

Triptolide reduces the viability of osteosarcoma cells by reducing MKP-1 and Hsp70 expression

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Abstract. Osteosarcoma is the most common type of malignant bone tumor found in adolescents and young adults. The aim of the present study was to determine whether triptolide, a diterpene epoxide extracted from the *Tripterygium* plant, was able effectively decrease the viability of osteosarcoma cells. The underlying molecular mechanisms are also investigated. The human osteosarcoma cell lines U-2 OS and MG-63 were used in this study. The U-2 OS and MG-63 cells were treated with 0, 5, 10, 25 or 50 nM triptolide. Cells treated with dimethyl sulfoxide only were used as the no drug treatment control. A commercial MTT kit was used to determine the effects of triptolide on cells. Mitogen-activated protein kinase phosphatase-1 (MKP-1) is frequently overexpressed in tumor tissues, possibly related to the failure of a number of chemotherapeutics. Heat shock protein 70 (Hsp70) is a chaperone molecule that is able to increase drug resistance. The protein expression levels of MKP-1 and Hsp70 were determined using western blot analysis. The results indicate that triptolide effectively reduced the viability of the osteosarcoma cells. Furthermore, triptolide was found to effectively reduce MKP-1 expression and Hsp70 levels. Further analysis showed that triptolide reduced MKP-1 mRNA expression in the U-2 OS and MG-63 cells. Triptolide reduced Hsp70 mRNA expression levels in U-2 OS and MG-63 cells. These results suggest that triptolide effectively decreases the viability of osteosarcoma cells. These effects may be associated with the decreased expression of MKP-1 and Hsp70 levels. These results suggest that triptolide may be used in the treatments of osteosarcoma.

Introduction

Osteosarcoma is the most frequently diagnosed type of the malignant bone tumor found in adolescents and young adults (1,2). Prognosis of patients with metastatic or advanced osteosarcoma is ~20%, despite treatments with single or combination of chemotherapy, radiotherapy, and surgery. Thus, novel therapies are necessary, particularly for patients that exhibit chemotherapy resistance (3,4).

Triptolide is a diterpene epoxide of *Tripterygium* extracts, which has been suggested to possess anti-cancer, anti-inflammatory, immunosuppressive and anti-cystogenic activities (5). Triptolide is effective against a number of malignancies, including ovarian cancer, breast cancer, pancreatic cancer and neuroblastoma (6). Triptolide suppresses the proliferation of prostate cancer cells by inhibition of expression of SUMO-specific protease 1 (7). Triptolide induces the apoptosis of pancreatic tumor cells by decreasing the expression of O-GlcNAc transferase to alter the distribution of transcription factor specificity protein 1 (8). However, it is not clear if triptolide can be used to treat osteosarcoma.

Mitogen-activated protein kinase phosphatases (MKPs) are protein phosphatases with dual specificity (9). MKPs can dephosphorylate the phospho-tyrosine and phospho-threonine residues on the mitogen-activated protein kinases (MAPKs) (10). Since the MAPK family members of the signaling molecules, such as c-Jun N-terminal kinase, p38 MAPK and the extracellular signal-regulated kinase, serve crucial functions in cellular signaling pathways, it may offer a potential therapeutic strategy to control the MAPK-related pathways (11). MKP-1 is an endogenous MAPK deactivator. MKP-1 is often overexpressed in tumors and is considered to be related to the failure of various chemotherapeutics (12,13).

Heat shock proteins (Hsps) are a group of proteins, including Hsp10, 27, 40, 60, 70, 90 and 110 (14), that perform various roles in the processes of all living organisms, from bacteria to humans. The members of this group are functionally related proteins involved in folding and unfolding of other proteins in the living organisms (15,16). Under the normal growth conditions, Hsp70s function as the ATP-molecular chaperones and facilitate protein folding (17). Under stress conditions, Hsp70 proteins cooperate with the increased concentrations of unfolded and denatured proteins, avoiding toxic aggregates via the induction of apoptosis (18,19). Their

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expression is often upregulated when cells are exposed to abnormal temperatures or extreme conditions. Changes in Hsp expression levels are often regulated in the transcriptional steps (20).

