

Triptolide overcomes dexamethasone resistance and enhanced PS-341-induced apoptosis via PI3k/Akt/NF- κ B pathways in human multiple myeloma cells

MIN YANG, JIAN HUANG, HAN-ZHANG PAN and JIE JIN

Department of Hematology, Institute of Hematology, The First Affiliated Hospital, College of Medicine, Zhejiang University, Key Lab of Combined Multi-Organ Transplantation, Ministry of Public Health, 79# Qingchun Road, Hangzhou 310003, P.R. China

Received March 17, 2008; Accepted June 10, 2008

DOI: 10.3892/ijmm_00000047

Abstract. Human multiple myeloma is a presently incurable hematological malignancy and novel biologically based therapies are urgently needed. Triptolide (TPL) is a purified diterpenoid isolated from the Chinese herb, *Tripterygium wilfordii* Hook. F that has shown antitumor activities in various cancer cell types. But its activity in Dex-resistant multiple myeloma cell lines and the main upstream signaling pathway has not been reported. Here we show that TPL induces apoptosis in dexamethasone-sensitive (MM.1S) and dexamethasone-resistant (MM.1R) cells, most importantly its main upstream signaling pathway is through the PI3k/Akt/NF- κ B pathway and is also associated with MAPK pathway, via mitochondrial apoptotic signaling and is also associated with the caspase and Bcl-2 family members. Moreover, TPL was able to enhance the activities of dexamethasone or bortezomib/PS-341 in multiple myeloma cell lines. Collectively, these findings provide the framework for a clinical evaluation of TPL, either alone or in combination with dexamethasone or bortezomib/PS-341, to overcome drug resistance and improve outcome for patients with this universally fatal hematological malignancy.

Introduction

Multiple myeloma (MM) is a malignancy of the plasma cell characterized by migration and localization to the bone marrow where cells then disseminate and facilitate the formation of bone lesions. Although conventional cytotoxic chemotherapy prolongs survival in symptomatic patients, the

prognosis of treated patients with MM remains poor (1). Compared with more conventional cytotoxic agents (2-4), relapses still invariably occur, indicating the need for continued investigation of novel agents in this disease.

Triptolide (TPL) is a purified component extracted from *Tripterygium wilfordii* Hook. F (TWHF) that has been demonstrated to be effective in patients with a variety of inflammatory and autoimmune diseases. Recent studies showed that TPL possessed potential antitumor properties (5-9). In leukemic cells, TPL induces caspase-dependent cell death mediated via the mitochondrial pathway (10). In our department, previous research also reported it induced apoptosis of MM cell lines 8226 and U266 without cytotoxic in PBMCs from normal volunteers (11).

Therefore, the objectives of this study were first to investigate its activity of the overcoming of the Dex-resistant MM cell line, confirm its cytotoxic to the drug-resistant MM cells, as well as to investigate how the drug acts on many important pathways involved in the drug-resistance of MM, its main upstream pathway is through the PI3k/Akt/NF- κ B pathway with rapid downregulation of phospho-AKT (p-AKT).

Materials and methods

Cell culture and drugs. Dexamethasone (Dex)-sensitive (MM.1S) and Dex-resistant (MM.1R) human MM cell lines were kindly provided by Dr Steven Rosen (Northwestern University, Chicago, IL). These cell lines were cultured in RPMI-1640 (Gibco BRL, USA), 10% fetal bovine serum (FBS Gibco BRL), 50 μ g/ml streptomycin, 50 IU/ml penicillin and 2 mM glutamine in a 5% humidified CO₂ atmosphere at 37°C. BM aspirates were obtained from two patients with high tumor burden. Plasma cell enriched preparations were obtained by centrifugation of mononuclear cells on a Percoll gradient, as described previously (12). Purity of plasma cells (containing >80% of plasma cells) was confirmed by monitoring cell-surface expression of CD138. All studies were performed with the patients' informed consent. TPL was obtained from Sigma (USA).

Growth inhibition assay. *In vitro* growth inhibition effect of TPL on MM cells was determined by measuring MTT [3-

Correspondence to: Dr Jie Jin, Department of Hematology, Institute of Hematology, The First Affiliated Hospital, College of Medicine, Zhejiang University, Key Lab of Combined Multi-Organ Transplantation, Ministry of Public Health, 79# Qingchun Road, Hangzhou 310003, P.R. China
E-mail: jiejin@hzcnc.com

Key words: triptolide, MM.1R and MM.1S cell lines, apoptosis, PI3k/Akt/NF- κ B

(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye absorbance of living cells. Briefly, cells (1×10^5) per well were seeded in 96-well microtiter plates. After exposure to the drug (0, 2, 4, 8 and 16 ng/ml) for 48 h, 20 μ l MTT (Sigma) solution (5 mg/ml in PBS) was added to each well and the plates were incubated for an additional 4 h at 37°C. MTT solution in medium was aspirated off. To achieve solubilization of the formazan crystal formed in viable cells, 200 μ l DMSO was added to each well before absorbance at 570 nm measured the density of 100,000 per well for MM cell lines and 50,000 for PBMCs and primary myeloma cells. Each sample point was assayed with six replica points per assay and cell lines experiments were performed at least three times.

Annexin V staining. Briefly, cells were incubated without or with TPL for 12 h and then harvested, washed in PBS and counted. About 1×10^6 cells were collected and washed in phosphate-buffered saline and resuspended in 100 μ l binding buffer containing annexin V (BD PharMingen). Cells were analyzed by flow cytometry after the addition of propidium iodide (PI). Data acquisition and analysis were performed on a BD (Becton Dickinson) FACSCalibur using CellQuest software (BD Biosciences). Annexin V binds to those cells that express phosphatidylserine on the outer layer of the cell membrane and PI stains the cellular DNA of those cells with compromised cell membrane.

Mitochondrial membrane potential. To evaluate the effect of TPL on alternations of mitochondrial membrane potential ($\Delta\psi_m$), MM.1R, MM.1S cells were treated with or without TPL for 6 h, with the addition of Mitocapture reagent (MitoCapture Apoptosis Detection kit, Biovision) for the last 20 min, followed by flow cytometric analysis (Cytomics FC500, Becton Dickinson, Franklin Lakes, NJ). Viable cells had low fluorescence intensity (FL-1), whereas cells with loss of $\Delta\psi_m$ had high FL-1.

