12 h before harvesting the cells. Figure 2a shows that proliferation of NCI-H929, OPM-2, RPMI-8226 and U266 is strongly decreased by NVP-BGT226. Inhibition of proliferation is not complete at 250 nmol/l only in U266 cells. However, together with apoptosis, U266 growth is completely abrogated at 500 nmol/l as demonstrated in Fig. 1a. As NVP-BGT226 strongly decreases myeloma cell proliferation, we hypothesized a cell cycle arrest due to PI3K inhibition with NVP-BGT226. Again, all four myeloma cells were incubated with 10 and 100 nmol/l of NVP-BGT226 for 48 h. After the incubation period, cells were harvested and further prepared for cell cycle analysis. Figure 2b and c clearly shows that myeloma cells accumulate in the G1 phase of the cell cycle (e.g. U266: 44 → 75%; OPM-2: 43 → 62%), whereas the amount of cells in the S-phase or G2/M-phase decreased.

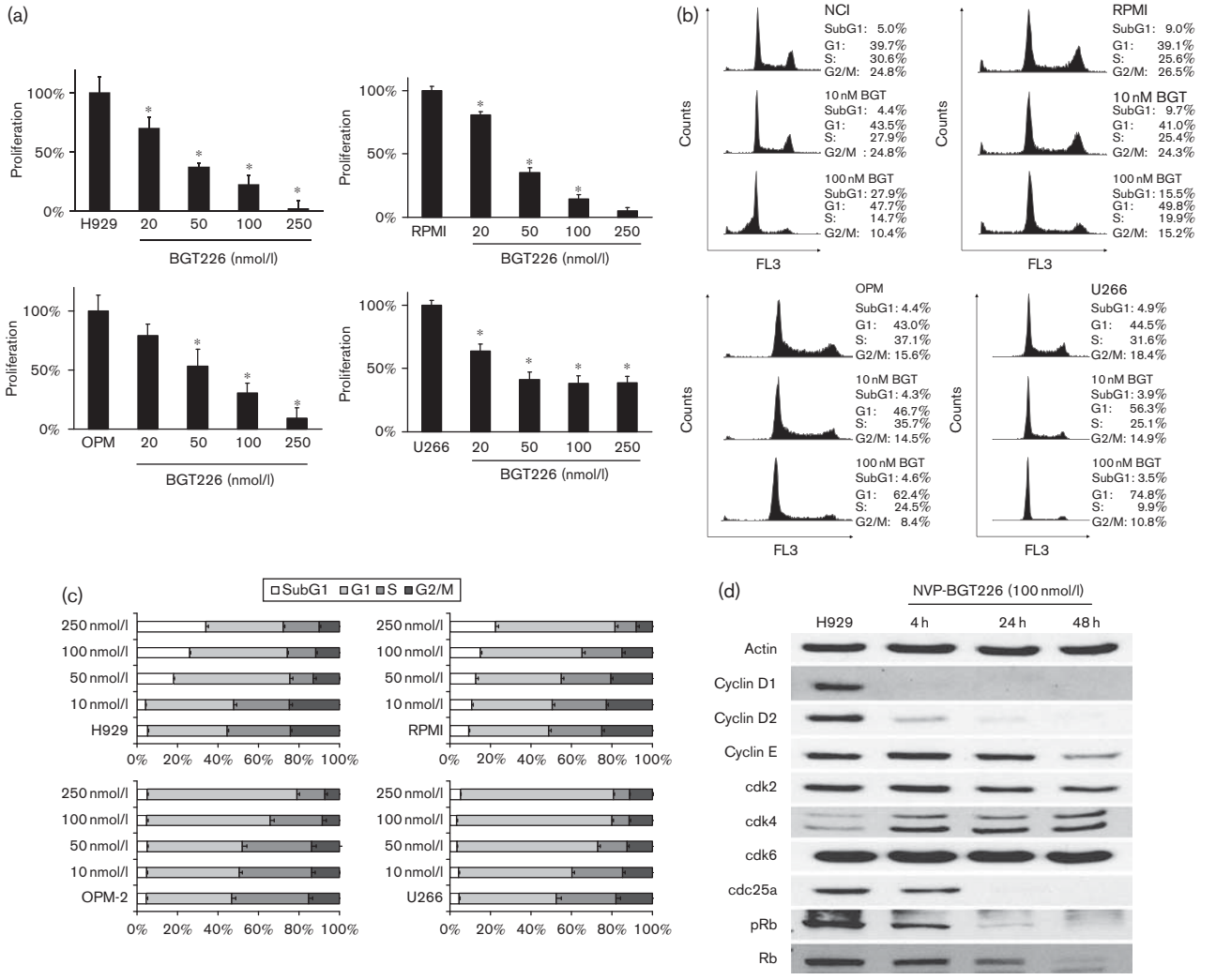
Fig. 1



NVP-BGT226 inhibits cell growth and induces apoptosis in MM cells. (a) The MM cell lines OPM-2, NCI-H929, RPMI-8226 and U266 were incubated with none, 5, 20, 100, 250 or 500 nmol/l of NVP-BGT226 for 48 and 96 h, and cell growth was determined by the WST-1 assay. Mean values and SDs are shown. The figures show results from two independent experiments. *$P<0.05$ versus control. (b) OPM-2, NCI-H929, RPMI-8226 and U266 myeloma cells were incubated with none, 5, 50, 100, 250 or 500 nmol/l of NVP-BGT226 for 48 h and induction of apoptosis was determined after annexin V-FITC/PI staining by flow cytometry. The figures