Hsp70 upregulation has been detected in patients with certain types of cancers, and therefore it is speculated that Hsp70 may contribute to resistance to chemotherapy (20). Inhibition of Hsp70 induction was previously used as a method to benefit the anti-leukemia activity of the Hsp90 inhibitor, 17-allylaminodemethoxy geldanamycin (21). Ibuprofen has been found to enhance the anti-tumor activities of cisplatin in lung cancer cells by inhibiting Hsp70 (22). In addition, the modulation of Hsp70 expression with quercetin increased the chemoresponsiveness of pancreatic cancer cells to gemcitabine (23). The aim of the present study was to investigate whether triptolide, a diterpene epoxide of *Tripterygium* extracts, can be used to treat osteosarcoma in human cell lines.

Materials and methods

Cell lines and reagents. The human osteosarcoma cell lines (U-2 OS and MG-63) were purchased from the American Type Culture Collection (Manassas, VA, USA). U-2 OS cells were cultured in McCoy's 5A medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C with 5% CO₂, supplemented with 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences, Logan, UT, USA), 100 U/ml penicillin (Invitrogen; Thermo Fisher Scientific, Inc.), and 100 mg/ml streptomycin (Invitrogen). MG-63 cells were cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Inc.) at 37°C with 5% CO₂, supplemented with 20 mM HEPES (Thermo Fisher Scientific, Inc.), 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin (Invitrogen) and 100 mg/ml streptomycin (Invitrogen). DNase and RNeasy isolation kits were purchased from Qiagen (Valencia, CA, USA). Triptolide was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. U-2 OS and MG-63 cells were treated with or without triptolide (0, 5, 10, 25 or 50 nM) at 37°C for 48 h. Triptolide was dissolved in DMSO. The cells treated with DMSO only were used as the no drug control (0 nM). Subsequent to the experiments, cells were incubated with 0.5 mg/ml MTT for 4 h at 37°C. The Vybrant MTT Cell Proliferation Assay kit (V13154; Thermo Fisher Scientific, Inc.) was used to perform the assay according to the manufacturer's manual. The absorbance values were determined at 540 nm using a Spectramax M2 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Viability of the treated cells relative to the control cells, which were treated with medium only, was determined.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Extraction of the total RNAs was performed using an RNeasy Protect Kit followed by DNase treatment according to the manufacturer's instructions (Qiagen, Inc., Valencia, CA, USA). RNAs were transcribed to cDNAs using the Superscript II kit according to the manufacturer's

instructions (Promega, Madison, WI, USA). Product detection was performed by measuring fluorescence signals, using a SYBR Green PCR kit (Takara Bio, Inc., Tokyo, Japan). Quantification of cDNA was conducted via qPCR to a final reaction volume of 20 µl, including 1 µl cDNA (equivalent to 50 ng input RNA), 0.4 µM each primer and 1X SYBR, according to the manufacturer's protocol. Thermal cycling was performed in a LightCycler 480 Real-Time PCR system (Roche Diagnostics, Basel, Switzerland) as follows: One cycle at 50°C for 2 min and 10 min at 95°C, followed by 40 cycles of amplification (denaturation for 15 sec at 95°C and annealing and extension for 1 min at 60°C). Template-negative and RT-negative controls were used. Cycle threshold values (Cq values) were calculated by using the LightCycler software v.3.5. Expression levels of the detected target genes were shown as the quantity relative to GAPDH, using the 2^{-ΔΔCq} method (24). The analyses were performed at least three times for each sample. Primers used in this study are listed in Table I.