Nuclear and cytosolic fractionation. Cells (1×10^7) were incubated in 400 μ l lysis buffer [10 mM HEPES (N-2-ethanesulfonic acid), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, pH 7.9] with 0.2% Nonidet P-40 (NP-40) and protease inhibitor cocktail for 1 min on ice. After being microcentrifuged for 1 min at 2,500 \times g, the supernatants were collected as cytoplasmic protein extracts. The pellets were washed with lysis buffer without NP-40, then resuspended in 150 μ l extraction buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 0.5 mM DTT, 0.2 mM EDTA and 25% glycerol) and incubated for 20 min on ice. After centrifugation at 12,000 \times g for 10 min, the supernatants were collected as nuclear protein extracts.

Western blot analysis. Cells were lysed by 10 mM Tris, 1 mM EDTA, 10 mM KCl and 0.3% Triton, pH 7.9. Proteins were separated on 12% SDS-polyacrylamide gels and electrophoretically transferred to immobilon-P transfer membranes (Millipore, USA). Membranes were blocked with 5% skimmed milk, incubated with the primary antibodies at 4°C overnight in TBS-T (10 mM Tris HCl pH 8.0, 150 mM NaCl, 0.1% Tween-20), followed by 2 h incubation with peroxidase-conjugated secondary antibodies. Signals were detected using

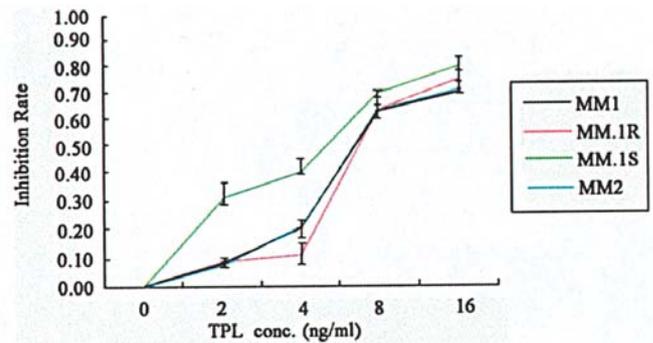


Figure 1. Effect of TPL on growth of Dex-resistant and Dex-sensitive MM cell lines and patient MM cells. Exponentially growing cell lines and fresh MM cells were treated with the indicated concentration of TPL for 48 h. Cell growth inhibition was assessed by MTT assay, as previously described. Values represent means (S.D.) of triplicate culture.

the ECL kit (Amersham, Little Chalfont, UK). Primary antibodies included caspase-3 (32 kDa, 19/17 kDa), poly (ADP-ribose) polymerase (PARP) (116/85 kDa), caspase-8 (57/43 kDa), caspase-9 (47, 37/35 kDa), total Akt, PI3Kp110 α , Ser 473 phosphorylated Akt antibodies (p-Akt) (60 kDa), CIAP1 (62 kDa), XIAP (53 kDa), NF- κ B (P65) (65 kDa). Caspase-9 inhibitor LEHD-FMK (BioVision, Mountain View, USA); Bad, Ser 136 phosphorylated Bad (p-Bad) (23 kDa), Bcl-2 (28 kDa), Bax (20 kDa), smac (23 kDa), total I κ B α and Ser 32 phosphorylated I κ B α B12TAM (40 kDa), Glucocorticoid Receptor (95 kDa) (Cell Signaling, Danvers, USA), Actin (42 kDa) and Lamin B (67 kDa) (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated secondary anti-mouse and anti-rabbit antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz). Bad (23 kDa), phospho-Bad (Ser136) (Cell Signaling). PI3K inhibitor: Wortmannin, NF- κ B inhibitor PDTC (Sigma), MEK1/2 inhibitor U0126 (Cell Signaling).

Statistical analysis. The significance of differences between experimental conditions was determined using the Student's t-test. The minimal level of significance was $P < 0.05$. Characterization of synergistic interactions was performed using the synergism quotient (SQ) (13). The SQ was defined as the net effect induced by the combination (Dex+TPL, PS-341+TPL) divided by the sum of the net individual effects of Dex+TPL, PS-341+TPL. A quotient > 1 indicated a synergistic effect, whereas a quotient < 1 indicated an antagonistic effect.

Results

Effect of TPL on the growth of dexamethasone (Dex)-sensitive (MM.1S) and Dex-resistant (MM.1R) human MM cell lines and the refractory cells of MM patients. We first found the effect of TPL on growth of dexamethasone (Dex)-resistant (MM.1R) and Dex-sensitive (MM.1S) human MM cell lines, and two cases, with MTT assay. Fig. 1 shows the dose-related effect of TPL in the 0-16 ng/ml range after 48 h of exposure. The TPL IC₅₀ for MM.1R cell lines was ~ 7.94 ng/ml and for MM.1S cell lines was ~ 4.54 ng/ml. Those Dex-resistant and sensitive cell lines were cultured in the presence

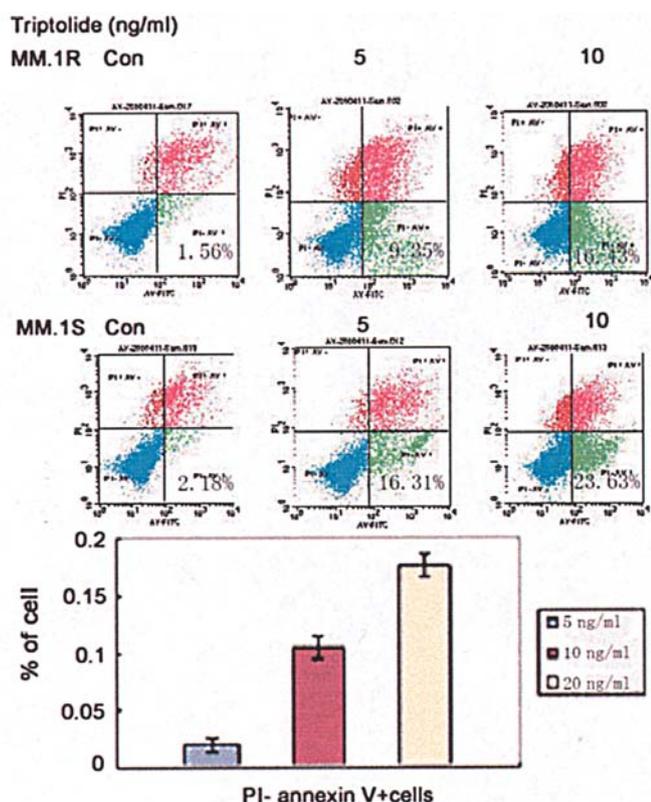


Figure 2. Apoptosis and non-apoptosis cell death induced by TPL. Flow cytometric analysis of TPL-induced cell death. After treatment with indicated concentrations of TPL (ng/ml) for 12 h, cells were collected and detected by the annexin V and propidium iodide (PI)-staining method. Results show a dose-dependent apoptosis defined as annexin V⁺ and PI⁻ group and non-apoptotic cell death defined as annexin V⁺ and PI⁺ population.

of different concentrations of TPL, a dose-dependent and time-dependent inhibition of cell growth was observed. TPL also induces dose-dependent cytotoxicity in tumor cells from two patients with relapsed multiple myeloma are refractory to conventional therapies, with IC₅₀ at 48 h of 7.70 and 7.78 ng/ml. In contrast, our former reported (11) TPL did not induce cytotoxicity in PBMCs from normal volunteers.

