SPANDIDOS PUBLICATIONS

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

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Abstract. Human multiple myeloma is a presently incurable hematological malignancy and novel biologically based therapies are urgently needed. Triptolide (TPL) is a purified diterpenod 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-κB 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 universially 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-κB 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 μg/ml streptomycin, 50 IU/ml penicillin and 2 mM glutamine in a 5% humidified CO_2 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 preciously (12). Purity of plasma cells (containing >80% of plasma cells) was confirmed by mornitoring 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-

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(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye absorbance of living cells. Briefly, cells ($1x10^5$) 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 μl 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 μl 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 $1x10^6$ cells were collected and washed in phosphate-buffered saline and resuspended in 100 μl 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 Mitocupture reagent (MitoCupture 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 ($1x10^7$) were incubated in 400 μl 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 x g, the supernatants were collected as cytoplasmic protein extracts. The pellets were washed with lysis buffer without NP-40, then resuspended in 150 μl 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 x 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 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), totle Akt, PI3Kp110α, Ser 473 phosphorylated Akt antibodies (p-Akt) (60 kDa), CIAP1 (62 kDa), XIAP (53 kDa), NF-κB (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 IκBα and Ser 32 phosphorylated IκBα 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 inihibitor: Wortmannin, NF-κB inhibitor PDTC (Sigma), MEK1/2 inhibitor U0126 (Cell Signaling).

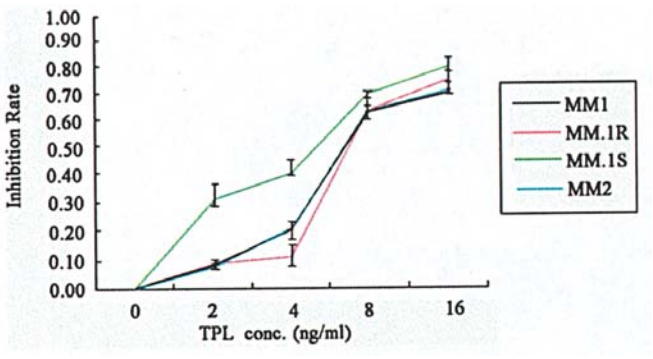

Figure 1. Effect of TPL on growth of Dex-resistant and Dex-sensitive MM cell lines and patient MM cells. Exponentially growing cell lines and fresh MM cells were treated with the indicated concentration of TPL for 48 h. Cell growth inhibition was assessed by MTT assay, as previously described. Values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

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 IC_{50} 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

