

Everolimus shows synergistic antimyeloma effects with bortezomib via the AKT/mTOR pathway

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ABSTRACT

Multiple myeloma (MM) is characterized by the proliferation of malignant plasma cells and a subsequent overabundance of monoclonal paraproteins (M proteins). Everolimus works similarly to sirolimus as a mammalian target of rapamycin (mTOR) inhibitor. Bortezomib was the first therapeutic proteasome inhibitor to be tested in humans with MM. However, the combination of these two drugs for the treatment of MM has been rarely reported. In this study, we compared the therapeutic effects of everolimus and bortezomib, as well as those of a combination of everolimus and bortezomib, using an in vitro MM cell line model and in vivo xenograft mouse model. Our results showed that the synergistic antitumor effects of everolimus and bortezomib have significant inhibitory effect through inhibition of the AKT/mTOR pathway in both the MM cell lines and MM-bearing mice model. Our results provided evidence that the mTOR inhibitor, everolimus, will be a potential drug in MM therapy.

INTRODUCTION

Multiple myeloma (MM) is a malignant clonal plasma cell disease. It is characterized by the proliferation of monoclonal plasma cells in the bone marrow, extensive infiltration, and the secretion and deposition of a large number of monoclonal immunoglobulins. Bone destruction, anemia, renal insufficiency, and hypercalcemia are the main clinical signs. The pathogenesis of MM is not completely understood to date. MM has been challenging to treat due to its low cure rate and treatment resistance. It is difficult to treat, and the prognosis is not ideal.

In the past 10 years, with the introduction of immune modulators and proteasome inhibitors, the median survival time of patients with MM has increased from 29.9 months to 44.8 months, which has fundamentally improved the clinical efficacy and prognosis of MM.¹ The latest data show that the 5-year survival rate of patients with MM has increased to 45%.² However, treatment with the aforementioned drugs is accompanied by new treatment-related complications, such as peripheral neurotoxicity, necessitating treatment with a reduced dose or even termination of treatment for some patients.³ Almost all patients with MM

Significance of this study

What is already known about this subject?

- ▶ Bortezomib inhibits the activity of NF- κ B, leading to apoptosis of myeloma cells, and upregulates the expression of Bcl-2 in an antiapoptotic role in multiple myeloma (MM) cells.
- ▶ Everolimus can inhibit tumor cell growth, metabolism and angiogenesis by blocking the PI3K-AKT-mTOR (mammalian target of rapamycin) pathway of the cancer cell.
- ▶ Bortezomib and everolimus are promising chemotherapeutic agents that are efficacious in the treatment of a variety of cancers.

What are the new findings?

- ▶ Everolimus shows synergistic antimyeloma effects with bortezomib.
- ▶ The mechanism of the effect is via AKT/mTOR pathway in in vitro and in vivo experiments.
- ▶ There are synergistic antimyeloma effects at a suitable drug concentration for human.

How might these results change the focus of research or clinical practice?

- ▶ Everolimus combined with bortezomib at a suitable concentration shows synergistic antimyeloma effects via the AKT/mTOR pathway, providing more benefits to patients with MM.

eventually experience refractory disease and relapse.⁴ To date, MM remains an incurable disease. Therefore, there is a pressing need for more effective therapeutic drugs with less toxic side effects to enhance the efficacy of the existing drugs and improve the quality of life and survival rate of patients with MM.

Bortezomib is a novel proteasome inhibitor. The threonine residue binds selectively to the proteasome active site, inhibiting the trypsin and chymotrypsin activity of the 20S proteasomal subunit. Bortezomib is the world's first drug to use an enzyme protein as a therapeutic target for cancer. It acts against myeloma cells through a variety of mechanisms: bortezomib inhibits the activity of nuclear factor κ B



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(NF- κ B), leading to apoptosis of myeloma cells,⁵ and upregulates the expression of Noxa, a member of the Bcl-2 family that interacts with the protein Bcl-2 in an antiapoptotic role in MM cells, thus inducing apoptosis.⁶ Clinical trials have shown that the most common adverse effects of bortezomib include thrombocytopenia, neutropenia, peripheral neuropathy, and anemia.^{7–9} These toxic side effects will undoubtedly reduce the quality of life for elderly patients.

Everolimus is an inhibitor of mammalian target of rapamycin (mTOR) for the 40-O-rapamycin (2-hydroxyethyl) derivatives. It interacts with FK506 binding protein-12 in cells, and forms an inhibitory complex, mTORC1, that inhibits the activation of mTOR kinase and the effect of mTOR on the regulation of downstream effectors. In addition, everolimus also inhibits the expression of vascular endothelial growth factor and hypoxia-inducible factor.¹⁰ Therefore, everolimus can inhibit tumor cell growth, metabolism and angiogenesis by blocking the phosphatidylinositol3-kinases (PI3K-AKT, protein kinase B) mTOR pathway of the cell. Everolimus was approved for the treatment of patients with advanced renal cell carcinoma in 2009 by the Food and Drug Administration, as well as for several malignancies such as subependymal giant cell astrocytoma, pancreatic neuroendocrine tumors, and gastroenteropancreatic cancer.^{11–14}

Bortezomib and everolimus are promising chemotherapeutic agents that are efficacious in the treatment of a variety of cancers.^{15–17} On the basis of existing research, we investigated the individual and combined therapeutic effects of bortezomib and everolimus in MM in vivo and in vitro, which provide a theoretical basis for the use of combination therapy involving bortezomib and everolimus in clinical trials.