Western blot assays. U-2 OS and MG-63 cells treated with various conditions were harvested by centrifugation at 7,200 x g for 5 min at 4°C, and washed twice with phosphate-buffered saline (PBS; Beyotime Institute of Biotechnology, Beijing, China). Cell lysates were prepared in radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology) supplemented with 0.5% cocktail protease inhibitor (Roche Diagnostics) and 0.5 mM phenylmethylsulfonyl fluoride. Cell lysates were subsequently sonicated for 15 sec and centrifuged at 12,000 x g for 10 min. Supernatants were collected and protein concentrations were determined according to the bicinchoninic acid protocol using bovine serum albumin as a standard, and the loading volumes of protein samples were adjusted accordingly. Using 5X loading buffer (250 mM Tris-HCl, 10% SDS, 0.5% BPB, 50% glycerol and 5% β-mercaptoethanol; pH 6.8; Proteintech; Wuhan Sanying Biotechnology, Wuhan, China) total proteins were separated on 10% SDS-PAGE gels (Beyotime Institute of Biotechnology), transferred onto polyvinylidene difluoride membranes (Amresco, LLC, Solon, OH, USA), and detected by immunoblot analyses. Membranes were blocked with 5% skimmed milk in Tris-buffered saline with Tween 20 (TBST; Proteintech), followed by incubation with primary antibodies overnight at 4°C. The primary antibodies against MKP-1, Hsp70, and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The antibody information and dilution ratios were as follows: Anti-MKP-1 (sc-370; 1:200), anti-Hsp70 (sc-32239; 1:200) and anti-GAPDH (sc-130301; 1:5,000). Following washing three times with TBST for 5 min, the membranes were subsequently incubated with goat anti-mouse (sc-2005; 1:10,000) or goat anti-rabbit horseradish peroxidase-conjugated IgG (sc-2004; 1:10,000; Santa Cruz Biotechnology, Inc.) secondary antibodies for 1 h at room temperature. Antibodies bound to the blots were detected using an ECL system (32209; Pierce Biotechnology; Thermo Fisher Scientific, Inc.). The immunoblot experiments were repeated at least 3 times. Blot quantifications were performed using an ImageQuant LAS 500 software (GE Healthcare Life Sciences). The resulting values are presented as the mean ± standard deviation (SD).

Table I. Primers used in this study.

Primer	Sequence
MKP-1	F, 5'-GTCGGTAGAGAGTCTACGCT-3' R, 5'-TCGTGTGGAACATTCATTC-3'
Hsp70	F, 5'-TACTATCTCCAGACTCTC-3' R, 5'-ATCCGCTGCTAAGCTGTG-3'
GAPDH	F, 5'-GTAATGCACCCAGAGGTATG-3' R, 5'-CACCAATCTCATGCGGAAC-3'

F, forward; R, reverse.

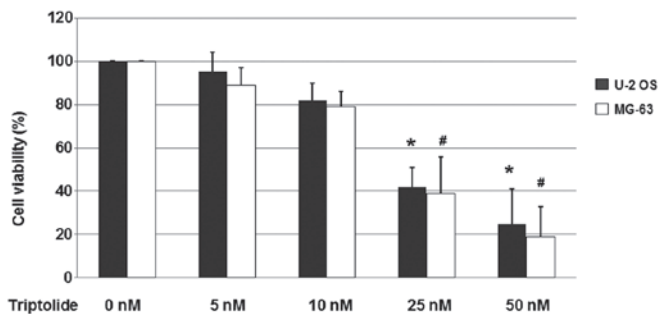


Figure 1. MTT assay of the U-2 OS and MG-63 cells treated with triptolide. Cells were treated with triptolide (0, 5, 10, 25 and 50 nM) at 37°C for 48 h. Values are presented as the mean \pm standard deviation obtained from three independent experiments. *P<0.05 vs. U-2 OS control group; #P<0.05 vs. MG-63 control group.

Statistical analysis. Experimental data are expressed as the mean \pm SD. SPSS 10.0 software (SPSS, Inc., Chicago, IL, USA) was used for independent sample tests, followed by one-way variance analyses. In these analyses, P<0.05 was considered to indicate statistically significant differences.

Results

Triptolide reduces the viability of osteosarcoma cells. To determine if triptolide was able to affect the viability of osteosarcoma cells, the U-2 OS and MG-63 cells were treated with triptolide (0, 5, 10, 25 or 50 nM) at 37°C for 48 h. The cells treated with DMSO only were used as the no drug treatment control (0 nM). Following the experiment, cells were incubated with 0.5 mg/ml MTT for 4 h at 37°C and the MTT kit was used to perform the assay.

