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Full Length Research Paper

Inhibition of HSP90 by triptolide (TPL) augments Bortezomib-induced U266 cells apoptosis

Dan Shen[1], Xiangmin Tong[1], Junyu Zhang[2], Panhan Zhang[1], Jie Jin[1], Xiangli Gao[1] and Chengjian Ma[1]*

[1]The First Affiliated Hospital, College of Medicine, Zhejiang University, China.
[2]Lishui Central Hospital, Lishui, China.

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HSP90 has high expression in myeloma cells and very important for prognosis, and that triptolide (TPL), the main active component of the traditional chinese herbal medicine *Tripterygium wilfordii* Hook F (Celastraceae) has the inhibition effects in the growth of myeloma cells. In this study, we test the ability of TPL to induce apoptosis in U266 human myeloma cells by reducing HSP90 expression, as well as the effect of TPL on Bortezomib sensitivity. The MTT cell viability assay was used to assess growth inhibition of U266 human myeloma cells due to different TPL concentrations and the combined use of different concentration of TPL and 0.01 mM Bortezomib. Annexin-V and Propidium iodide (PI) labeling were used to detect cell death and determine the mode of death. TPL alone inhibited U266 cell growth and apoptosis inducement in a concentration-dependent manner. When TPL is combined with 0.01 mM Bortezomib, both effects increase. Western blotting revealed that caspase 3 and caspase 9 gene expression increased, while nuclear factor (NF)-KB and HSP 90 protein expression decreased, related with apoptosis and growth inhibition. TPL increases U266 cell sensitivity to Bortezomib, inducing apoptosis, which might be the result of suppress NF-KB and HSP90 activity.

Key words: Bortezomib, multiple myeloma, heat shock protein gp90, triptolide.

INTRODUCTION

Multiple myeloma (MM), a common malignant hematological tumor, is a monoclonal malignant plasmocyte proliferative disease, and is the second most common hematological malignant tumors. Proteasome inhibitors, like Bortezomib, and immunomodulators, like thalidomide, have been used as multiple targeting therapeutic drugs to treat multiple myeloma (Gertz et al., 2008). Bortezomib is currently one of the most effective drugs for treating multiple myeloma (Ludwig et al., 2005). However, in clinical applications, there are very unpleasant side effects, such as neurotoxicity and hemopoietic system toxicity. Hence, new combined treatments to improve MM patient survival and prognosis are still needed. Triptolide (TPL) is a diterpenoid compound extracted from *Tripterygium wilfordii*, a traditional Chinese medicinal herb known as lei gong teng. TPL possesses significant anti-inflammatory, anti-tumor and immunoregulatory effects. In clinical applications, it is widely used to treat autoimmune diseases, such as rheumatoid arthritis, nephritis, and systemic lupus erythrematosus (SLE) (Chen, 2001; Feng et al., 2007). Recently, our research demonstrated that TPL inhibits MM cell proliferation of and induce apoptosis (Min et al., 2008).

Heat shock proteins (HSPs) are a group of proteins which are highly expressed when cells are exposed to stressors like high temperature, radiation, oxygen deficiency, and toxins. They are divided into five families: HSP110, HSP90, HSP70, HSP60 and small HSPs. HSP90 has a series of specific substrate proteins, most of which are closely linked with the occurrence and development of tumors, such as hypoxia inducible factor-1α (HIF-1α), most receptor tyrosine kinases (RTKs) and

*Corresponding author. E-mail: machengjian2010@hotmail.com. Tel: +86-571-87236746.

Table 1. Group of U266 cells treated differently in this study.