MATERIALS AND METHODS

Cells

Human MM cell lines U266 and LP-1 were purchased from the Tumor Cell Bank of the Chinese Academy of Medical Sciences (Beijing, China). U266 cells were cultured in dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. LP-1 cells were cultured in RPMI 1640 containing 10% fetal bovine serum with 100 units/mL penicillin and 100 μ g/mL streptomycin at 37°C in a humidified incubator with 5% CO₂.

Proliferation assay (CCK-8 assay)

The cell lines were randomly divided into four groups, namely the control group (C), everolimus treatment group (E), bortezomib treatment group (B), and the everolimus combined with bortezomib treatment group (E+B). The dosage of bortezomib was fixed at 10 nM, and the dosage of everolimus increased from 0 nM to 5 nM, 10 nM, 15 nM, 20 nM, and 25 nM.^{18 19} A Cell Counting Kit-8 (CCK-8) assay was performed to observe cell proliferation at 0 hour, 12 hours, 24 hours, 48 hours, 60 hours, and 72 hours.

The CCK-8 assay (Dojindo Molecular Technologies, Shanghai, China) was performed, according to the manufacturer's recommendations, to evaluate the MM cell viability in all groups. Cells were seeded in 96-well plates (Corning, Steuben County, USA). The different groups of cells were

treated by the addition of 20 μ L of the corresponding drug, with the control group cells receiving 20 μ L RPMI 1640 culture solution. Each group of cells was simultaneously seeded in three wells, with a blank control well containing no cell culture solution, and then incubated for 48 hours. Four hours before culture termination, 10 mL of CCK-8 solution was added to the culture. The optical density was read on a 96-well plate reader at a wavelength of 450 nm within 15 min. The experiment was repeated three times, and each sample was run in triplicate. Data from the cell proliferation experiments were expressed as the percentage of cell proliferation compared with that in untreated cells. The everolimus concentration resulting in 50% inhibition of cell growth (IC₅₀) was determined by fitting to a dose–response curve. Cell viability was calculated using the following equation: cytotoxicity (%) = (1–OD₄₅₀ of isogarcinol group/OD₄₅₀ of control group) \times 100%.²⁰

Cell apoptosis assays

Cell apoptosis was detected by flow cytometry, and cells were stained with fluorescein-conjugated annexin V and propidium iodide (PI).²¹ The cells were washed twice with phosphate buffered saline (PBS), centrifuged, and the supernatant was discarded. Cells were resuspended in binding buffer (10 mmol/L N-2-hydroxyl piperazine-N0-2-ethane sulfonic acid/NaOH, pH 7.4, 140 mmol/L NaCl, 2.5 mmol/L CaCl₂). Then, 100 μ L cell suspension was incubated with 10 μ L annexin V-fluorescein isothiocyanate (FITC) and 10 μ L PI and gently mixed away from light for 15 min at room temperature (RT). Cells were detected by flow cytometry within 30 min, and the percentage of apoptotic cells relative to the total number of cells in each group was compared. A tube without annexin V-FITC and PI was used as the negative control, and each sample had three replications.

Western blot

Cells and tumor tissue for all groups were collected and added to sufficient lysis buffer (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 100 mM NaF, 1% NP40, 1 μ g/mL leupeptin, 1 μ g/mL antipain, and 1 mM phenylmethylsulfonyl fluoride), and protein quantification was performed by BCA Assay (Pierce, Rockford, Illinois, USA). Samples were analyzed by western blotting, as described previously,²² using a β -actin monoclonal antibody as the internal control. The primary antibodies used were the PTEN antibody (1:500, Abcam, ab32199, Cambridge, Massachusetts), AKT1 antibody (1:1000, Abcam, ab81283), mTOR antibody (1:600, Abcam, ab2732), P70S6K1 antibody (1:1000, Abcam, ab32529), 4EBP1 antibody (1:1000, Abcam, ab32024), caspase 3 antibody (1:500, Abcam, ab13847), NF- κ B antibody (1:1000, Abcam, ab207297), and β -actin antibody (1:1000, Abcam, ab8227). All gray value of protein expression was determined by computer scanning.

Animal model

All animals were housed in the Animal Experimental Center of the Fifth Central Hospital of Tianjin at a humidity of 50% \pm 5% at 20°C–25°C. Nude mice aged 6 weeks were inoculated subcutaneously with 1 \times 10⁷ U266 and LP-1

cells (100 μ L tumor cell suspension) on both sides of the back and divided into four groups (n=8): the control group (C), the everolimus treatment group (E), the bortezomib treatment group (B), and the everolimus combined with bortezomib treatment group (E+B). Each group received the corresponding concentration of drug by intraperitoneal injection every other day for 4 weeks. The rate of tumor formation and the change in the tumor were observed every day; the tumor volume (mm^3) was estimated to evaluate tumor growth by measuring the longest perpendicular tumor diameter using calipers every alternate day and calculated with the following formula: $1/2 \times (\text{major axis}) \times (\text{minor axis})$.²²³ Animals were euthanized when mice appeared moribund to prevent unnecessary morbidity. The subcutaneous nodules were taken out for volume measurement and fixed in 10% neutral buffered formalin for immunohistochemistry (IHC). All mice received care according to the standard animal care protocols and guidelines.