show results from two independent experiments. *P less than 0.05 versus control. (c) NCI-H929 myeloma cells were incubated with none, 5, 50, 100, 250 or 500 nmol/l of NVP-BGT226 for 48 h, and induction of apoptosis was determined after annexin V-FITC/PI staining by flow cytometry. The figures show a representative result. (d) Primary myeloma cells from seven myeloma were incubated with none, 5, 50, 100, 250 or 500 nmol/l of NVP-BGT226 for 48 h, and cell growth was determined with the WST-1 assay. Mean values and SDs are shown. *P less than 0.05 versus control. In all figures, the used cell line is indicated in the control experiment. (e, f) MM cells from the H929 cell line were incubated with 100 nmol/l of NVP-BGT226 for 0, 4, 24 or 48 h. After the incubation period, cells were lysed and directly subjected to SDS-PAGE, transferred to membranes and blotted with the indicated antibodies. FITC, fluorescein isothiocyanate; PI, propidium iodide.

We further asked why NVP-BGT226 induces a G1 cell cycle arrest, and therefore performed western-blotting experiments. For this, NCI-H929 cells were incubated with 100 nmol/l of NVP-BGT226 for 4, 24 or 48 h, and then harvested and processed for western blotting. We noticed that the expression of both cyclins D1 and D2 and pRb and cdc25a declined in a time-dependent manner after incubation with NVP-BGT226. The effects on ck4, cdk2 and cyclin E were less pronounced; whereas cdk4 and cdk2 were upregulated, cyclin E was downregulated upon incubation with NVP-BGT226 (Fig. 2d).