As shown in Fig. 1, 25 and 50 nM triptolide reduced cell viability significantly in the U-2 OS and MG-63 cell populations. Treatment with 25 nM triptolide reduced the cell viability to 42 and 39% in the U-2 OS and MG-63 cells, respectively. Furthermore, treatment with 50 nM triptolide led to cell viabilities of 25 and 19% in the U-2 OS and MG-63 cells, respectively. These results indicated that triptolide reduces the viability of osteosarcoma cells.

Triptolide effectively reduce MKP-1 expression. As MKP-1 is frequently overexpressed in tumor tissues and possibly related to the chemoresistance of many chemotherapeutics (12,13),

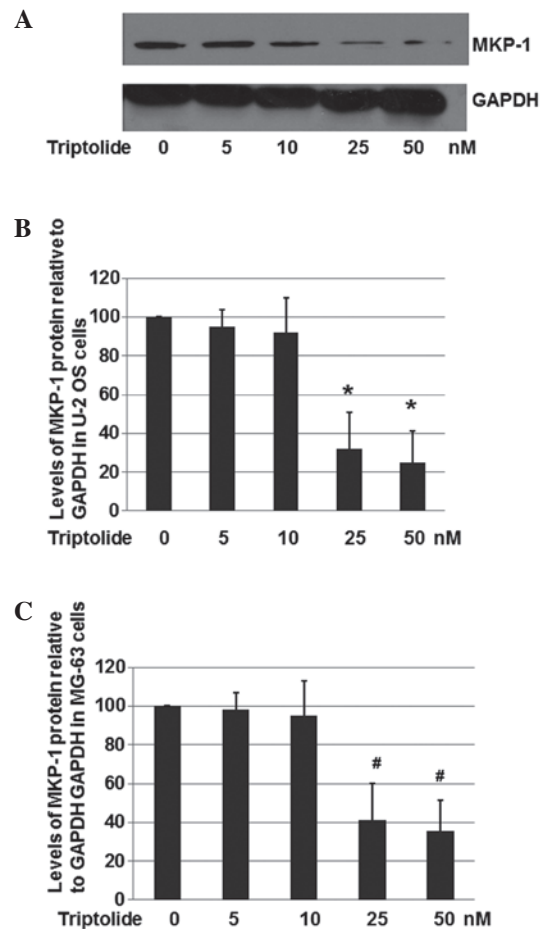


Figure 2. Triptolide decreases the expression of MKP-1 protein. U-2 OS cells (A and B) or MG-63 cells (C) were treated with triptolide (0, 5, 10, 25 and 50 nM) at 37°C for 48 h. (A) Representative blot using U-2 OS cell lysates was shown. (B) Protein expression levels of MKP-1 relative to GAPDH levels in the U-2 OS cells treated with triptolide. (C) Similar experiments were performed using the MG-63 cells. Data were from at least three independent experiments. *P<0.05 vs. U-2 OS control group; #P<0.05 vs. MG-63 control group. MKP-1, mitogen-activated protein kinase phosphatase-1.

the effects of triptolide on MKP-1 expression were determined. U-2 OS cells were treated with triptolide (0, 5, 10, 25 and 50 nM) at 37°C for 48 h. Cells treated with DMSO only were used as the no drug treatment control (0 nM). Total proteins were harvested and subjected to western blot analyses. GAPDH was used as a loading control.

As shown in Fig. 2A and B, triptolide with a concentration of 25 nM significantly repressed the expression of MKP-1 to ~30.6% compared with the U-2 OS cells treated with DMSO only. In the cells treated with 50 nM triptolide, the expression of MKP-1 was decreased to 24.2%. These results suggest that triptolide is able to reduce MKP-1 expression in U-2 OS cells.

The effect of triptolide on MKP-1 expression in the human osteosarcoma cell line MG-63 was also evaluated. As shown in Fig. 2C, triptolide at a concentration of 25 nM inhibited the expression of MKP-1 to ~41.2%, when compared with the level in the MG-63 cells treated with DMSO only. In the cells treated with 50 nM triptolide, the expression of MKP-1 was decreased to 35.6%. These results suggest that triptolide is able to decrease MKP-1 expression in MG-63 cells. However, the inhibitory effects on MKP-1 are slightly less than in U-2 OS cells.