Immunohistochemistry

Tumor tissues were fixed and stored in 10% neutral buffered formalin at 4°C for approximately 24 hours and then processed in paraffin wax. Eight samples in each group were randomly selected for IHC studies. Serial paraffin tumor tissue sections (50 μ m) were made to observe the IHC staining. Sections were dewaxed in water, washed three times in buffer for 15 min each, immersed in newly prepared 0.3% hydrogen peroxide in methanol at RT for 15 min to block endogenous peroxidase activity, and washed three times in buffer for 5 min. Then, non-specific binding sites were blocked with normal goat serum in Tris-buffered saline at RT for 30 min and then treated overnight at 4°C with primary antibodies: namely rabbit polyclonal antibodies against PTEN, AKT1, mTOR, P70S6K1, 4EBP1, caspase 3, and NF- κ B (all are Abcam). After the sections were washed, they were incubated in a goat antirabbit secondary antibody for 30 min at RT. The staining in the sections was visualized using a horseradish peroxidase-conjugated compact polymer system. Dimethylaminoazobenzene was used as the chromogen. Positive IHC staining was detected by light microscopy.

Statistical analysis

All experiments were repeated at least three times. Data are expressed as mean \pm SEM and were analyzed by Student's t-test or two-way analysis of variance. Statistical analyses were performed using SPSS V.17.0 statistical software program/package. The level of statistical significance was set at $p \leq 0.05$.

RESULTS

Everolimus shows synergistic antitumor effect with bortezomib in MM cell lines

To provide a basis for the use of everolimus and bortezomib as combination therapy agents against MM, we first used a series of combination of different concentrations of everolimus with 10 nM bortezomib to test the effects on the proliferation of the MM cell lines U266 and LP-1. The experimental results showed that treatment with everolimus in combination with bortezomib suppressed cell proliferation; treatment with 20 nM everolimus in combination with

10 nM bortezomib achieved the lowest cell survival rate for both cell lines (figure 1).

Proliferation of U266 and LP-1 cells treated with everolimus alone, bortezomib alone, and everolimus in combination with bortezomib was analyzed using the CCK-8 assay. The optical density was measured at 12, 24, 48, 60, and 72 hours of treatment. Proliferation was markedly lower in both cell lines treated with the drugs for 72 hours than that in the control group (figure 1C,D).

To evaluate the effects of combining bortezomib and everolimus on the cell survival rate, we used the IC50 for everolimus and bortezomib in all of the groups, using the fixed ratios of 1:8, 2:8, 4:8, 6:8, and 8:8 IC50 for everolimus and bortezomib. The combination of everolimus and bortezomib was synergistic in both cell lines, indicated by combination index (CI) values of < 0.8 (figure 1E,F).²⁴ For example, the CI value in U-266 cells ranged from 0.46 to 0.63 with everolimus and bortezomib simultaneously. No antagonism ($CI > 1.2$) was observed in any cell line.

Combination therapy with everolimus and bortezomib induces apoptosis in vitro more effectively

Our study showed that combination therapy with everolimus and bortezomib could inhibit myeloma cell proliferation more effectively than either treatment alone.

Flow cytometry was used to determine the effect on apoptosis of bortezomib in combination with everolimus. U266 cells were treated with everolimus (20 nM), bortezomib (10 nM), or a combination of everolimus and bortezomib for 72 hours, followed by double staining with annexin V/PI. As shown in figure 2, the combination of everolimus and bortezomib had a synergistic effect on apoptosis in U266 and LP-1 cells. Compared with apoptosis observed in control cells, on treatment with everolimus alone and bortezomib alone, 22.5% and 17.2% apoptosis, respectively, were observed. However, combination treatment induced 28.8% apoptosis in U266 cells, and a similar trend was observed in LP-1 cells. Collectively, these results show that bortezomib and everolimus combination therapy induced myeloma cell apoptosis in vitro more effectively.

AKT/mTOR signal transduction pathway

To explore the mechanisms whereby apoptosis is triggered, we examined the expression of the primary apoptotic molecule, caspase 3, in U266 and LP-1 cells in each group. In this experiment, the two cell lines were incubated with the various drugs for 72 hours and were then lysed to extract the proteins for further analysis.