Fig. 2

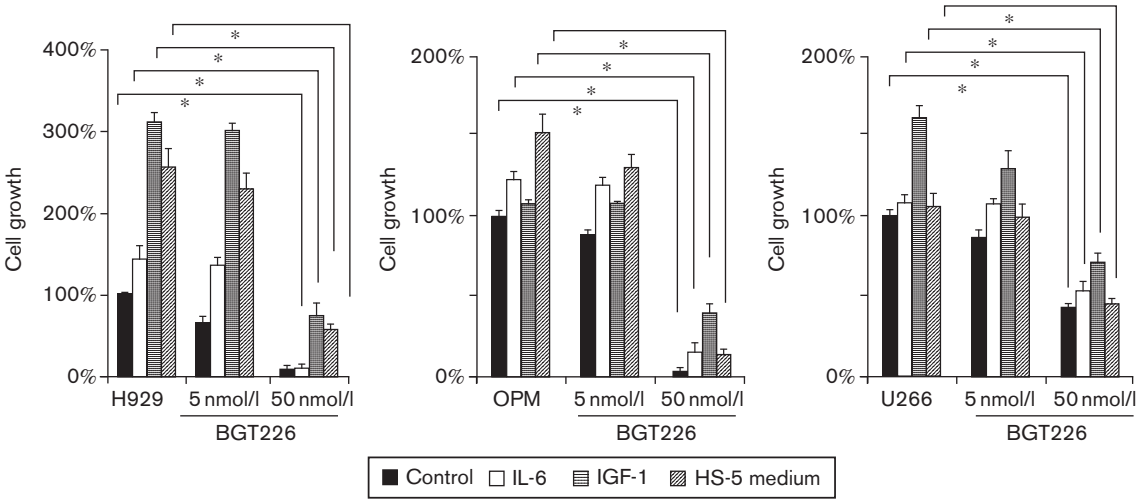


BEZ inhibits MM cell proliferation and induces a G1 cell cycle arrest. (a) OPM-2, NCI-H929 and U266 cells were incubated with none, 20, 50, 100 and 250 nmol/l of NVP-BGT226 for 48 h. After the incubation period, cell proliferation was determined using the BrdU proliferation assay. The figures show results from two independent experiments. **P* less than 0.05 versus control. (b, c) OPM-2, NCI-H929 and U266 myeloma cells were incubated with increasing concentrations of NVP-BGT226 as indicated in the figure. The DNA content was determined by flow cytometry after fixation with ethanol and staining the cells with propidium iodide. The figures show results from two independent experiments performed in triplicates. In all figures, the used cell line is indicated in the control experiment. (d) NCI-H929 myeloma cells were incubated with 100 nmol/l of NVP-BGT226 for the indicated time periods. After the incubation period, cells were lysed and directly subjected to SDS-PAGE, transferred to membranes and blotted with the indicated antibodies. MM, multiple myeloma.

NVP-BGT226 inhibits IL-6-induced, IGF-1-induced and stromal cell medium-induced cell growth

MM cells accumulate in the bone marrow and adhere to bone marrow stromal cells. Several research groups have shown that cytokines and growth factors such as IL-6 and IGF-1 stimulate MM cell growth and induce proliferation, cell survival and drug resistance by activating the PI3K/Akt pathway. To investigate whether NVP-BGT226-induced PI3K inhibition can diminish the stimulatory effect of cytokines and growth factors, we performed cell growth stimulation assays. NCI-H929, OPM-2 and U266 myeloma cells were incubated with 5 and 50 nmol/l NVP-BGT226, and 15 ng/ml IL-6 or 200 ng/ml IGF-1 in RPMI1640 without FCS. Additionally, MM cells were stimulated with medium containing 50% HS-5 stromal cell conditioned medium. Cell growth of all three cell lines was augmented, and NVP-BGT226 was able to suppress this stimulation (Fig. 3). Cell lines significantly differ in their dependence on the type of soluble factors, but overall growth all cell lines could be strongly and significantly stimulated: IGF-1 led to an increase of +211% in NCI-H929, +8% in OPM-2 and +61% in U266 cells, whereas IL-6 stimulated NCI-H929 by +45%, OPM-2 by +22% and U266 by +8%. HS-5 conditioned

Fig. 3



NVP-BGT226 inhibits cytokine-induced increase of myeloma cell growth. NCI-H929, OPM-2 and U266 myeloma cells were incubated with 200 ng/ml rhIGF-1, 15 ng/ml IL-6 or 50% conditioned medium obtained from a 48-h-old HS-5 stromal cell culture for 48 h. Additionally, cell growth was inhibited by adding 5 or 50 nmol/l of NVP-BGT226 to the culture. Cell growth was determined using the WST-1 assay. The figures show results from two independent experiments. *P less than 0.05 versus control.

medium increased cell growth of NCI-H929 by +154%, OPM-2 by +53% and U266 by +6%. Upon treatment with NVP-BGT226, cell growth was strongly inhibited, and the stimulatory effect on cell growth exerted by the cytokines and growth factors was abrogated at low nanomolar concentrations.

NVP-BGT226 inhibits the PI3K/Akt signalling pathway

It has been shown before that myeloma cell growth is closely related to the activation of the PI3K/Akt pathway. As NVP-BGT226 is claimed to be a PI3K/mTOR inhibitor, we performed western-blotting experiments in order to show the inhibitory effects of this substance on the modulation of the PI3K pathway in MM. We therefore cultured NCI-H929 myeloma cells for 4, 24 and 48 h in the presence of 100 nmol/l of NVP-BGT226, harvested and prepared the cells for western blotting. In our experiments, the amount of phosphorylated Akt, mTOR, P70S6k and 4E-BP-1 were significantly reduced upon incubation with NVP-BGT226 (Fig. 4a).