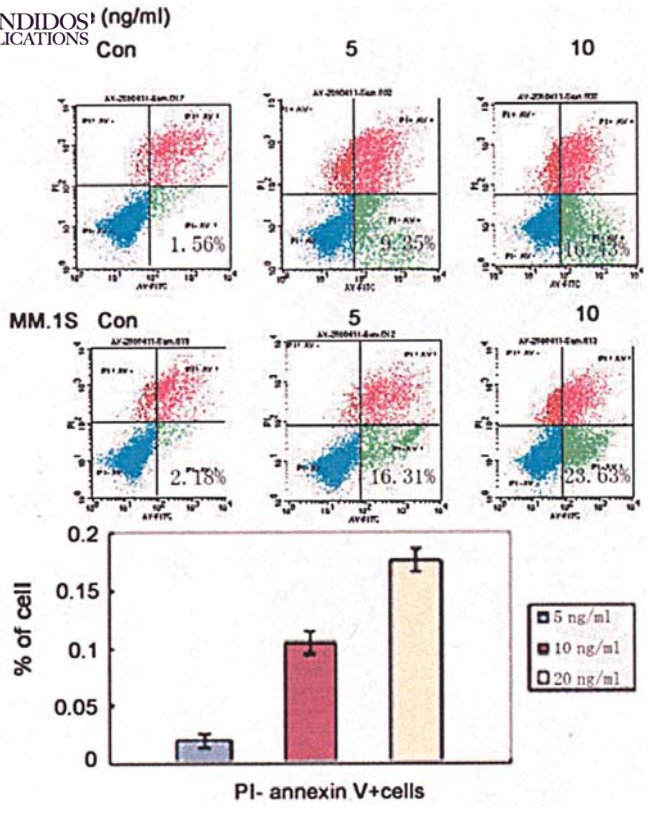

Figure 2. Apoptosis and non-apoptosis cell death induced by TPL. Flow cytometric analysis of TPL-induced cell death. After treatment with indicated concentrations of TPL (ng/ml) for 12 h, cells were collected and detected by the annexin V and propidium iodide (PI)-staining method. Results show a dose-dependent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

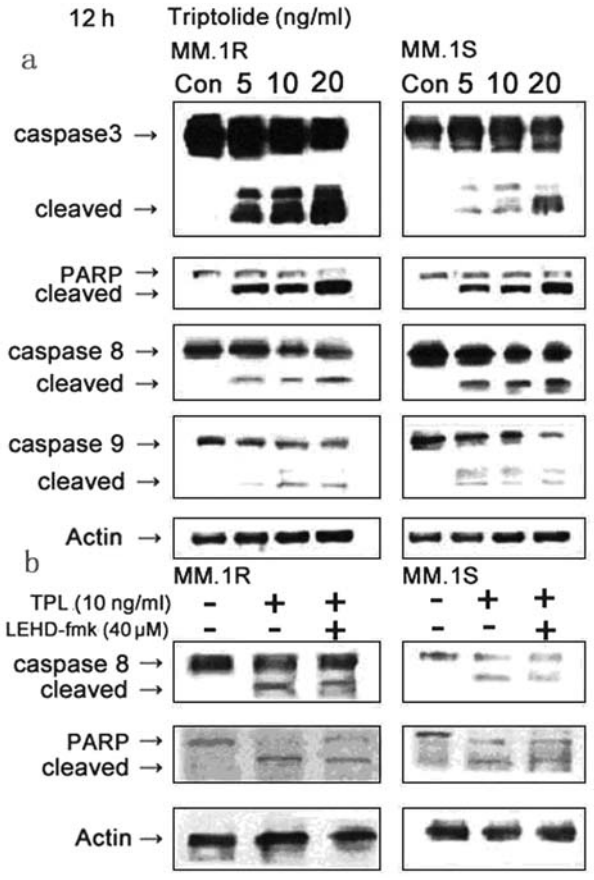

Figure 3. TPL-induced apoptosis in Dex-resistant and Dex-sensitive MM cells. (a) Western blot analysis of caspase-3 (32 kDa, 19/17 kDa), PARP (116/85 kDa), caspase-8 (57/43 kDa), caspase-9 (47 kDa, 37/35 kDa) and their cleaved fractions with Actin (42 kDa) as a loading control in MM.1R and MM.1S cells. (b) Pretreatment of MM.1R cells with 40 μM 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.

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 IC_{50} 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 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