As shown in figure 3, the expression of AKT1, mTOR, p70S6K1, and 4EBP1 in the E-treated and E+B-treated groups was significantly lower than that in the control group ($p < 0.05$ for all comparisons), while in the B-treated group, there was no significant change in the expression of these proteins ($p > 0.05$). In the drug-treated groups, the protein expression of caspase 3 was significantly higher than that in the control group ($p < 0.05$), and the protein expression of NF- κ B was significantly lower than that in the control group ($p < 0.05$), while there was no significant difference in the protein expression of PTEN in any of the groups. The results of the IHC further showed that the expression of mTOR, p70S6K1, and 4EBP1 in the E+B-treated groups was

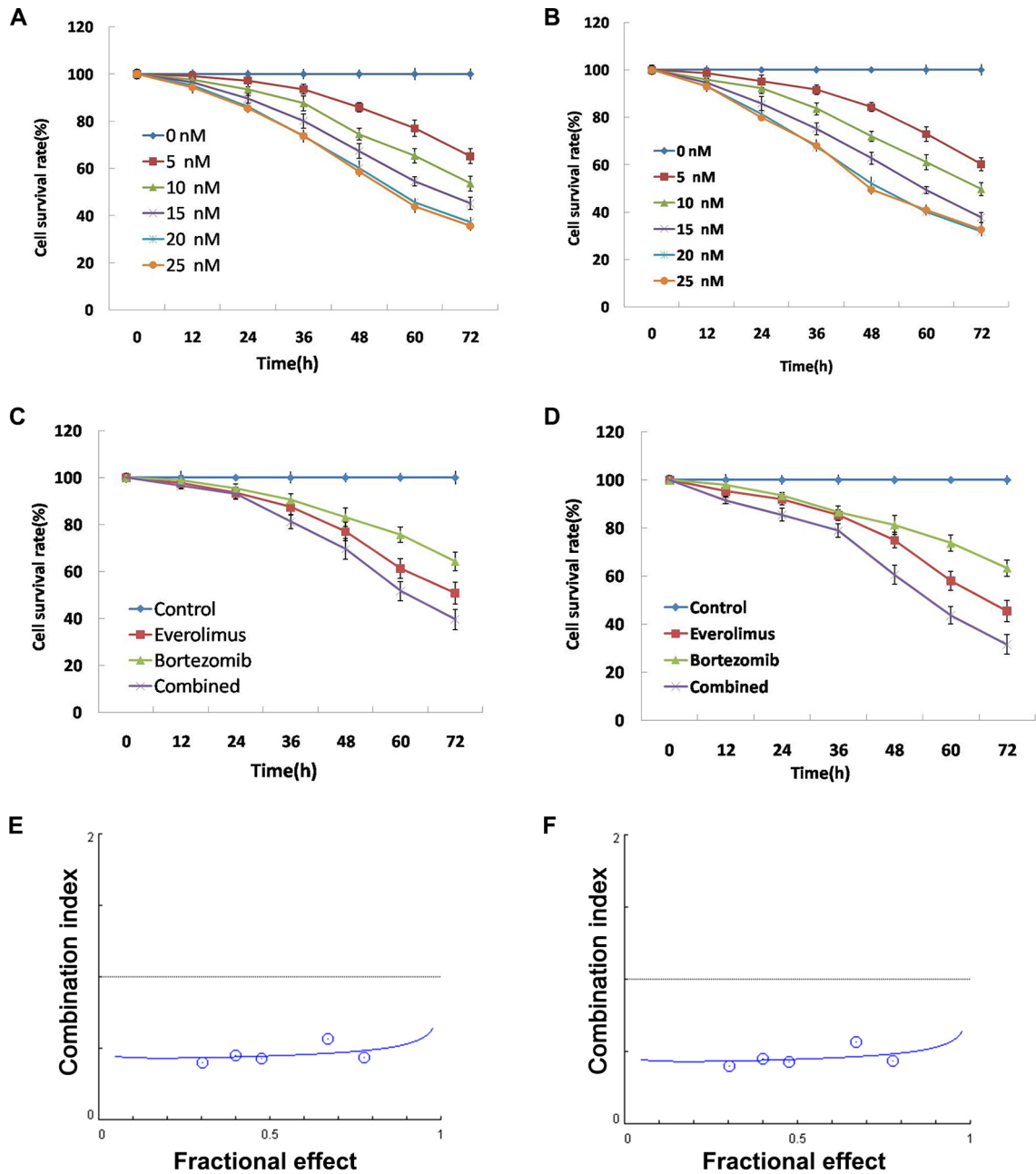


Figure 1 Effects of bortezomib and everolimus on the cell survival rate. (A) Effect of the different doses of everolimus from 0 nM to 25 nM in combination with 10 nM bortezomib on the survival rate in the U266 cell line at 12, 24, 36, 48, 60 and 72 hours. (B) Effect of different doses of everolimus from 0 nM to 25 nmol/L in combination with 10 nm bortezomib on the survival rate in the LP-1 cell line at 12, 24, 36, 48, 60 and 72 hours. Both U266 cells and LP-1 cells were treated with 10 nM bortezomib alone, 20 nM everolimus alone, and 10 nM bortezomib in combination with 20 nM everolimus for 72 hours. (C) The survival rate of U266 cells in each treatment group. (D) The survival rate of LP-1 cells in each treatment group. (E) CI plots of the everolimus and bortezomib combinations in the U266 cell lines. (F) CI plots of the everolimus and bortezomib combinations in the LP-1 cell lines.

significantly lower than that in the control group, and the protein expression of caspase 3 was significantly higher than that in the control group (figure 4).