Triptolide induces apoptosis. In order to further characterize the cytotoxicity of TPL against Dex-resistant and Dex-sensitive MM cell lines, we performed *in vitro* the dual staining of cells with annexin V and PI analysed by flow cytometry to evaluate whether TPL treatment could induce apoptosis both in MM.1R and MM.1S cells. The results showed that TPL caused a dose-dependent increase of the frequency of cells with annexin V⁺ and PI⁻, which is characteristic of early apoptosis (Fig. 2). TPL induces apoptosis in Dex-resistant and Dex-sensitive cell lines, MM.1R and MM.1S cells.

TPL also induced caspase -8, -9 and -3 activation and cleavage of PARP. Caspases, especially caspase-3, participate in apoptosis. The activation of caspase-3 plays a key role in the induction of apoptosis by a variety of stimuli. As shown in Fig. 3a, we found dose-dependent activation of caspases-3, -8 and -9.

As seen in Fig. 3a, our data show that TPL induces caspase-9 activation in MM cells. Interestingly, TPL also

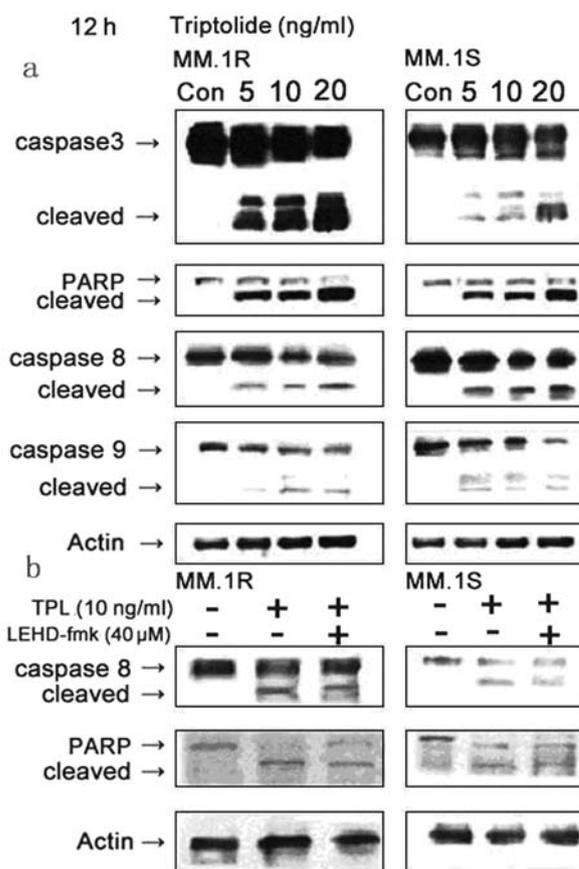


Figure 3. TPL-induced apoptosis in Dex-resistant and Dex-sensitive MM cells. (a) Western blot analysis of caspase-3 (32 kDa, 19/17 kDa), PARP (116/85 kDa), caspase-8 (57/43 kDa), caspase-9 (47 kDa, 37/35 kDa) and their cleaved fractions with Actin (42 kDa) as a loading control in MM.1R and MM.1S cells. (b) Pretreatment of MM.1R cells with 40 μ M caspase-9-specific inhibitor (LEHD-fmk) for 1 h before the treatment of 10 ng/ml TPL for 12 h. Only modest decreased effects on caspase-8 activity, along with similar decreases in both PARP cleavage.

triggers caspase-8 activation, which is consistent with a previous report (14). We therefore examined the mechanisms of how TPL triggered caspase-8 activation occur through a caspase-9-mediated signaling pathway. In Fig. 3b, pretreatment of MM.1R cells with a caspase-9-specific inhibitor (LEHD-fmk) showed only modest effects on caspase-8 activity, along with similar decreases in both PARP cleavage and viability. These results suggest that TPL induced caspase-8 activation is partly dependent on caspase-9 activation.

Triptolide-induced apoptosis involves Bcl-2 and IAP family in Dex-resistant and Dex-sensitive MM cell lines. We first examined whether treatment with TPL induced a loss in mitochondria transmembrane potential ($\Delta\psi_m$) as evidence of mitochondria-mediated apoptosis (15,16). As seen in Fig. 4a, TPL causes a decrease in $\Delta\psi_m$ in a dose-dependent manner. Since the loss of $\Delta\psi_m$ is associated with translocation of mitochondrial proteins, such as cytochrome c and Smac/Diablo, to the cytosol, we then examined whether translocation of Smac/Diablo to the cytosol is induced by TPL. As seen in Fig. 4b, treatment with TPL induces the release of Smac/Diablo to the cytosol. These results indicate that cytotoxicity