Groups	A	B	C	D	E	F	G	H
TPL (ng/ml)	20	10	5	0	0	5	10	20
Bortezomib (0.01 mM)	+	+	+	+	-	-	-	-

signal transduction proteins like nuclear factor (NF)-KB, Src, Raf-1, Akt and others (Solit and Rosen, 2006; Koga et al., 2009). HSP90 expressed more highly in myeloma cells than thao of in normal cells, suggesting that it plays an important role in promoting tumor cells' proliferation. Mosser and colleagues (Mosser and Morimoto, 2004) indicated that HSP90 could be anti-apoptotic. HSP90 inhibitors like tanespimycin (17-AAG/KOS-953) function as strong anti-MM agents (Mitsiades et al., 2006). Therefore, HSP90 is a new target spot for myeloma treatment.

Herein we test the ability of TPL to induce apoptosis in U266 human myeloma cells by reducing HSP90 expression, as well as the effect of TPL on Bortezomib sensitivity. This research aims to explore whether TPL can enhance Bortezomib sensitivity and identify a potential mechanism, which could provide a theoretical foundation for the use of TPL to treat myeloma.

MATERIALS AND METHODS

Bortezomib was bought from Millennium Pharmaceuticals (Cambridge , MA, USA). TPL, PI kit and MTT were bought from Sigma (St. Louis, MO, USA). Annexin V-FITC kit was bought from BD Company (Suzhou, China). Anti-p-NF-KB, p65 (phospho-p65), HSP-90, cleaved-caspase 3, and cleaved-caspase 9 using whole cellular protein extraction kits (Active Motif Inc. Lake Placid, New York, USA), horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG were purchased from Cell Signaling Technology (Danvers, MA, USA).

The actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). RPMI1640 culture medium and fetal calf serum (FCS) were from Gibco (Carlsbad , CA, USA). HSP90 antibody and NF-KB antibodies were bought from Fuzhou Maxim IHC World (Fujian, China).

Cell culture

U266 cells were purchased from the Shanghai Institute of Biochemistry and Cell Biology (SIBCB; Shanghai, China). Cells were suspended in RPMI1640 medium containing 10% FCS, placed in an incubator with saturated humidity and 5% CO_2 at 37℃. The cells were passaged once every 3 to 4 days. For experiments, cells in logarithmic growth phase were used.

Cells were plated in 96-well culture plates at a density of 1 x 10^5 cells/ml, and different concentrations of the drugs were added. Three wells were treated for each treatment group. The specific experimental groups are in Table 1.

MTT assay

The untreated control group was treated with serum-free RPMI l640. The total volume of each well was 0.2 ml, and each was treated for 24 h. The MTT working solution (20 µl/pore) was added to a final concentration of 0.5 mg/ml. 0.5 x 10^4/well cells were incubated in the incubator with 5% CO_2 at 37℃ for 4 h. The medium was removed and centrifuged at 1000 rpm/min. The supernatant was removed carefully and discarded. Dimethyl sulfoxide (DMSO) was added (0.2 ml/well) and mixed to dissolve the indigo blue formazan precipitate. The absorbance (A) was read at 570 nm using a Biotek Elx800 ELISA plate reader (Biotek, Vermont, USA). The results are expressed as the mean. The survival rate was calculated as follows: survival rate = [A (experimental)/A (control)] *100%.

Flow cytometry

U266 cells were inoculated into culture flasks at 5 x 10^5 cells/ml. After 16 h, the cells were harvested and suspended in1 ml 1x Binding buffer solution from the kit. Cells (100 µl) were placed in special flow cytometry tubes. Annexin V-FITC (5 µl) and propidium iodide (PI; 5 µl) were added and the cells mixed gently. The solution was incubated for 15 min at room temperature in the dark. Binding buffer solution (1x; 300 µl) was added, and fluorescence detected immediately with a flow cytometry (BD, MD, USA). The results were analyzed using CellQuest v. l.2 analysis software.