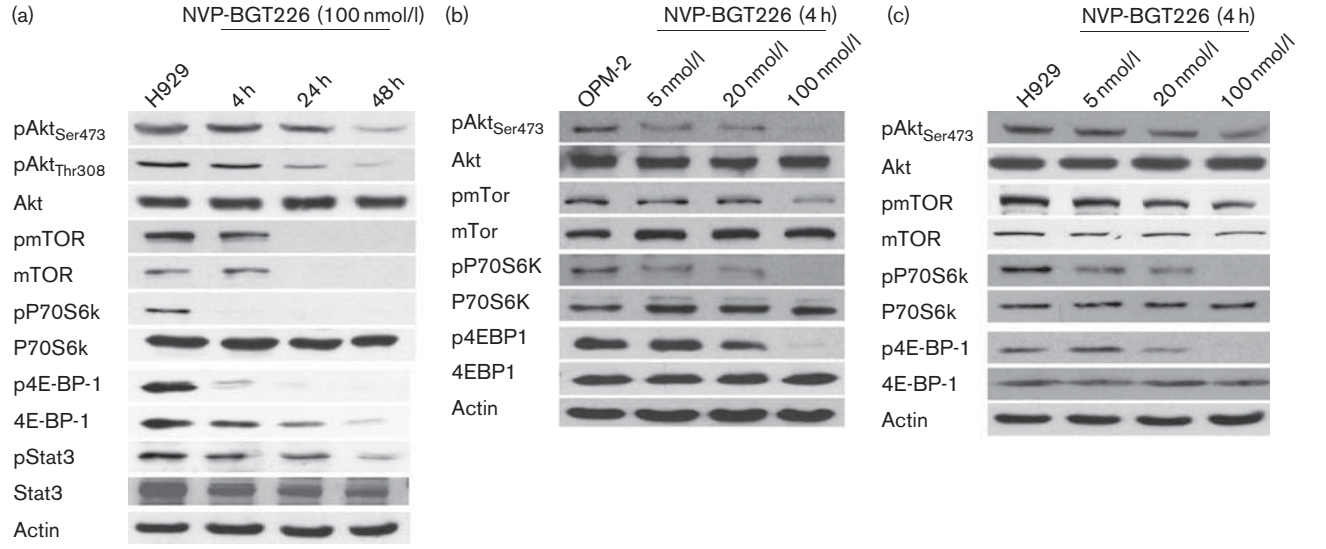
In order to confirm this finding, we incubated OPM-2 (Fig. 4b) and NCI-H929 (Fig. 4c) with 5, 20 and 100 nmol/l for 4 h and performed western-blotting experiments showing a strong dephosphorylation of Akt, mTOR, P70S6k and 4E-BP-1, whereas total protein levels of Akt and P70S6k remained unchanged.

Discussion

Both noncellular and cellular components of the bone marrow microenvironment are essential in the pathophysiology of MM disease [20]. Elevated levels of cytokines such as IL-6 and growth factors such as IGF-1 lead to activation of signalling pathways that induce cell survival, growth and drug resistance. Elevated IL-6 is mostly due to cellular adhesion of MM cells to bone marrow stromal cells, which are stimulated to secrete IL-6 upon adhesion [21] of MM cells [22]. IL-6 and many other cytokines and growth factors induce the activation of PI3K, which in turn activates Akt and therefore mTOR [23]. Akt activation is considered to be responsible for cell survival and proliferation of MM cells. Thus, both PI3K and mTOR are favourable targets in MM.