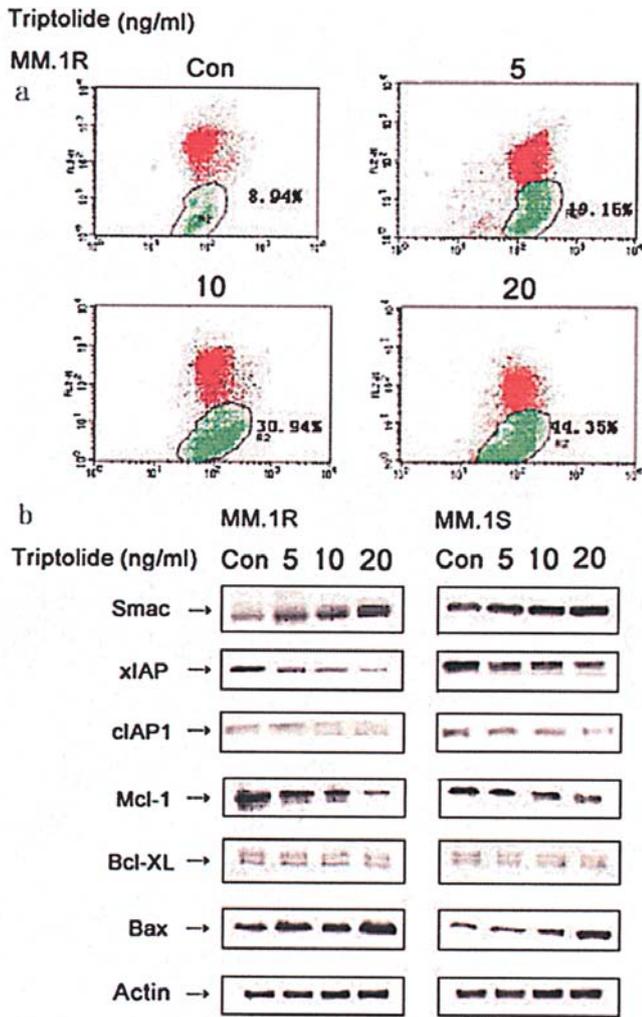


Figure 4. TPL-mediated apoptosis involves mitochondria. (a) Effects of TPL on mitochondrial membrane potential ($\Delta\psi_m$). MM.1R cells were treated with or without TPL (5, 10 and 20 ng/ml) for 6 h, with Mitocapture reagent added for the last 20 min, followed by analysis by flow cytometry. Values in percent indicate percentage of FL-1 high cells (green fraction). (b) MM.1R and MM.1S cells were cultured with TPL (5, 10 and 20 ng/ml) for 12 h, cytosolic extracts were obtained as described as the methods and through Western blotting using anti-Smac/Diablo antibodies (23 kDa). MM.1R and MM.1S cells were cultured with TPL (5, 10 and 20 ng/ml) for 12 h. Total cell lysates were analysed by Western blotting using anti-Smac (23 kDa), xiAP (53 kDa), cIAP1 (62 kDa), Mcl-1 (40 kDa), Bcl-XL (30 kDa), Bax (20 kDa) and Actin (42 kDa) antibodies.

triggered by TPL, as novel agents bortezomib (17), As2O3 (18) and 2-methoxyestradiol (19), is mediated via the activation of caspase-8, -3, and -9, alterations in mitochondrial membrane potential ($\Delta\psi_m$), release of mitochondria proteins and PARP cleavage. Because Bcl-2 family proteins regulate mitochondria-mediated apoptosis, we determined whether Bcl-2 family protein levels changed in Dex-resistant and Dex-sensitive multiple myeloma cells after treatment with TPL. As seen in Fig. 4b, antiapoptotic proteins Bcl-2, Bcl-xL and Mcl-1 proteins were decreased after 12 h of treatment with TPL; however, proapoptotic Bax was increased at 12 h.

We then examined the IAP family. The cIAP1 and XIAP protein levels in MM cell lines correlate with sensitivity to TPL. As seen in Fig. 4b, the cIAP1 and XIAP expression levels were down-regulated.

Triptolide prevented NF- κ B nuclear translocation and induced I κ B α cleaved in Dex-resistant and Dex-sensitive MM cell lines. The Bcl-2 and IAP families are known to be transcriptionally regulated by NF- κ B (20-23). Constitutive activation of the NF- κ B signal transduction pathway has been implicated in several hematological malignancies, including multiple myeloma (24,25). NF- κ B remains inactivated in the cytoplasm due to its complex formation with inhibitory protein I κ B α . Upon growth or survival stimulation, I κ B α is phosphorylated, ubiquitinated and degraded by proteasomes, leading to the disassociation of p50/65 and its translocation to the nucleus (26). Activated NF- κ B binds to the consensus sites present within the promoter region of various growth factors and thereby induces their transcription and secretion (24,27). To assess whether TPL influenced the status of NF- κ B, we conducted Western blot analysis to examine the NF- κ B (p65) nuclear translocation. The nuclear content of NF- κ B was decreased with its accumulation in cytoplasm after TPL treatment. To assess the effect of TPL on the nuclear NF- κ B, nuclear protein extracts were prepared from MM.1R and MM.1S cells. A decrease in P65 was seen in the nucleus of MM.1R and MM.1S cells treated with TPL for 24 h (Fig. 5).

We also detected that TPL induced I κ B α cleavage to a 40 kDa product (p-I κ B α), which may produce a super-repressive effect on NF- κ B by stably sequestering it within the cytosol.

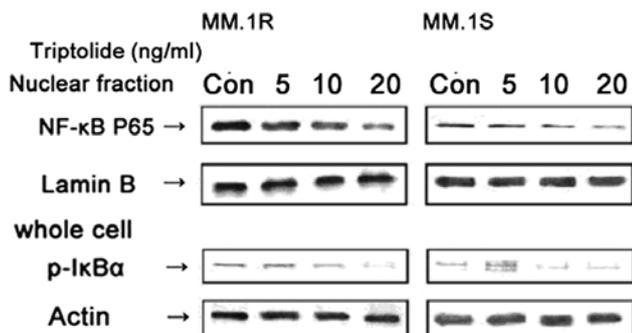


Figure 5. TPL induced the decrease of NF- κ B P65 in the nucleus and the cleavage of I κ B α . MM.1R and MM.1S cells were treated with 5, 10 and 20 ng/ml TPL for 24 h. Nuclear proteins were extracted and subjected to Western blotting for P65 detection. I κ B α proteins in whole cell lysates were determined with anti-phosphorylated form of I κ B α (p-I κ B α) (40 kDa).

Triptolide induces apoptosis in MM.1R and MM.1S cells mainly via PI3K/AKT pathway. We investigated the ability of TPL to inhibit the PI3K/AKT signal pathway including phosphorylation of PI3Kp110 α and Akt in MM cells. Akt directly phosphorylates several components of the cell death machinery (28). The detection of Ser473 p-Akt and total Akt in MM.1R and MM.1S cells was performed following exposure to TPL for 12 h. Fig. 6 shows that 20 ng/ml TPL inhibited Ser473 phosphorylation, with no change in total Akt at 12 h. Its downstream factors including p53, Bad (29) were all altered. We evaluated the change in the expression of the p53 and Bad level were upregulated at the same conditions.