Western blotting

The whole-cell protein extraction kit (Active Motif, CA, USA) was used to extract U266 cellular proteins from different treatment groups. The protein content was measured by the Bradford assay (Pierce, Rockford, IL, USA) by DU640 UV analyzer (Beckman, CA, USA). Protein samples (40 µg) were loaded on 100 to 150 g/L polyacrylamide gels. After electrophoretic separation, the proteins were transferred to a PVDF membrane, and blocked for 1 h in TBST containing 50 g/L non-fat powdered milk. The membrane was washed 3 times for 5 min each prior to exposure to primary antibodies (cleaved-caspase 3, cleaved-caspase 9, p-p65 NF-KB, HSP90 or β-actin; 1:200). The membranes were incubated overnight at 4℃. The membrane was washed again, 3 times for 5 min each, prior to addition of the corresponding secondary antibodies (goat anti-rabbit, 1:1000), and shaken gently for 1 h. TBST was used to wash the membrane three times, and then the membrane was exposed to SuperSignal West Dura ECL reagents (Super Signal Dura kit, Pierce, IL, USA), and the labeled bands detected using X-ray film. A gray-scale scan using a laser density scanner and Image J software was acquired for each blot. The density value of each band of the target protein was normalized to the density value of the β-actin band for that sample.

Statistical analysis

SPSS 12.0 software (Chicago, USA) was used for analysis and

Table 2. The survival rate of U266 cells treated with TPL and Bortezomib. The survival rate decreased in a dose dependent.

Group	OD570	Survival rate (%)	P
A	0.40 ± 0.02	25.25 ± 1.15	<0.05
B	0.59 ± 0.04	37.49 ± 0.62	<0.05
C	0.98 ± 0.07	62.47 ± 5.41	<0.05
D	1.47 ± 0.04	92.28 ± 4.46	>0.05
E	1.57 ± 0.09	100	——
F	1.37 ± 0.04	72.36 ± 2.91	<0.05
G	0.90 ± 0.09	57.52 ± 6.07	<0.05
H	0.75 ± 0.02	47.78 ± 2.03	<0.05

(A) 20 ng/ml TPL+ 0.01 mM Bortezomib, (B) 10 ng/ml TPL+ 0.01 mM Bortezomib, (C) 5 ng/ml TPL+ 0.01 mM Bortezomib, (D) 0.01 mM Bortezomib, (E) Blank control, (F) 5 ng/ml TPL, (G) 10 ng/ml TPLI, (H) 20 ng/ml TPL.

treatment. Experimental data are expressed as the mean ± the standard deviation (SD). One-way ANOVA was used to compare many groups of data. Regression analysis was used for the relationship between growth inhibition rate and drug concentration. A P value less than 0.05 was considered significant.

RESULTS

TPL combined with Bortezomib had a synergistic effect on cell growth

Treat U266 cells for 24 h by 0 to 20 ng/ml TPL lower than IC_{50} dosage. The results showed that TPL alone inhibited U266 cell growth in a dose-dependent manner (r = 0.991, P < 0.01). The differences between various concentrations were statistically significant (P < 0.05). No significant growth inhibition was observed when cells were exposed to (0.01 mM) Bortezomib (P > 0.05). When Bortezomib was used with 5 - 20 ng/L TPL, the survival rate of U266 cells decreased significantly (P < 0.05 compared with F, G and H groups (Table 2).

Apoptosis induced by single used TPL and combined used TPL and 0.01 mmol/L Bortezomib

U266 cells were exposed to 5, 10 or 20 ng/L TPL for 16 h (Figure 1). Flow cytometry revealed that (3.48 ± 0.65)%, (11.15 ± 1.86)%, or (19.2 ± 2.69)% of the cells were Annexin V positive but PI negative (early stage apoptosis), respectively, compared to (1.80 ± 0.16)% of the untreated cells. Early stage apoptosis positively correlated with TPL concentration (r = 0.981, P < 0.01). Bortezomib alone (0.01 mM) resulted in (3.36 ± 0.57)% Annexin V positive, PI negative cells (early stage apoptosis). Combining 5, 10 or 20 ng/L TPL with 0.01 mM Bortezomib for 16 h resulted in (7.37 ± 1.32), (36.53 ± 0.53), or (46.30 ± 2.97)% U266 cells, respectively, were Annexin V positive, PI negative cells (early stage apoptosis). Early stage apoptosis was positively correlated with the TPL concentration (r = 0.935, P < 0.01). Combined exposure to TPL (5, 10 or 20 ng/L) and Bortezomib, (0.01 mM) increased U266 cell apoptosis compared with TPL alone (P < 0.05 compared with F, G and H groups). The actual apoptosis induced by TPL and Bortezomib combined was higher than the theoretically calculated additive effect by 5.62% (5 ng/L TPL), 12.08% (10 ng/L TPL) and 19.3% (20 ng/L TPL), which suggested that TPL and Bortezomib have a synergistic effect.