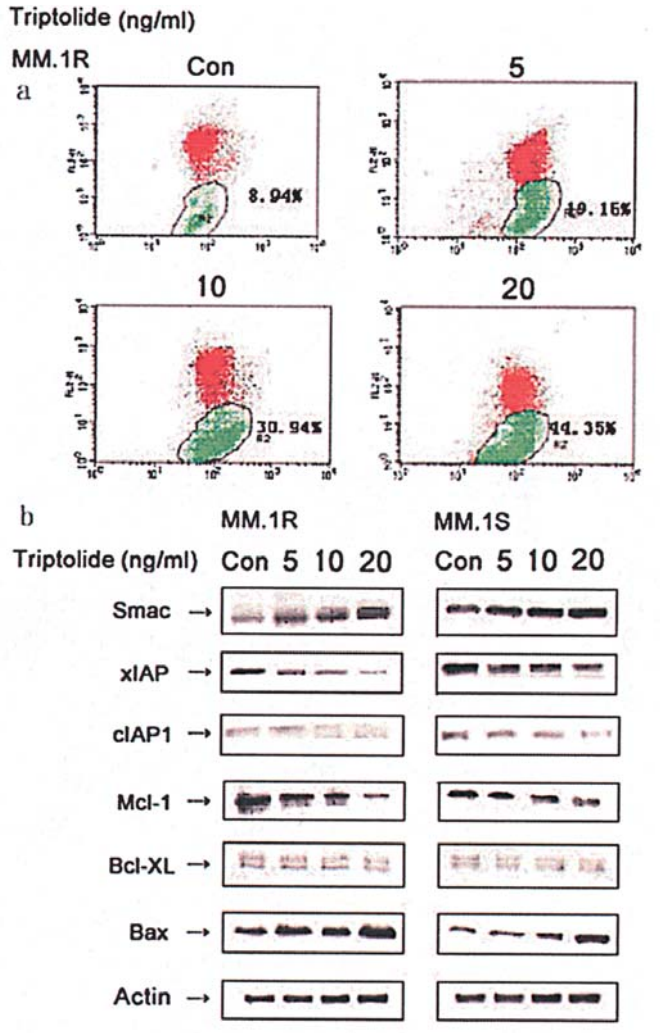

Figure 4. TPL-mediated apoptosis involves mitochondria. (a) Effects of TPL on mitochondrial membrane potential (Δψm). MM.1R cells were treated with or without TPL (5, 10 and 20 ng/ml) for 6 h, with Mitocupture reagent added for the last 20 min, followed by analysis by flow cytometry. Values in percent 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.

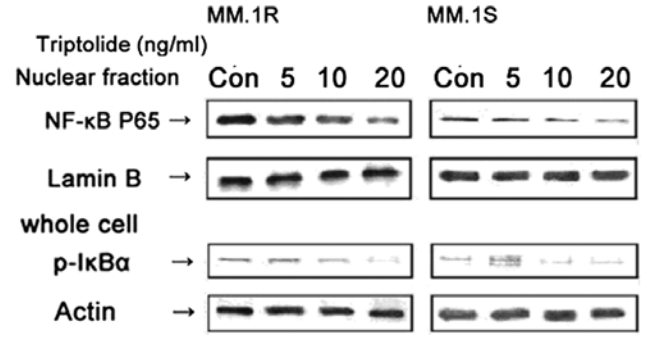

Figure 5. TPL induced the decrease of NF-κB P65 in the nucleus and the cleavage of IκBα. MM.1R and MM.1S cells were treated with 5, 10 and 20 ng/ml TPL for 24 h. Nuclear proteins were extracted and subjected to Western blotting for P65 detection IκBα proteins in whole cell lysates were determined with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

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 (Δψm), 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-κB nuclear translocation and induced IκBα cleavged in Dex-resistant and Dex-sensitive MM cell lines. The Bcl-2 and IAP families are known to be transcriptionally regulated by NF-κB (20-23). Constitutive activation of the NF-κB signal transduction pathway has been implicated in several hematological malignancies, including multiple myeloma (24,25). NF-κB remains inactivated in the cytoplasm due to its complex formation with inhibitory protein IκBα. Upon growth or survival stimulation, IκBα is phosphorylated, ubiquitinated and degraded by proteasomes, leading to the disassociation of p50/65 and its translocation to the nucleus (26). Activated NF-κB 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-κB, we conducted Western blot analysis to examine the NF-κB (p65) nuclear translocation. The nuclear content of NF-κB was decreased with its accumulation in cytoplasm after TPL treatment. To assess the effect of TPL on the nuclear NF-κB, 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 IκBα cleavage to a 40 kDa product (p-IκBα), which may produce a super-repressive effect on NF-κB by stably sequestering it within the cytosol.