In addition to the PI3K/Akt pathway, the mitogen-activated protein kinase (MAPK) pathways are also activated in MM, as previously reviewed (30,31). As seen in Fig. 6, we also detected a rapid decrease of ERK1/2 phosphorylation in Dex-

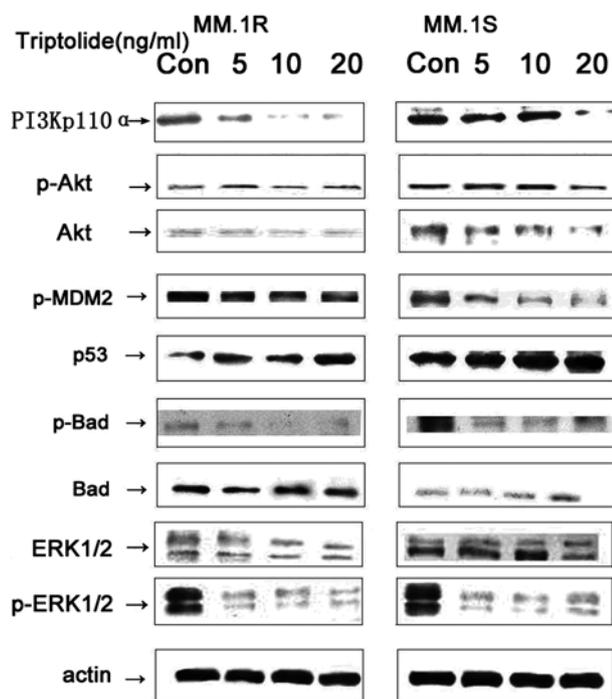


Figure 6. Change in expression of Akt, p-Akt and downstream effector, p-MDM2 (90 kDa), p53 (53 kDa), p-Bad (Ser136) (23 kDa) and TPL decreased ERK (44/42 kDa) phosphorylation in MM.1R and MM.1S cells. Representative Western blot analysis of PI3Kp110 α , p-Akt (Ser473 phosphorylation) and Akt (60 kDa) expression from cell lysates of MM.1R and MM.1S cells, which were exposed to 5, 10 and 20 ng/ml TPL for 12 h. TPL inhibited the Akt, MDM2 phosphorylation form and increased the expression of p53 and Bad. TPL inhibited the phosphorylation of ERK. MM.1R and MM.1S cells were exposed to 5, 10 and 20 ng/ml TPL for 12 h.

resistant and Dex-sensitive MM.1R and MM.1S cells treated by TPL for 12 h. Extracellular signal-regulated kinase 1/2 (ERK1/2) signaling cascade mediates human multiple myeloma (MM) growth and survival.

Since TPL induced apoptosis in MM.1R and MM.1S cells via multiple mechanisms including PI3K/AKT and MEK/MAPK signaling pathways and also associated with IAP and Bcl-2 family proteins. Therefore the mechanisms of mainly signal pathway seems most importantly.

As seen in Fig. 7, the effect of phosphorylation of AKT inhibited by TPL in MM.1R and MM.1S cells was intensified by Wortmannin pretreatment. Moreover, when combination TPL with either U0126 or Wortmannin, the level of ERK1/2 phosphorylation was inhibited similarly to each other. TPL showed analogously synergistic inhibition of phosphorylation of ERK1/2 in combination with either Wortmannin or U0126. While Wortmannin alone could not inhibit the phosphorylation of ERK1/2.

We further studied TPL induced alteration of these kinases in MM.1R and MM.1S cells by Western blotting with phospho-I κ B α , phospho-AKT Abs, with or without the NF- κ B inhibitor PDTC and/or Wortmannin. Importantly the effect of phosphorylation of AKT inhibited by TPL was enhanced by Wortmannin, but not by PDTC. Conversely, the degree of the phosphorylation of I κ B α , the inhibitory protein I κ B α active formation, similarly reduced by the combination TPL with

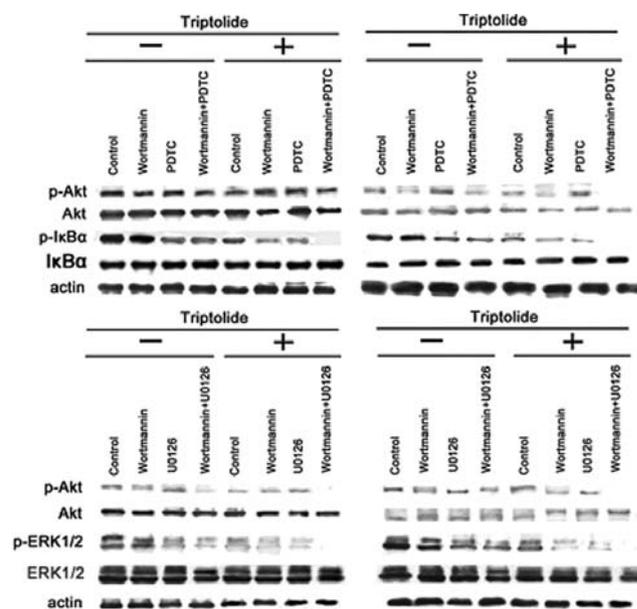


Figure 7. Effect of TPL inhibited PI3-K activation on MEK/MAPK and NF- κ B signaling pathway in MM.1R and MM.1S cells. (a) MM.1R cells were pretreated with Wortmannin (5 μ M, 30 min) and/or U0126 (5 μ M, 1 h), PDTC (50 μ M, 1 h) then with TPL (10 ng/ml, 12 h) and subjected to Western blotting using anti-phospho-Akt (Ser473), Akt, phospho-ERK1/2, phospho-I κ B α (p-I κ B α) Abs.

either Wortmannin or PDTC. Although Wortmannin alone did not inhibit the phosphorylation of I κ B α .

These results suggest cross talk between PI3K/AKT and MEK/MAPK, NF- κ B signaling pathways. PI3K/AKT may influence the MEK/MAPK and NF- κ B signaling pathways.

Up-regulation of glucocorticoid receptor (GR) protein contributes to overcoming drug resistance induced by Triptolide and Triptolide enhances Dex and PS-341 cytotoxicity against MM.1 cells. Although the GR protein is a cell membrane-associated receptor, it is primarily expressed intracellularly (32), while the expression of GR protein plays a key role in MM.1R cells. To elucidate whether this is one of the mechanisms of TPL overcoming dexamethasone resistance, we performed Western blot assay to evaluate the effect of TPL. The results showed that TPL induced a slight increase in the glucocorticoid-receptor protein expression in MM.1R and MM.1S cells (Fig. 8a).