Here, we show significant preclinical activity of NVP-BGT226 as an agent targeting MM cells in a collection of MM cell lines and primary myeloma cells from patients. NVP-BGT226 is a second-generation combined PI3K/mTOR inhibitor. Inhibition of only mTOR in malignant cell leads to an compensatory activation of upstream signalling molecules such as Akt [24,25]. This finding provides the rationale for developing dual inhibitors such as NVP-BGT226. We show that inhibition of PI3K/Akt/mTOR signalling using the small molecule NVP-BGT226 is highly effective in MM. It induces cell growth inhibition and apoptosis even at low nanomolar concentrations. Induction of apoptosis is accompanied by decreased expression of capases 3 and 9 and cleavage of caspases 3 and 9, indicating that the intrinsic pathway of apoptosis is activated. Furthermore we observed an increased expression of proapoptotic bim and bak and downregulation of bcl-XL, bcl-2 and Noxa. This is partially in agreement with previous results with the first-generation PI3K/mTOR inhibitor NVP-BEZ235 [26]. In contrast to the first-generation kinase inhibitor, NVP-BEZ235, NVP-BGT226 shows remarkably lower IC_{50} values considering cell viability and cell apoptosis, underlining its higher efficacy.

Fig. 4



Incubation of myeloma cells with NVP-BGT226 leads to decreased phosphorylation of mediators of the PI3 kinase/Akt pathway. (a) H929 myeloma cells were incubated with 100 nmol/l of NVP-BGT226 for 4, 24 and 48 h. After the incubation period, cells were lysed and directly subjected to SDS-PAGE, transferred to membranes and blotted with the indicated antibodies. (b) OPM-2 and (c) NCI-H929 myeloma cells were incubated with increasing concentrations of NVP-BGT226 for 4 h. After the incubation period, cells were lysed and directly subjected to SDS-PAGE, transferred to membranes and blotted with the indicated antibodies. PI3, phosphoinositol-3-kinase.

Malignant plasma cells are stimulated by cytokines and growth factors that lead to activation of the JAK/STAT pathway, the MEK/ERK pathway and the PI3K/Akt signalling cascade [27,28]. In our studies, NVP-BGT226 overcame the pro-proliferative stimuli of IL-6, IGF-1 and conditioned medium of HS-5 stromal cells.

We hypothesized that the inhibition of PI3K/mTOR by NVP-BGT226 leads to decreased cell proliferation. As expected, NVP-BGT226 strongly decreased proliferation as shown by the BrdU uptake assay. This is in accordance with results of other groups [23,26]. Cell cycle analysis revealed that decreased cell proliferation was due to a phase G1 arrest. Similar results have been shown for PI3K inhibition in other cell types [29,30].

The cell cycle machinery has been observed to be commonly deregulated in MM. Cyclins D1, D2 or D3 are deregulated in all MM patients and expressed more than in normal cells [28]. Furthermore, the 11q13 and the 6p21 IgH translocations are accompanied by cyclin D1 and cyclin D3 overexpression, respectively [28]. Our western-blotting experiments show that G1 cell cycle arrest was due to decreased expression of cyclin D1, cyclin D2, pRb and Cdc25.

Activated PI3K stimulates Akt kinase, exerting multiple effects on myeloma cells by modulating several signalling pathways. Besides its antiapoptotic role, phosphorylated Akt has stimulatory effects on cell growth and cell proliferation. Akt activates mTOR, a protein related to cell growth. mTOR in turn augments activation of 4E-BP-1, which leads to increased protein translation [31,32]. Furthermore, it activates P70S6k, which leads to cell cycle progression [33] and inhibition of apoptosis [34]. Our western-blotting experiments revealed that NVP-BGT226 strongly inhibits phosphorylation of Akt, P70S6k and 4E-BP-1. Similar observations were made by Liu *et al.* [29] and Chiarini *et al.* [35], who treated glioma cell lines and T-cell acute lymphoblastic leukaemia cell lines, and observed a hypophosphorylation of Akt, P70S6k and 4E-BP-1.

Summing up, the small molecule NVP-BGT226, which is the first representative of the novel group of second-generation dual PI3K/mTOR inhibitors, strongly inhibits myeloma cell growth and induces apoptosis. The results provide the rationale for further preclinical and clinical development of this drug.

Acknowledgements

P.B. wrote the paper and designed research, L.S. and S.M.-W. performed the experiments, F.O. revised the paper and R.S. analysed data and revised the paper.

Conflicts of interest

There are no conflicts of interest.