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 PI3Kp110α 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-

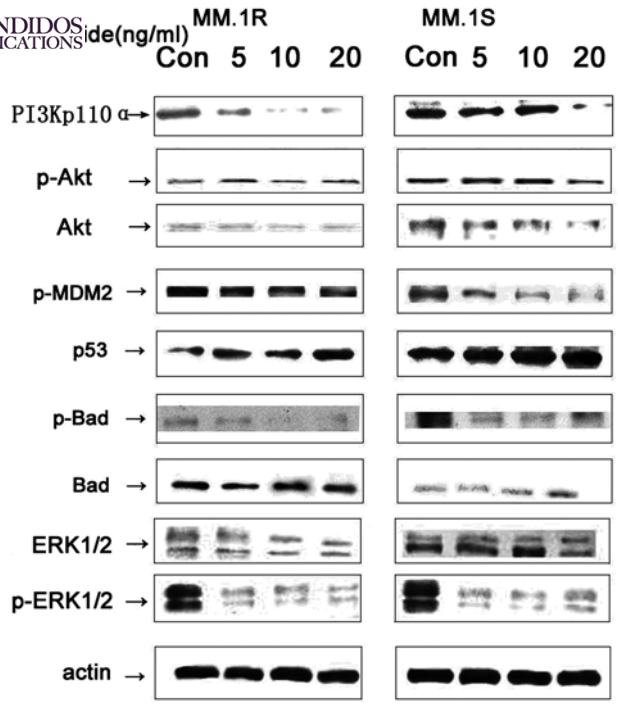

Figure 6. Change in expression of Akt, p-Akt and downstream effector, p-MDM2 (90 kDa), p53 (53 kDa), p-Bad (Ser136) (23 kDa) and TPL decreased ERK (44/42 kDa) phosphorylation in MM.1R and MM.1S cells. Representative Western blot analysis of PI3Kp110α, p-Akt (Ser473 phosphorylation) and Akt (60 kDa) expression 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.

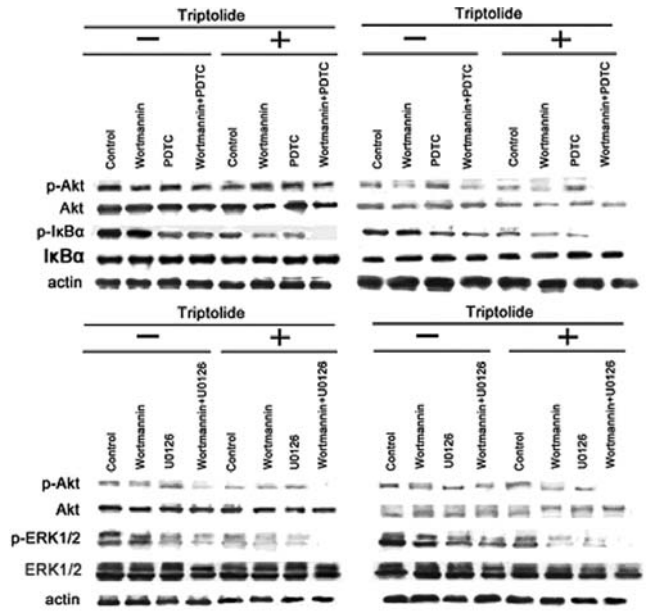

Figure 7. Effect of TPL inhibited PI3-K activation on MEK/MAPK and NF-κB signaling pathway in MM.1R and MM.1S cells. (a) MM.1R cells were pretreated with Wortmannin (5 μM, 30 min) and/or U0126 (5 μM, 1 h), PDTC (50 μM, 1 h) then with TPL (10 ng/ml, 12 h) and subjected to Western blotting using anti-phospho-Akt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

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-IκBα, phospho-AKT Abs, with or without the NF-κB 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 IκBα, the inhibitory protein IκBα active formation, similarly reduced by the combination TPL with either Wortmannin or PDTC. Although Wortmannin alone did not inhibit the phosphorylation of IκBα.