We further examined whether TPL could enhance the growth inhibitory effect of Dex and PS-341. Dex-resistant and Dex-sensitive MM cell lines were cultured for 24 h with Dex (0.5 μ g/ml) or PS-341 (0.001-0.01 \times 10 $^{-6}$ M) in media with or without sub-IC $_{50}$ concentrations of TPL (1.25-5 ng/ml). As shown in Fig. 8b and c, TPL enhances growth inhibition mediated by Dex and PS-341, as analyzed by MTT assay. In our results after treatment with 0.5 μ g/ml dexamethasone the inhibition rates of MM.1R and MM.1S cells for 24 h were 1.19 and 7.55%, while combination with 2.5 ng/ml TPL the inhibition rates of MM.1R and MM.1S cells increased rapidly to 66.42 and 79.68% respectively. The 2.5 ng/ml TPL treated MM.1R and MM.1S cells in our previous data showed inhibition rates of only 2.79 and 3.96%, respectively. The

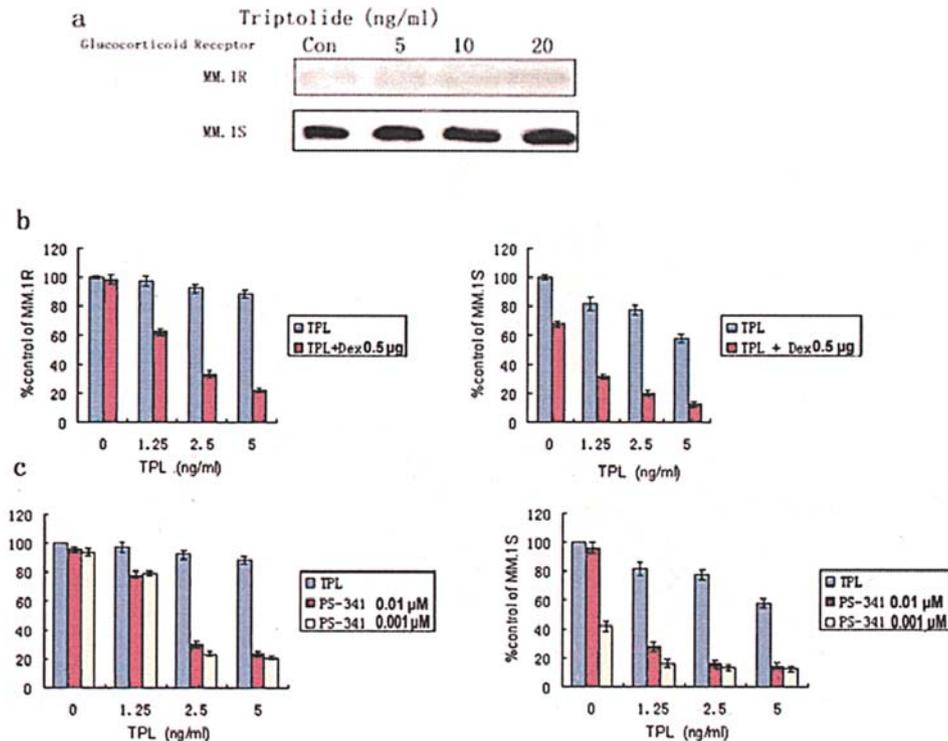


Figure 8. Effect of TPL induced the expression of glucocorticoid receptor. MM.1R and MM.1S cells were treated with 5, 10 and 20 ng/ml TPL for 24 h. Western blotting for glucocorticoid receptor proteins in whole cell lysates were determined with anti-phosphorylated form of the glucocorticoid receptor (95 kDa). (a) TPL sensitizes Dex-resistant and Dex-sensitive MM cells to dexamethasone (b) and protease inhibitor, PS-341 (c). MTT assay was performed for analysis of cell growth. Values are means of three experiments \pm S.D.

inhibition rates of MM.1R and MM.1S cell lines treated with 0.001 μ M PS-341 for 24 h were \sim 22.31 and 4.29%, respectively. Using combination of 0.001 μ M PS-341 with 2.5 ng/ml TPL, the inhibition rates were increased significantly to 69.72 and 84.31%, respectively ($P < 0.01$). Therefore, combined treatment of MM.1R and MM.1S cells with TPL and dexamethasone or PS-341 enhances the cytotoxicity compared with treatment with each drug alone.

Discussion

In this study, we have shown that TPL has a potent cytotoxic activity against Dex-resistant and Dex-sensitive multiple myeloma cell lines in a dose-dependent manner as well as primary MM cells and is likely to mediate cell death in drug-resistant MM cells by acting on multiple pathways mainly through the PI3K/AKT/NF- κ B signaling pathway. Previous studies have reported that TPL could inhibit the growth of leukemias cells (10). We evaluated its activity on dexamethasone-resistant cell lines to elucidate the main signal pathway.

Herein, we extensively investigated the mechanisms of TPL-induced cell growth inhibition in dexamethasone-resistant and sensitive multiple myeloma cell lines. We first demonstrated that TPL could induce apoptosis in MM.1R and MM.1S cell lines as well as freshly isolated from tumor cells from multiple myeloma patients who did not response to conventional agents. TPL acts through multiple signal pathways. It downregulated the expression of phospho-Akt and its downstream targets, such as MDM2 and caspase-9 and increased in the levels of p53 and phospho-Bad. TPL also

inhibited NF- κ B translocation and phospho-ERK1/2 expression. Furthermore, TPL modulated the alteration of proteins of the IAP and Bcl-2 families, activated caspases in the extrinsic and mitochondrial pathways.

Our data showed that blockade of Akt signaling by the PI3K inhibitor Wortmannin (5 μ M) resulted in the inhibition of Akt phosphorylation in MM.1R and MM.1S cells. Of note, when TPL was combined with Wortmannin, the ability of TPL to induce apoptosis of MM.1R and MM.1S cells was potentiated and the phosphorylation of Akt was significantly downregulated, which indicated that the inactivation of PI3K/Akt survival pathway played an important role in TPL-induced MM.1 cell death. Wortmannin enhanced the effect of TPL inhibiting the phosphorylation of ERK1/2 in MM.1R and MM.1S cells, which was similar to TPL combination with U0126. Consistent with this observation is in the alteration of the phosphorylation of I κ B α . TPL combination with Wortmannin inhibited the level of phosphorylation of I κ B α , which was similar to TPL combination with PDTC. However, TPL combination with U0126 and PDTC did not affect the level of phosphorylation of AKT more than the effect of TPL alone. Therefore, these data suggest cross talk between PI3K/AKT, MEK/MAPK, and NF- κ B signaling pathways, the PI3K/AKT pathway being the main one.