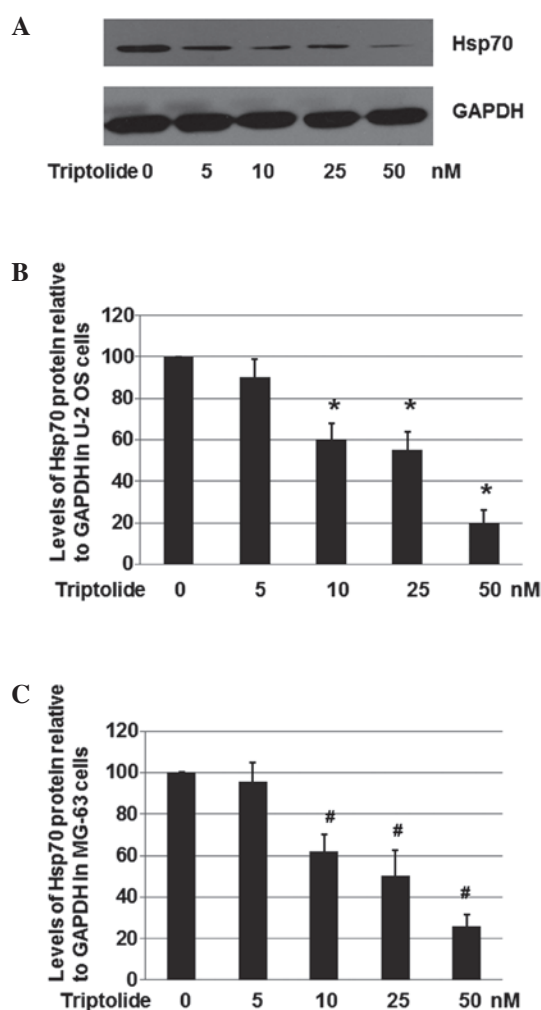


Figure 3. Triptolide decreases the expression of Hsp70 protein. (A) Representative blot using U-2 OS cell lysates. (B) Levels of Hsp70 protein relative to GAPDH levels in the U-2 OS cells treated with triptolide. Data were from at least three independent experiments. * $P < 0.05$ vs. U-2 OS cell control group. (C) Similar experiments were performed using the MG-63 cells. # $P < 0.05$ vs. MG-63 cell control group. Data were from at least three independent experiments.

Triptolide effectively reduces Hsp70 levels. As Hsp70 is a chaperone molecule which can increase drug resistance, the effects of triptolide on Hsp70 expression were determined (23). U-2 OS cells were treated with triptolide (0, 5, 10, 25 and 50 nM) at 37°C for 48 h. Cells treated with DMSO alone were used as the no drug treatment control (0 nM). Total proteins were harvested and subjected to western blot analyses, with GAPDH used as a loading control.

As shown in Fig. 3A and B, triptolide at a concentration of 10, 25 or 50 nM significantly repressed expression of Hsp70 to ~60.1, 54.9 and 20.6%, respectively, when compared with the level in the U-2 OS control cells treated with DMSO only. These results suggest that triptolide is able to decrease Hsp70 expression in U-2 OS cells.

The present study also aimed to determine whether triptolide decreases Hsp70 expression in MG-63 cells. As shown in Fig. 3C, triptolide with a concentration of 10, 25 or 50 nM significantly repressed the expression of Hsp70 to ~62.2, 50.3 and 25.7%, respectively, when compared with the level in the MG-63 cells treated with DMSO only. These results

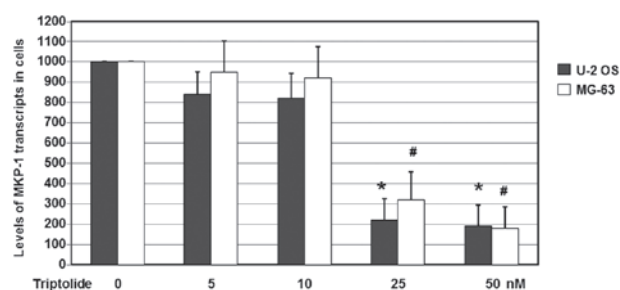


Figure 4. Effects of triptolide on the mRNA expression levels of MKP-1. U-2 OS and MG-63 cells were treated with triptolide (0, 5, 10, 25 and 50 nM) at 37°C for 24 h. All of the experiments were conducted in three independent experiments. Values are presented as the mean \pm standard deviation. * $P < 0.05$ vs. U-2 OS cell control group; # $P < 0.05$ vs. MG-63 cell control group.