Synergistic antitumor effect of everolimus and bortezomib on myeloma cell proliferation in vivo

Our in vitro study suggested that treatment with everolimus induced apoptosis in MM cells. These observations led us to investigate the ability of everolimus to inhibit the tumor

growth of myeloma cells in a nude mouse xenograft model. Eight nude mice were chosen to analyze the similar shape and size of the tumor in each experimental group.

As shown in figure 5A, when the animal model carrying the myeloma xenograft was successfully established, we observed the effect of drug treatment on the volume (mm^3) of the tumor in vivo. In figure 5B, drug treatment substantially reduced the size of the tumor compared with the tumor size in the control group mice bearing myeloma xenografts

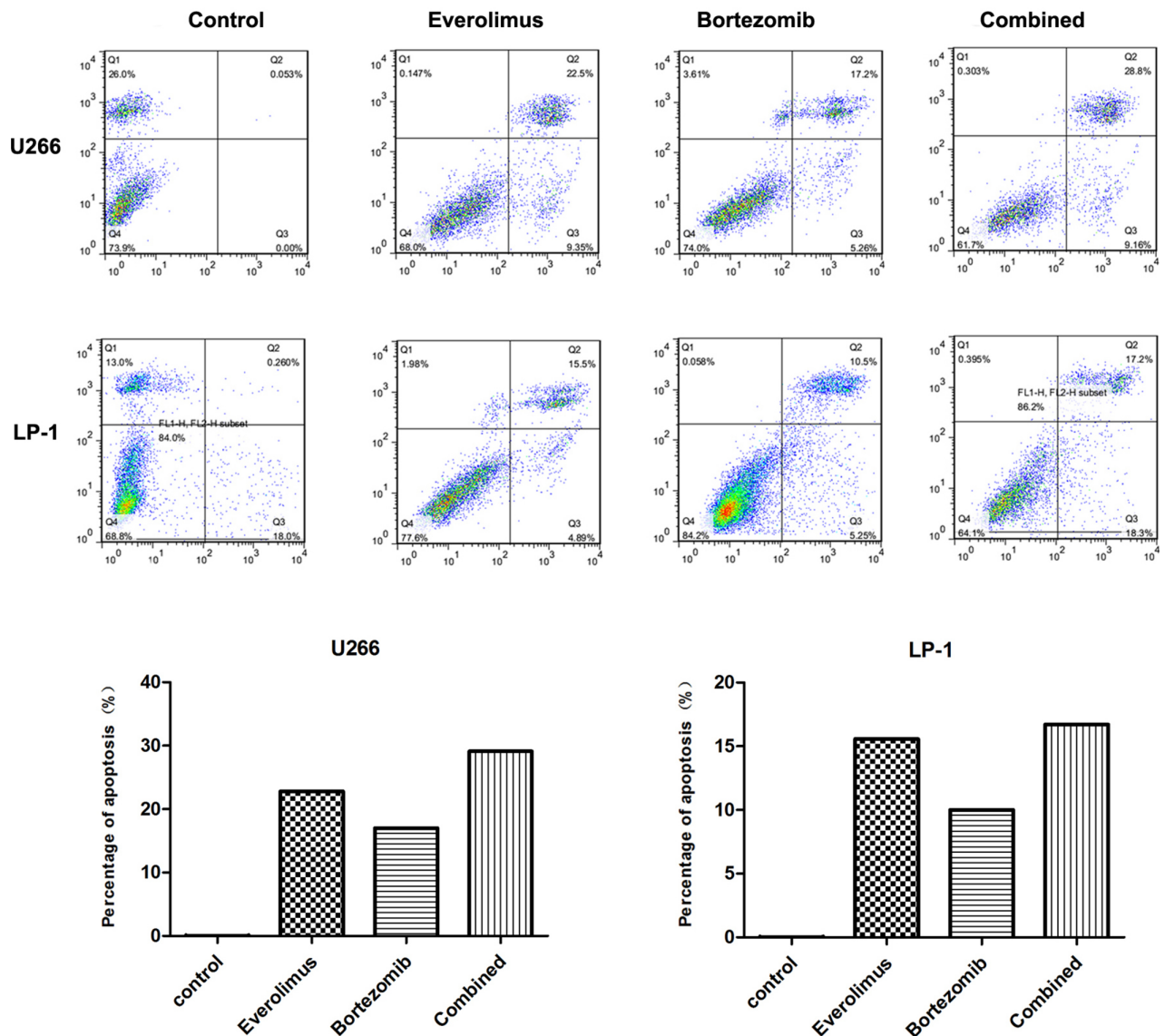


Figure 2 Cell apoptosis in the U266 and LP-1 cell lines detected by flow cytometry using annexin V-Fluorescein isothiocyanate (FIT)/propidium iodide (PI) staining.

treated with PBS. On the 25th day after tumor implantation of U266 cells, statistical reduction in the tumor volume was observed when comparing the volume in the drug-treated and control groups ($p < 0.05$). More significantly, the tumor volume in the E+B-treated group of the U266 xenograft model on day 30 was also less than that in the other groups.

DISCUSSION

Previous studies have investigated the effects of bortezomib alone on MM cells. Everolimus has been recommended for the treatment of advanced renal cell carcinoma, advanced breast cancer, esophageal cancer and gastric cancer. However, few studies have examined the effect of everolimus alone and the synergistic effects of bortezomib and everolimus on MM cells.