PI3K/AKT signal pathway maybe the main pathway resulting in significant cell death. The phosphoinositide-3-kinase (PI3K)/Akt signaling pathway is important for the survival and growth of MM cells, as well as mediating MM cell resistance to conventional therapeutics (33,34). Activation of Akt phosphorylates and inactivates several downstream targets, including the Bcl-2 family member BAD (34) and

p53, and caspase-9 (24), which promote cell survival. PI3K/AKT pathway is known to be constitutively activated in many primary myeloma cells and cell lines (35) and has been shown to play a key role in the survival and proliferation of MM cells in response to interleukin-6 (IL-6) and insulin-like growth factor-1 (IGF-1) (34,36). The PI3K/AKT pathway could be a target in the treatment of MM (37).

Triptolide modulated the expression of antiapoptotic proteins of the Bcl-2 family and IAP family, increased expression of the XIAP, cIAP1 and decreased expression of the Smac, which were correlated with poor outcome and chemotherapy-induced drug resistance in MM (38). Many of the two family members are located downstream of Akt and NF- κ B. Dan *et al* reported that IAP family are physiological substrates of Akt that are stabilized to inhibit programmed cell death. IAPs have direct effects on caspase-9 and -3 (39). One of the Bcl-2 family members, Bcl-xL is also known to promote cell survival and a report suggests that expression of this protein in MM may reflect disease severity and serve as an indicator of patient chemoresistance (40). Mcl-1 is also an antiapoptotic member of the Bcl-2 family; decreased Mcl-1 by caspases counteracts the function of residual intact Mcl-1 (41). While Derenne *et al* reported that Bcl-2 or Mcl-1, both Bcl-2 family members, down-regulation sensitizes myeloma cells to Dex-induced apoptosis. Mcl-1 down-regulation induces rapid and strong apoptosis of human myeloma cells (42). Therefore, our data also showed that TPL inhibited the expression levels of the Mcl-1, Bcl-2 and Bcl-xL in a dose-dependent manner. Thus, we found that TPL was cytotoxic to Dex-resistant and Dex-sensitive MM cell lines associated with the related protein change, down-regulation of the expression of XIAP, and cIAP1a decrease of the expression of the Smac/DIABLO. Therefore, this is one of the mechanisms of TPL's potent cytotoxic activity against MM.1R and MM.1S cells.

We found that TPL suppressed constitutive NF- κ B activation and influenced its downstream target proteins in two MM cell lines. Dong *et al* demonstrated that bcl-xL plays a key role in the control of apoptosis and transcription of bcl-xL is induced by constitutive RelA/NF- κ B activity in human pancreatic cancer cells (43). Rena *et al* (44) reported that NF- κ B activity is an early event in Dex-induced apoptosis. Our data showed that NF- κ B was a downstream target of PI3K/AKT pathway in TPL-induced apoptosis in MM.1 cells. Therefore, we propose that PI3K/AKT could have a central role in the TPL effect on the dexamethasone-resistant and sensitive multiple myeloma cell lines. We also found that TPL increased the expression of glucocorticoid receptor protein. This is probably one of the mechanisms by which TPL overcomes dexamethasone resistance. TPL was able to induce apoptosis either in MM.1R or MM.1S cells.

However, as the survival and proliferation of MM depend on multiple pathways, such pleiotropic effects of TPL likely contribute to its efficacy and potential for synergy with many other agents, such as Dex and PS-341. Our data provide a rationale for clinical investigation of TPL in refractory MM to evaluate whether TPL, alone and combined with conventional and novel therapies, could improve patient outcome in multiple myeloma.

Acknowledgements

This study was supported by the National Natural Science Foundation of P.R. China (no. 30572154).

References

- Alexanian R, Barlogie B and Tucker S: VAD-based regimens as primary treatment for multiple myeloma. *Am J Hematol* 33: 86-89, 1990.
- Rajkumar SV, Blood E, Vesole D, Fonseca R and Greipp PR: Phase III clinical trial of thalidomide plus dexamethasone compared with dexamethasone alone in newly diagnosed multiple myeloma: a clinical trial coordinated by the Eastern Cooperative Oncology Group. *J Clin Oncol* 24: 431-436, 2006.
- Richardson PG, Blood E, Mitsiades CS, *et al*: A randomized phase 2 study of lenalidomide therapy for patients with relapsed or relapsed and refractory multiple myeloma. *Blood* 108: 3458-3464, 2006.
- Richardson PG, Sonneveld P, Schuster MW, *et al*: Bortezomib or high-dose dexamethasone for relapsed multiple myeloma. *N Engl J Med* 352: 2487-2498, 2005.
- Shamon LA, Pezzuto JM, Graves JM, *et al*: Evaluation of the mutagenic, cytotoxic, and antitumor potential of triptolide, a highly oxygenated diterpene isolated from *Tripterygium wilfordii*. *Cancer Lett* 112: 113-117, 1997.
- Chen BJ: Triptolide, a novel immunosuppressive and anti-inflammatory agent purified from a Chinese herb *Tripterygium wilfordii* Hook F. *Leuk Lymphoma* 42: 253-265, 2001.
- Yang S, Chen J, Guo Z, *et al*: Triptolide inhibits the growth and metastasis of solid tumors. *Mol Cancer Ther* 2: 65-72, 2003.
- Kiviharju TM, Lecane PS, Sellers RG and Peehl DM: Antiproliferative and proapoptotic activities of triptolide (PG490), a natural product entering clinical trials, on primary cultures of human prostatic epithelial cells. *Clin Cancer Res* 8: 2666-2674, 2002.
- Yang Y, Liu Z, Tolosa E, Yang J and Li L: Triptolide induces apoptotic death of T lymphocyte. *Immunopharmacology* 40: 139-149, 1998.
- Carter BZ, Mak DH, Schober WD, *et al*: Triptolide induces caspase-dependent cell death mediated via the mitochondrial pathway. *Blood* 108: 630-637, 2006.
- Yinjun L, Jie J and Yungui W: Triptolide inhibits transcription factor NF- κ B and induces apoptosis of multiple myeloma cells. *Leuk Res* 29: 99-105, 2005.
- Iwato K, Kawano M, Asaoku H, Tanabe O, Tanaka H and Kuramoto A: Separation of human myeloma cells from bone marrow aspirates in multiple myeloma and their proliferation and M-protein secretion in vitro. *Blood* 72: 562-526, 1988.
- Tassone P, Neri P, Burger R, *et al*: Combination therapy with interleukin-6 receptor superantagonist Sant7 and dexamethasone induces antitumor effects in a novel SCID-hu in vivo model of human multiple myeloma. *Clin Cancer Res* 11: 4251-4258, 2005.
- Deng Y, Ren X, Yang L, Lin Y and Wu X: A JNK-dependent pathway is required for TNF α -induced apoptosis. *Cell* 115: 61-70, 2003.
- Chauhan D, Hideshima T, Rosen S, *et al*: Apaf-1/cytochrome c-independent and Smac-dependent induction of apoptosis in multiple myeloma (MM) cells. *J Biol Chem* 276: 24453-24456, 2001.
- Chauhan D, Li G, Podar K, *et al*: Targeting mitochondria to overcome conventional and bortezomib/proteasome inhibitor PS-341 resistance in multiple myeloma (MM) cells. *Blood* 104: 2458-2466, 2004.
- Mitsiades N, Mitsiades CS, Poulaki V, *et al*: Molecular sequelae of proteasome inhibition in human multiple myeloma cells. *Proc Natl Acad Sci USA* 99: 14374-14379, 2002.
- Hayashi T, Hideshima T, Akiyama M, *et al*: Arsenic trioxide inhibits growth of human multiple myeloma cells in the bone marrow microenvironment. *Mol Cancer Ther* 1: 851-860, 2002.
- Chauhan D, Catley L, Hideshima T, *et al*: 2-Methoxyestradiol overcomes drug resistance in multiple myeloma cells. *Blood* 100: 2187-2194, 2002.
- Sun Y: E3 ubiquitin ligases as cancer targets and biomarkers. *Neoplasia* 8: 645-654, 2006.
- Lee R and Collins T: Nuclear factor- κ B and cell survival: IAPs call for support. *Circ Res* 88: 262-264, 2001.