Both TPL alone and combined with Bortezomib alters caspase 3, caspase 9, NF-K B protein, and HSP90 protein expression in U266 cells

Western blotting for caspase 3, caspase 9, NF-KB, and HSP90 protein expression (Figure 2) revealed that Bortezomib alone (0.01 mM) did not significantly affect cleaved-caspase 3, cleaved-caspase 9, p-p65NF-KB, or HSP90 protein expression (P > 0.05 compared with E group). TPL alone (5, 10 or 20 ng/ml) increased caspase 3 and caspase9 expression (P < 0.05 compared with E group) which positively correlated with apoptosis. NF-KB protein and HSP90 protein expression decreased (P < 0.05 compared with E group), which negatively correlated with apoptosis. While combined TPL and Bortezomib remarkably increased caspase 3 and caspase 9 expression but decreased NF-KB and HSP90 protein expression (P < 0.05 compared with F, G and H groups). The higher the concentration of TPL in the combined samples, the more obvious effect.

DISCUSSION

Therapeutic strategies for MM consist of conventional

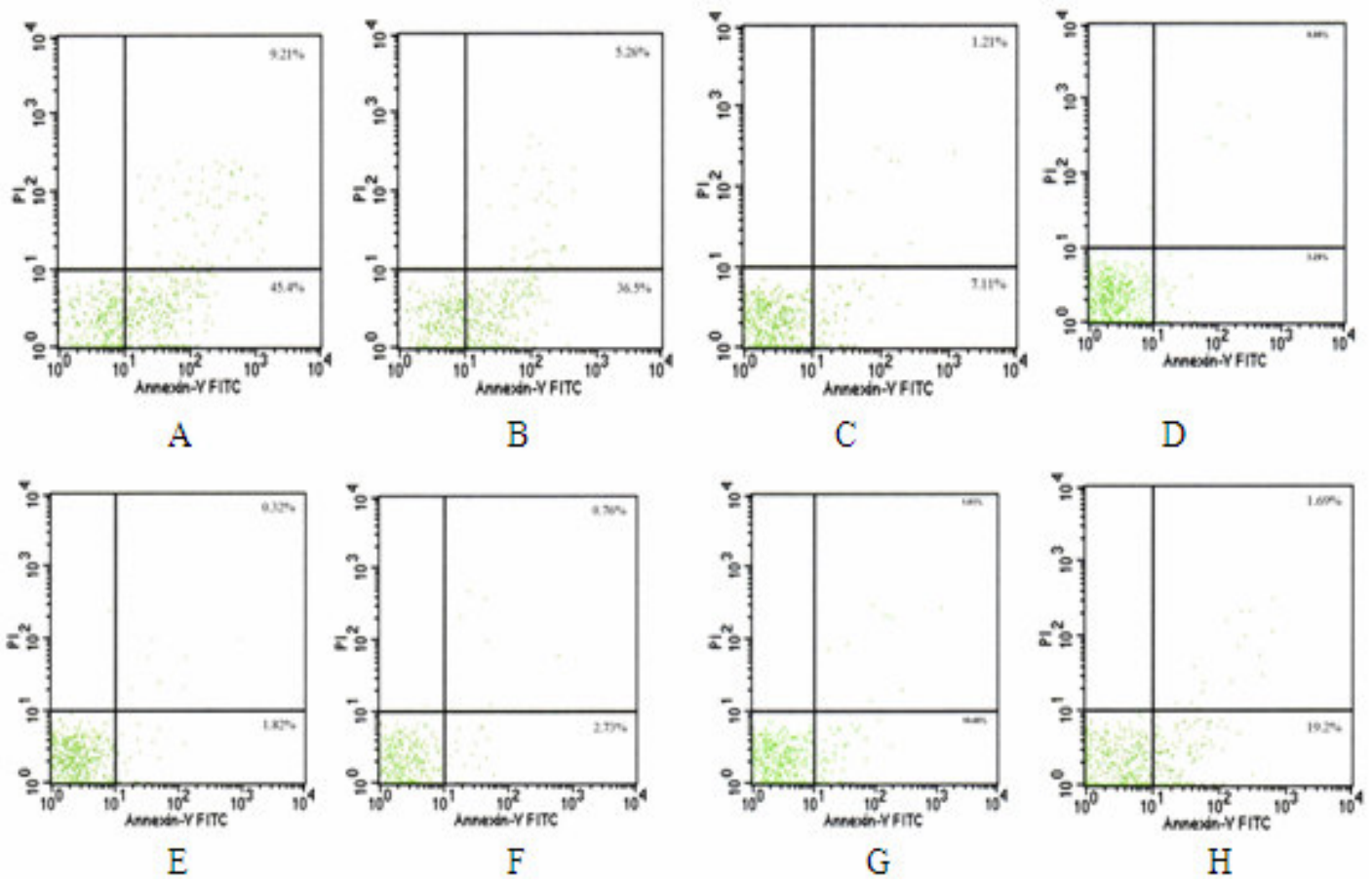

Figure 1. Inhibition of TPL and Bortezomib triggered apoptosis in U266 cells. In the present of TPL and Bortezomib, apoptosis induction increased significantly in a dose-dependent. (A) 20 ng/ml TPL+ 0.01 mmol/l Bortezomib, (B) 10 ng/ml TPL+ 0.01 mmol/l Bortezomib, (C) 5 ng/ml TPL+ 0.01 mmol/l Bortezomib, (D) 0.01 mmol/l Bortezomib, (E) blank control, (F) 5 ng/ml TPL, (G) 10 ng/ml TPLl ; H , 20 ng/ml TPL.