In this study, we investigated the combined effects of bortezomib and everolimus. Our results indicated that the two drugs synergistically suppressed the growth of MM

cells and induced MM cell apoptosis, even at low concentrations. The CI values were calculated according to the Chou-Talalay equation.²⁴ The mean CIs of the combined bortezomib and everolimus group were lower than 1 in the U266 and LP-1, which suggests good synergy with both drugs. Thus, everolimus shows synergistic antimyeloma effects with bortezomib. This finding provides a powerful evidence for the future clinical combinatorial application of bortezomib and everolimus in patients with MM.

The results from flow cytometry showed that bortezomib and everolimus played a cytotoxic role by mediating cell apoptosis. Both bortezomib and everolimus participated in the activation of cell death pathways mediated by caspase 3, and a low concentration of everolimus significantly enhanced the cytotoxic effect of bortezomib.

Cell cycle progression, growth, differentiation, and apoptosis of cells are known to be directly controlled by a set of genes. Extracellular molecules indirectly interfere with gene

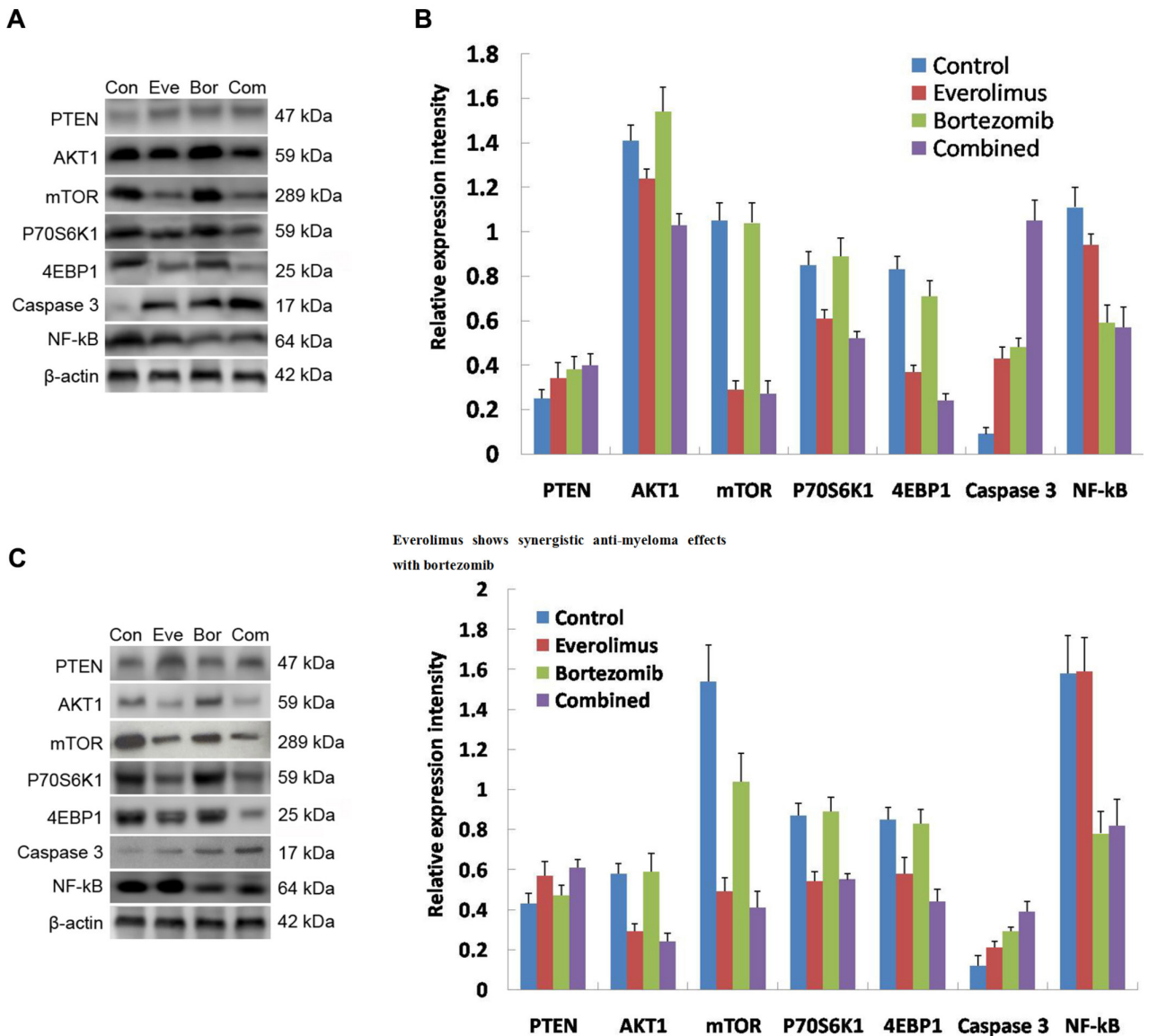


Figure 3 AKT/mTOR signal transduction pathway in both U266 and LP-1 cell lines following treatment with bortezomib and everolimus. (A,C) Western blot analysis of PTEN, AKT1, mTOR, P70S6K1, 4EBP1, caspase 3, and NF-κB. (B,D) The relative expression of the proteins. Bor, bortezomib; Com, combined; Con, control; Eve, everolimus; mTOR, mammalian target of rapamycin.