22. Stehlik C, de Martin R, Kumabashiri I, Schmid JA, Binder BR and Lipp J: Nuclear factor (NF)-kappaB-regulated X-chromosome-linked iap gene expression protects endothelial cells from tumor necrosis factor alpha-induced apoptosis. *J Exp Med* 188: 211-216, 1998.
23. Catz SD and Johnson JL: Transcriptional regulation of bcl-2 by nuclear factor kappa B and its significance in prostate cancer. *Oncogene* 20: 7342-7351, 2001.
24. Chauhan D, Uchiyama H, Akbarali Y, Urashima M, Yamamoto K, Libermann TA and Anderson KC: Multiple myeloma cell adhesion-induced interleukin-6 expression in bone marrow stromal cells involves activation of NF-kappa B. *Blood* 87: 1104-1112, 1996.
25. Gilmore T, Gapuzan ME, Kalaitzidis D and Starczynowski D: Rel/NF-kappa B/I kappa B signal transduction in the generation and treatment of human cancer. *Cancer Lett* 181: 1-9, 2002.
26. Alkalay I, Yaron A, Hatzubai A, *et al*: In vivo stimulation of I kappa B phosphorylation is not sufficient to activate NF-kappa B. *Mol Cell Biol* 15: 1294-1301, 1995.
27. Karin M, Yamamoto Y and Wang QM: The IKK NF- κ B system: a treasure trove for drug development. *Nat Rev Drug Discov* 3: 17-26, 2004.
28. Brunet A, Bonni A, Zigmond MJ, Lin MZ, *et al*: Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* 96: 857-868, 1999.
29. Zhou BP, Liao Y, Xia W, Zou Y, Spohn B and Hung MC: HER-2/neu induces p53 ubiquitination via Akt mediated MDM2 phosphorylation. *Nat Cell Biol* 3: 973-982, 2001.
30. Lonial S, Waller EK, Simons JW, *et al*: Signal transduction and myeloma: new targets, new hope. *Cancer Biol Ther* 2: 310-319, 2003.
31. Mitsiades CS, Mitsiades N, Munshi NC, *et al*: Focus on multiple myeloma. *Cancer Cell* 6: 439-444, 2004.
32. Sánchez-Vega B, Krett N, Rosen ST and Gandhi V: Glucocorticoid receptor transcriptional isoforms and resistance in multiple myeloma cells. *Mol Cancer Ther* 5: 3062-3070, 2006.
33. Hideshima T, Nakamura N, Chauhan D and Anderson KC: Biologic sequelae of interleukin-6 induced PI3-K/Akt signaling in multiple myeloma. *Oncogene* 20: 5991-6000, 2001.
34. Hsu J, Shi Y, Krajewski S, *et al*: The AKT kinase is activated in multiple myeloma tumor cells. *Blood* 98: 2853-2855, 2001.
35. Pene F, Claessens YE, Muller O, *et al*: Role of the phosphatidylinositol 3-kinase/Akt and mTOR/P70S6-kinase pathways in the proliferation and apoptosis in multiple myeloma. *Oncogene* 21: 6587-6597, 2002.
36. Mitsiades N, Mitsiades CS, Poulaki V, *et al*: Biologic sequelae of nuclear factor-kappaB blockade in multiple myeloma: therapeutic applications. *Blood* 99: 4079-4086, 2002.
37. Harvey RD and Lonial S: PI3 kinase/AKT pathway as a therapeutic target in multiple myeloma (Review). *Future Oncol* 3: 639-647, 2007.
38. Nakagawa Y, Abe S, Kurata M, Hasegawa M, *et al*: IAP family protein expression correlates with poor outcome of multiple myeloma patients in association with chemotherapy-induced overexpression of multidrug resistance genes. *Am J Hematol* 81: 824-831, 2006.
39. Dan HC, Sun M, Kaneko S, *et al*: Akt phosphorylation and stabilization of X-linked inhibitor of apoptosis protein (XIAP). *J Biol Chem* 279: 5405-5412, 2004.
40. Tu Y, Renner S, Xu F, *et al*: BCL-X expression in multiple myeloma: possible indicator of chemoresistance. *Cancer Res* 58: 256-262, 1998.
41. Herrant M, Jacquet A, Marchetti S, Belhacène N, Colosetti P, Luciano F and Auberger P: Cleavage of Mcl-1 by caspases impaired its ability to counteract Bim-induced apoptosis. *Oncogene* 23: 7863-7873, 2004.
42. Derenne S, Monia B, Dean NM, *et al*: Antisense strategy shows that Mcl-1 rather than Bcl-2 or Bcl-x(L) is an essential survival protein of human myeloma cells. *Blood* 100: 194-199, 2002.
43. Dong QG, Sclabas GM, Fujioka S, *et al*: The function of multiple I kappa B: NF-kappaB complexes in the resistance of cancer cells to Taxol-induced apoptosis. *Oncogene* 21: 6510-6519, 2002.
44. Feinman R, Koury J, Thames M, Barlogie B, Epstein J and Siegel DS: Role of NF-kappaB in the rescue of multiple myeloma cells from glucocorticoid-induced apoptosis by bcl-2. *Blood* 93: 3044-3052, 1999.