chemotherapy, stem cell transplantation, Bortezomib, and thalidomide, among others (Hideshima and Anderson, 2002). Due to age and other medical conditions, there are only a few patients for whom bone marrow transplantation is a viable option. The efficacy of conventional chemotherapy is limited, and median survival time is just 3 to 4 y (Shirato et al., 2008; Kyle and Rajkumar, 2004). TPL alone (10 to 80 ng/ ml) inhibits the proliferation of MM cell lines RPMI8226 and U266 in a dose-dependent manner (Yin et al., 2005). Our experiments confirm that TPL inhibits U266 growth in a dose-dependent manner. TPL successfully decreased cell viability by Bortezomib, as well as increasing expression of caspases 3 and 9. Therefore, TPL have similar effect with small dose of Bortezomib, which may reduce TPL side effect, thereby allowing for a full course of treatment.

TPL possesses a broad spectrum antitumor effect, significantly inhibiting the growth of B16 melanoma (B16M), human breast cancer cells (MDA-435), prostatic cancer (TSU), colon cancer (HIC/S) and gastric cancer cells (MGC80-3) in a mouse xeneotransplantation model, as well as inhibiting melanoma metastasis (Yang et al., 2003). But TPL toxicity restricts its clinical application as an anti-tumor drug. Therefore, current research focuses on reducing drug resistance and side effects of MM treatment, reducing the disease burden, prolonging survival time, and relieving symptoms to improve quality of life.