References

1 Jagannath S, Kyle RA, Palumbo A, Siegel DS, Cunningham S, Berenson J. The current status and future of multiple myeloma in the clinic. *Clin Lymphoma Myeloma Leuk* 2010; **10**:28–43.
2 Kyle RA, Rajkumar SV. Multiple myeloma. *Blood* 2008; **111**:2962–2972.

3 Attal M, Harousseau JL. Standard therapy versus autologous transplantation in multiple myeloma. *Hematol Oncol Clin North Am* 1997; **11**:133–146.
4 Attal M, Harousseau JL, Facon T, Guilhot F, Doyen C, Fuzibet JG, *et al*. Single versus double autologous stem-cell transplantation for multiple myeloma. *N Engl J Med* 2003; **349**:2495–2502.
5 APEX (Assessment of Proteasome inhibition for Extending remissions) trial: phase III randomized, multicenter, placebo-controlled trial to evaluate the efficacy and safety of bortezomib versus dexamethasone in patients with recurrent or treatment-resistant multiple myeloma. *Clin Adv Hematol Oncol* 2003; **1**:190.
6 Gregory WM, Richards MA, Malpas JS. Combination chemotherapy versus melphalan and prednisolone in the treatment of multiple myeloma: an overview of published trials. *J Clin Oncol* 1992; **10**:334–342.
7 Singhal S, Mehta J, Desikan R, Ayers D, Roberson P, Eddlemon P, *et al*. Antitumor activity of thalidomide in refractory multiple myeloma. *N Engl J Med* 1999; **341**:1565–1571.
8 Richardson PG, Barlogie B, Berenson J, Singhal S, Jagannath S, Irwin D, *et al*. Clinical factors predictive of outcome with bortezomib in patients with relapsed, refractory multiple myeloma. *Blood* 2005; **106**:2977–2981.
9 Kawano M, Hirano T, Matsuda T, Taga T, Horii Y, Iwato K, *et al*. Autocrine generation and requirement of BSF-2/IL-6 for human multiple myelomas. *Nature* 1988; **332**:83–85.
10 Klein B, Tarte K, Jourdan M, Mathouk K, Moreaux J, Jourdan E, *et al*. Survival and proliferation factors of normal and malignant plasma cells. *Int J Hematol* 2003; **78**:106–113.
11 Jelinek DF, Witzig TE, Arendt BK. A role for insulin-like growth factor in the regulation of IL-6-responsive human myeloma cell line growth. *J Immunol* 1997; **159**:487–496.
12 Ferlin M, Noraz N, Hertogh C, Brochier J, Taylor N, Klein B. Insulin-like growth factor induces the survival and proliferation of myeloma cells through an interleukin-6-independent transduction pathway. *Br J Haematol* 2000; **111**:626–634.
13 Freund GG, Kulas DT, Mooney RA. Insulin and IGF-1 increase mitogenesis and glucose metabolism in the multiple myeloma cell line, RPMI 8226. *J Immunol* 1993; **151**:1811–1820.
14 Ge NL, Rudikoff S. Expression of PTEN in PTEN-deficient multiple myeloma cells abolishes tumor growth in vivo. *Oncogene* 2000; **19**: 4091–4095.
15 Baumann P, Mandl-Weber S, Emmerich B, Straka C, Schmidmaier R. Activation of adenosine monophosphate activated protein kinase inhibits growth of multiple myeloma cells. *Exp Cell Res* 2007; **313**:3592–3603.
16 Schmidmaier R, Baumann P, Bumeder I, Meinhardt G, Straka C, Emmerich B. First clinical experience with simvastatin to overcome drug resistance in refractory multiple myeloma. *Eur J Haematol* 2007; **79**:240–243.
17 Serra V, Markman B, Scaltriti M, Eichhorn PJ, Valero V, Guzman M, *et al*. NVP-BEZ235, a dual PI3K/mTOR inhibitor, prevents PI3K signaling and inhibits the growth of cancer cells with activating PI3K mutations. *Cancer Res* 2008; **68**:8022–8030.
18 Inoki K, Li Y, Zhu T, Wu J, Guan KL. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat Cell Biol* 2002; **4**:648–657.