expression through signal transduction pathways regulating differentiation, development, proliferation and apoptosis. Among these signal transduction pathways, the PI3K/AKT/mTOR signaling pathway is the most important one. A variety of mechanisms could result in abnormal alterations of the PI3K/AKT/mTOR signaling pathway, which can further result in malignant tumors.²⁵

Many studies have shown that apoptosis can be induced in MM cells through a combination of agents blocking target proteins in the PI3K/AKT/mTOR signaling pathway. mTOR is an AKT substrate downstream of the PI3K/AKT/mTOR signaling transduction pathway. It functions as a Ser/Thr protein kinase and a member of the PI3K family. mTOR is an effector of the PI3K/AKT signaling pathway and plays a key role in the regulation of multiple cellular functions, including proliferation, survival, apoptosis, migration, and angiogenesis.²⁶ P70S6

and 4E-BP1 are two target proteins of mTOR that can be phosphorylated by mTOR. 4EBP is a eukaryotic translation initiation factor-inhibiting protein, and the phosphorylation of 4EBP by mTOR frees eIF4E, prompting mRNA translation. P70S6K1 is a ribosomal 40S small subunit S6 protein kinase, and phosphorylation of the 40S ribosomal protein S6 can promote the translation of mRNA containing a 5' TOP structure.²⁷ A study by Tam *et al*²⁸ showed that everolimus enhanced the cytotoxicity of cisplatin, an alternate alkylating agent, which regulated the expression of P70S6K and 4EBP-1 in liver cancer cells.

mTOR regulates cell growth by controlling protein biosynthesis. It has been found that after blocking the mTOR signaling pathway with rapamycin or its analog CCI-779, the phosphorylation of PI3K/AKT is significantly enhanced in MM cells and the activation of AKT is