These results suggest cross talk between PI3K/AKT and MEK/MAPK, NF-κB signaling pathways. PI3K/AKT may influence the MEK/MAPK and NF-κB 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 μg/ml) or PS-341 (0.001-0.01x10^{-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 μg/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

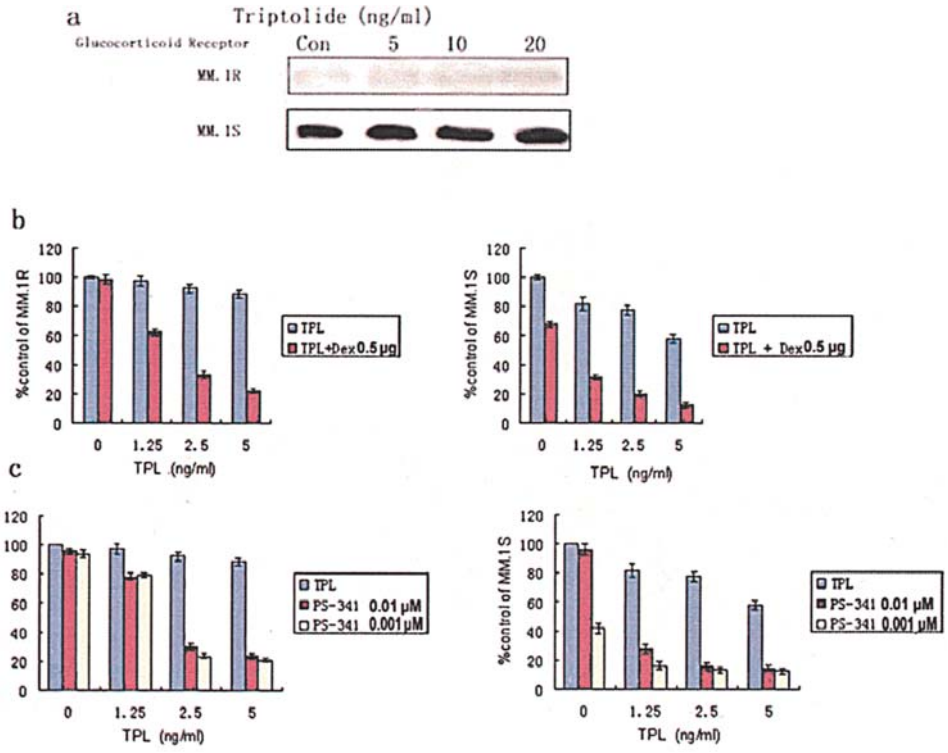

Figure 8. Effect of TPL induced the expression of glucocorticoid receptor. MM.1R and MM.1S cells were treated with 5, 10 and 20 ng/ml TPL for 24 h. Western blotting for glucocorticoid receptor proteins in whole cell lysates were determined with anti-phosphorylated form of the glucocorticoid receptor (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 ±S.D.

inhibition rates of MM.1R and MM.1S cell lines treated with 0.001 μM PS-341 for 24 h were ~22.31 and 4.29%, respectively. Using combination of 0.001 μM 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-κB 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-κB 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 μM) resulted in the inhibition of Akt phosphorylation in MM.1R and MM.1S cells. Of note, when TPL was combined with Wortmannin, the ablity 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 IκBα. TPL combination with Wortamannin inhibited the level of phosphorylation of IκBα, 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-κB 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

 caspase-9 (24), which promote cell survival. T 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 insuline-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-κB. 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 agaist MM.1R and MM.1S cells.

We found that TPL suppressed constitutive NF-κB 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-κB activity in human pancreatic cancer cells (43). Rena *et al* (44) reported that NF-κB activity is an early event in Dex-induced apoptosis. Our data showed that NF-κB 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.

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