19 Fan QW, Knight ZA, Goldenberg DD, Yu W, Mostov KE, Stokoe D, *et al*. A dual PI3 kinase/mTOR inhibitor reveals emergent efficacy in glioma. *Cancer Cell* 2006; **9**:341–349.
20 Podar K, Chauhan D, Anderson KC. Bone marrow microenvironment and the identification of new targets for myeloma therapy. *Leukemia* 2009; **23**:10–24.
21 Huston A, Leleu X, Jia X, Moreau AS, Ngo HT, Runnels J, *et al*. Targeting Akt and heat shock protein 90 produces synergistic multiple myeloma cell cytotoxicity in the bone marrow microenvironment. *Clin Cancer Res* 2008; **14**:865–874.
22 Shain KH, Yarde DN, Meads MB, Huang M, Jove R, Hazlehurst LA, *et al*. Beta1 integrin adhesion enhances IL-6-mediated STAT3 signaling in myeloma cells: implications for microenvironment influence on tumor survival and proliferation. *Cancer Res* 2009; **69**:1009–1015.
23 McMillin DW, Ooi M, Delmore J, Negri J, Hayden P, Mitsiades N, *et al*. Antimyeloma activity of the orally bioavailable dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor NVP-BEZ235. *Cancer Res* 2009; **69**:5835–5842.
24 Shi Y, Yan H, Frost P, Gera J, Lichtenstein A. Mammalian target of rapamycin inhibitors activate the AKT kinase in multiple myeloma cells by up-regulating the insulin-like growth factor receptor/insulin receptor substrate-1/phosphatidylinositol 3-kinase cascade. *Mol Cancer Ther* 2005; **4**:1533–1540.
25 O'Reilly KE, Rojo F, She QB, Solit D, Mills GB, Smith D, *et al*. mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. *Cancer Res* 2006; **66**:1500–1508.
26 Baumann P, Mandl-Weber S, Oduncu F, Schmidmaier R. The novel orally bioavailable inhibitor of phosphoinositol-3-kinase and mammalian target of rapamycin, NVP-BEZ235, inhibits growth and proliferation in multiple myeloma. *Exp Cell Res* 2009; **315**:485–497.
27 Hideshima T, Richardson P, Anderson KC. Novel therapeutic approaches for multiple myeloma. *Immunol Rev* 2003; **194**:164–176.
28 Hideshima T, Bergsagel PL, Kuehl WM, Anderson KC. Advances in biology of multiple myeloma: clinical applications. *Blood* 2004; **104**:607–618.
29 Liu TJ, Koul D, Lafortune T, Tiao N, Shen RJ, Maira SM, *et al*. NVP-BEZ235, a novel dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor, elicits multifaceted antitumor activities in human gliomas. *Mol Cancer Ther* 2009; **8**:2204–2210.
30 Wierod L, Rosseland CM, Lindeman B, Oksvold MP, Grosvik H, Skarpen E, *et al*. CDK2 regulation through PI3K and CDK4 is necessary for cell cycle progression of primary rat hepatocytes. *Cell Prolif* 2007; **40**:475–487.
31 Xu RH, Pelicano H, Zhang H, Giles FJ, Keating MJ, Huang P. Synergistic effect of targeting mTOR by rapamycin and depleting ATP by inhibition of glycolysis in lymphoma and leukemia cells. *Leukemia* 2005; **19**:2153–2158.
32 Ikezoe T, Nishioka C, Bandobashi K, Yang Y, Kuwayama Y, Adachi Y, *et al*. Longitudinal inhibition of PI3K/Akt/mTOR signaling by LY294002 and rapamycin induces growth arrest of adult T-cell leukemia cells. *Leuk Res* 2007; **31**:673–682.
33 Lane HA, Fernandez A, Lamb NJ, Thomas G. p70s6k function is essential for G1 progression. *Nature* 1993; **363**:170–172.
34 Harada H, Andersen JS, Mann M, Terada N, Korsmeyer SJ. p70S6 kinase signals cell survival as well as growth, inactivating the pro-apoptotic molecule BAD. *Proc Natl Acad Sci USA* 2001; **98**:9666–9670.
35 Chiarini F, Grimaldi C, Ricci F, Tazzari PL, Evangelisti C, Ognibene A, *et al*. Activity of the novel dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor NVP-BEZ235 against T-cell acute lymphoblastic leukemia. *Cancer Res* 2010; **70**:8097–8107.