Bortezomib is the first proteasome inhibitor used clinically. The median time for Bortezomib effects to manifest clinically when treating MM is 38 days, which is a rapid effect. But, there are also unfortunate side effects like nausea, diarrhea, fatigue, thrombocytopenia, constipation, vomiting, apocleisis, fever, peripheral nervous pathological changes and anemia (Richardson et al., 2006). Low Bortezomib concentration (0.01 mM) does not induce significant myeloma apoptosis; while TPL alone induced limited apoptosis. However, the combined used low concentration Bortezomib and TPL remarkably enhanced apoptosis, demonstrating that the two drugs have synergistic effect when used together. Mitsiades and colleagues (Mitsiades et al., 2002; Adams, 2003) found that Bortezomib affected apoptosis by adjusting

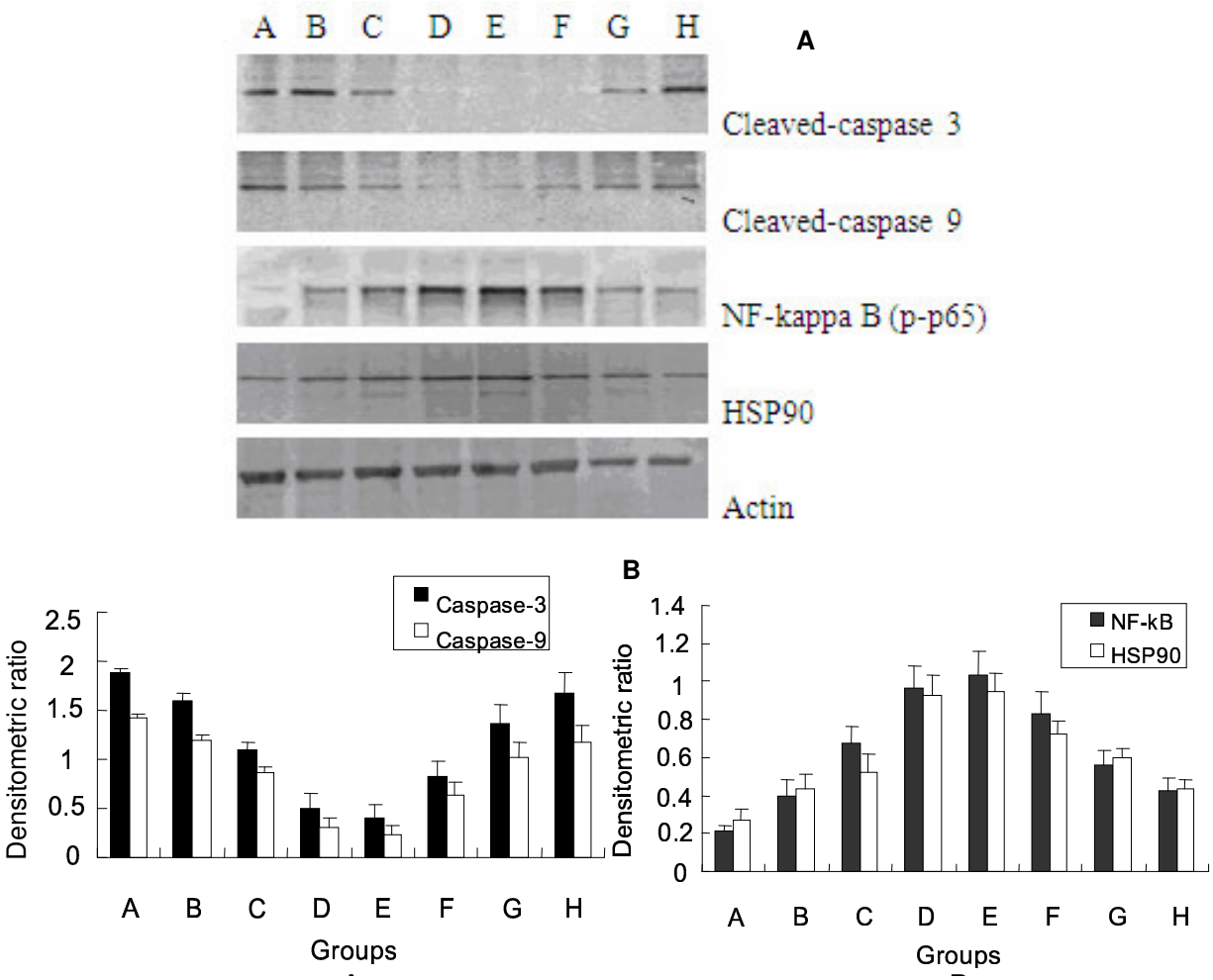

Figure 2. Western blot analysis for cleaved-caspase 3, cleaved-caspase 9, p-p65NF-kB and HSP90 protein expression in U266 cells (densitometric ratio with actin) (A) TPL combined with Bortezomib significantly increased caspase-3 and caspase-9 protein expression in U266 cells, in a dose-dependent manner. Densitometric analysis (B) was performed as described in material and methods. (A) 20 ng/ml TPL+ 0.01 mmol/L Bortezomib, (B) 10 ng/ml TPL+ 0.01 mmol/L Bortezomib, (C) 5 ng/ml TPL+ 0.01 mmol/L Bortezomib, (D) 0.01 mmol/L Bortezomib, (E) blank control, (F) 5 ng/ml TPL, (G) 10 ng/ml TPLl, (H) 20 ng/ml TPL.

Fas and Fas ligand (FasL) expression, as well as regulating caspase activation. In this work, we found that TPL alone increases caspase 3 and caspase 9 expression, both of which are positively correlated with apoptosis, but a low concentration Bortezomib alone had no effect. When used together, caspase 3 and caspase 9 expression increased. The higher the TPL concentration, the more obvious the effect.

NF-KB protein expression is high in MM cell lines and patients (Sun et al., 2006). Therapeutic doses of Bortezomib inhibit myeloma cell growth primarily by blocking NF-KB signaling (Adams, 2004). The research shows that low concentration Bortezomib has no effect on NF-KB expression, and has no significant effect on apoptosis or cell proliferation. When