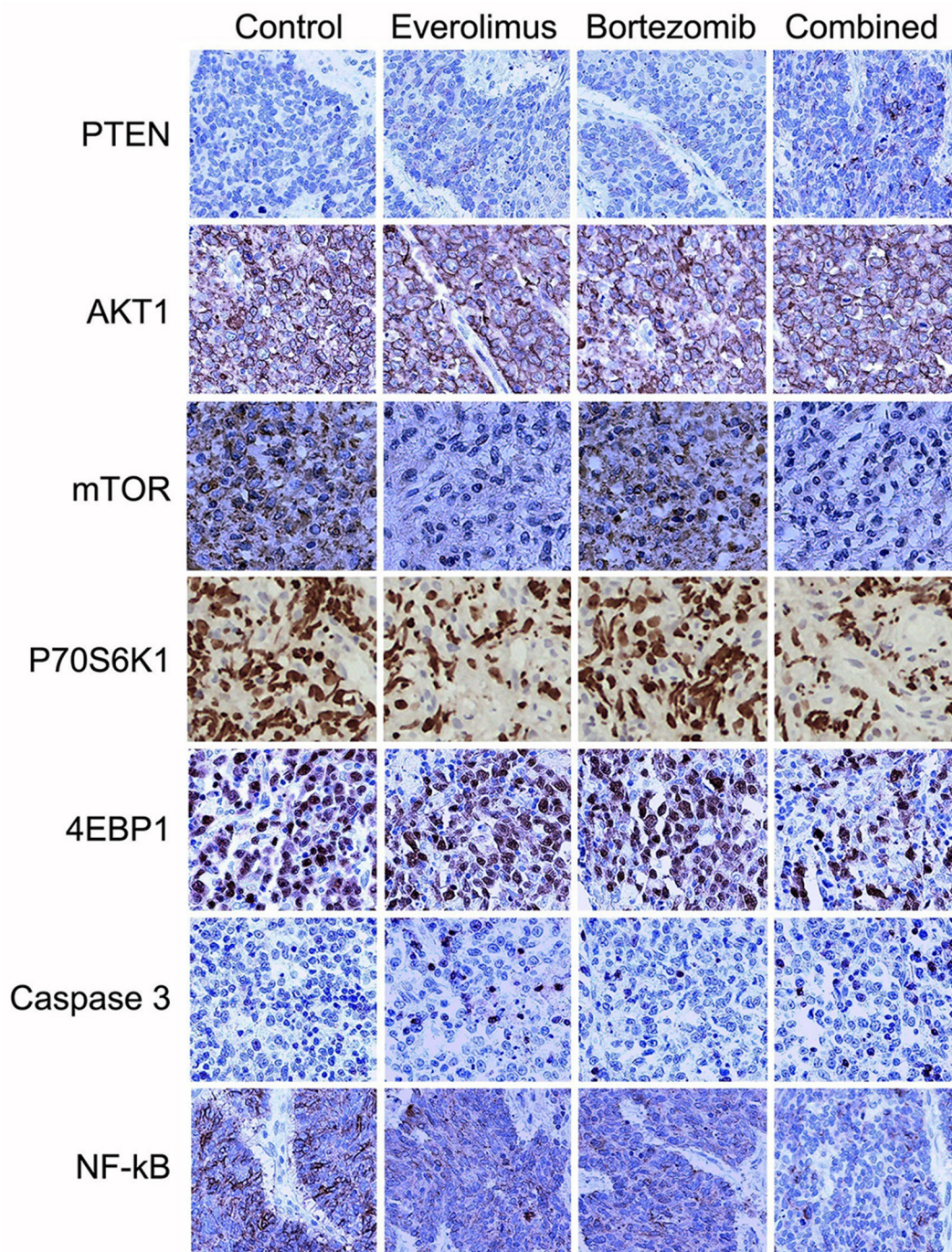


Figure 4 Results of immunohistochemical analysis of tumors in mice bearing multiple myeloma cells. mTOR, mammalian target of rapamycin.

prolonged.²⁹ Therefore, drugs can inhibit proliferation of MM cells through this mechanism.

In our study, the expression of P70S6K and 4EBP-1 was significantly downregulated when cells of either cell line were treated with everolimus alone or in combination with bortezomib. This result indicates that everolimus inhibits the AKT/mTOR pathway, while bortezomib had no effect on the AKT/mTOR pathway. Although many studies have confirmed the feedback activation effects of mTOR inhibitors on AKT1, we did not observe a

significant activation in the experiment in the U266 cell line. This might be dose-dependent, with a small dose of everolimus resulting in activation and a larger dose having an inhibitory effect.³⁰ Moreover, combination therapy with bortezomib and everolimus treatment slowed the tumor progression in vivo using the myeloma nude mice model, and the effect of everolimus alone was better than the effect of bortezomib. This observation is in accordance with the in vitro results. These results suggest that bortezomib should be further tested, studied

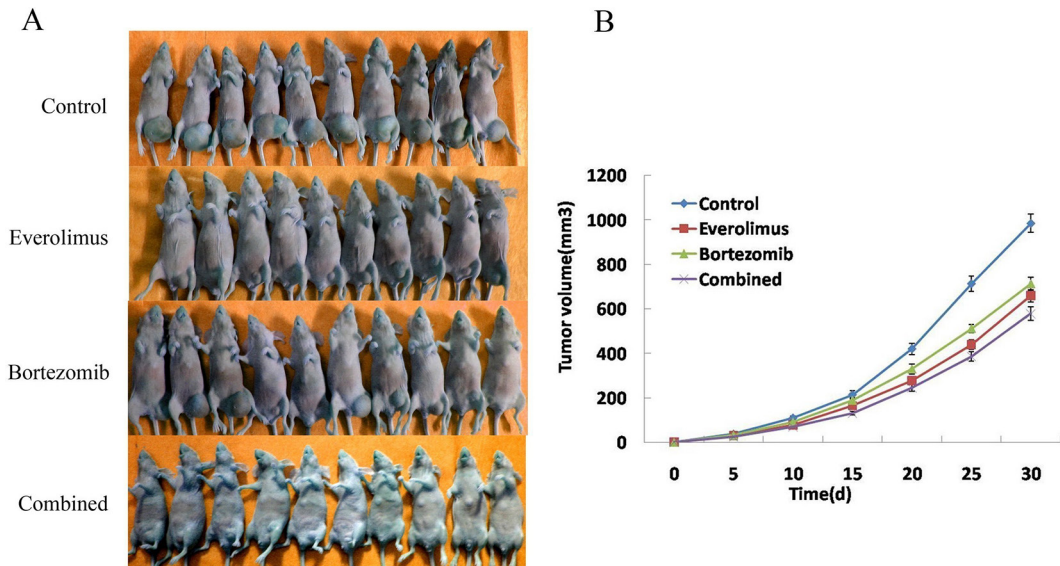


Figure 5 Everolimus and bortezomib treatment reduced myeloma cell growth in vivo. (A) Combination therapy with everolimus and bortezomib significantly delayed the growth of subcutaneous tumors in mice bearing U266 and LP-1 cells. (B) Tumor volume of myeloma xenografts in the different treatment groups.

and investigated in combination with everolimus to treat patients with MM.

In conclusion, we demonstrated that everolimus targeted the AKT/mTOR signal transduction pathway to inhibit proliferation and induce apoptosis in vitro and delayed the growth of human myeloma cells in vivo. The present work revealed that everolimus acted synergistically with bortezomib to treat MM.

Contributors ZL carried out the molecular genetic studies. YL and QS carried out the immunoassays. JC, JF and QJ participated in the cell culture and performed the statistical analysis. RF conceived of the study and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Competing interests None declared.

Patient consent Not required.

Ethics approval This article does not contain any studies with human participants performed by any of the authors. All of the experimental procedures on animals were carried out with strict adherence to the rules and guidelines for the ethical use of animals in research, according to the principles outlined in the Guide for Care and Use of Laboratory Animals (National Institutes of Health, Publication No 86–23, revised 1985), and are approved by the Tianjin Municipal Science and Technology Commission and the ethics committee of the Fifth Central Hospital of Tianjin (approval no: 2016012).

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