Bortezomib is combined with TPL, NF-KB expression decreases significantly, more than with TPL alone. Therefore, Bortezomib improves TPL sensitivity to NF-KB block, thereby increasing apoptosis. Successful anti-myeloma treatment may be achieved by combining low dose Bortezomib and TPL, which could reduce the toxic side effect caused by using higher Bortezomib doses.

HSP90 is one of the most active molecular chaperones participating in multiple cell signal transduction pathways, hormone responses and transcriptional control, and has an important role in cell survival under stress conditions. HSP90 is highly expressed in myeloma cells (Andrulis et al., 2007). HSP90 substrate proteins, such as hypoxia inducible factor-1α (HIF-1α), most receptor tyrosine kinase (RTKs) and signal transduction proteins NF-KB, Src, Raf-1, and Akt have important roles in many signal transduction pathways that promote tumor growth and

metastasis (Ammirante et al., 2008). Our experiments showed that low concentration Bortezomib has no effect on HSP90. TPL alone reduced HSP90 expression, which was significantly increased by adding Bortezomib. The higher the TPL concentration used the more obvious decrease, and stronger induction of apoptosis, observed. Therefore, we consider TPL and Bortezomib have a synergistic influence on HSP90 expression in U266 cells. Westerheide et al. (2006) found that TPL inhibits the heat shock response by blocking heat shock transcription factors, and that the effect of TPL occurred after HSF1 trimerization, nuclear localization and association with heat shock gene promoter but before mRNA transcription. Hence, TPL may reduce HSP90 levels in U266 cells by blocking the heat shock response, thereby inducing apoptosis and improving cell sensitivity to Bortezomib.

In conclusion, the data presented here demonstrate that TPL can induce apoptosis by reducing HSP90 and NF-KB expression, as well as enhancing caspase 3 and caspase 9 expression, in myeloma cells, which sensitizes the cells to Bortezomib. Identifying potential targets of TPL provides a mechanistic basis for the combined use of TPL and Bortezomib in clinical MM treatment.

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