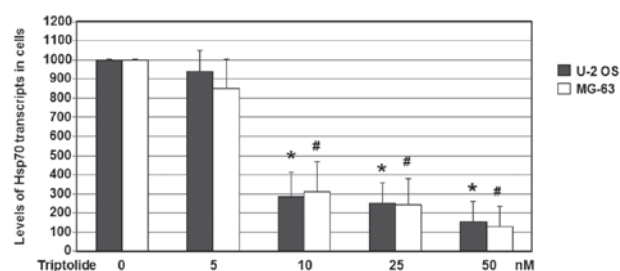


Figure 5. Effects of triptolide on the mRNA expression levels of Hsp70. U-2 OS and MG-63 cells were treated with triptolide (0, 5, 10, 25 and 50 nM) at 37°C for 24 h. All of the experiments were conducted in three independent experiments. Values are presented as the mean \pm standard deviation. * $P < 0.05$ vs. U-2 OS cell control group; # $P < 0.05$ vs. MG-63 cell control group.

suggested that triptolide is capable of decreasing Hsp70 levels in MG-63 cells.

Triptolide effectively reduces MKP-1 mRNA expression levels in U-2 OS and MG-63 cells. In order to examine the possible effects of triptolide on MKP-1 mRNA expression, U-2 OS cells were treated with triptolide (0, 5, 10, 25 and 50 nM) at 37°C for 24 h. Cells treated with DMSO only were used as the no drug treatment control (0 nM). Total RNA was harvested and subjected to RT-qPCR analyses, with GAPDH used as a loading control. The gene expression levels of MKP-1 was determined using RT-qPCR detection.

As shown in Fig. 4, following treatment with various concentrations of triptolide, the mRNA expression levels of MKP-1 in the U-2 OS cells were reduced significantly. When compared with the untreated cells, the MKP-1 mRNA levels were reduced to 22.4 and 19.1% by treatment with 25 and 50 nM triptolide, respectively ($P < 0.05$). When compared with the untreated MG-63 cells, the MKP-1 mRNA levels were reduced to 32.2 and 18.1% ($P < 0.05$). These results suggest that triptolide decreases the mRNA expression levels of MKP-1 genes in U-2 OS and MG-63 cells.

Triptolide effectively reduces Hsp70 mRNA expression levels in U-2 OS and MG-63 cells. In order to examine the effects of triptolide on Hsp70 mRNA levels, U-2 OS and MG-63 cells were treated with triptolide (0, 5, 10, 25 and 50 nM) at 37°C for 24 h. Cells treated with DMSO only were used as the no drug treatment control. Total RNA was harvested and subjected to

RT-qPCR analyses, with GAPDH used as a loading control. The gene expression of Hsp70 was determined using RT-qPCR detection.

As shown in Fig. 5, with treatments of various concentrations of triptolide, the mRNA expression levels of Hsp70 in the U-2 OS and MG-63 cells were reduced significantly. When compared with the untreated cells, the Hsp70 mRNA levels were reduced to 15.6% following treatment with 50 nM triptolide ($P < 0.05$). Hsp70 mRNA levels in the 50 nM triptolide-treated MG-63 cells were reduced to 12.8% ($P < 0.05$) of the control expression levels. These results suggest that triptolide is able to decrease the mRNA expression levels of Hsp70 in U-2 OS and MG-63 cells.

Discussion

Osteosarcoma is the most common type of the malignant bone tumors found in adolescents and young adults. Current treatments, including chemotherapy, radiotherapy and surgery have not achieved satisfactory effects in clinical settings (4). Triptolide has been suggested to have anti-cancer, anti-inflammatory, immunosuppressive and anti-cystogenic activity (5). MKP-1 protein is often overexpressed in tumors and is considered to be related to chemoresistance (12,13). Cancer cells express high levels of Hsps that are closely correlated with poor prognosis (25). Under non-stress conditions, Hsp70 has multiple functions, including protein folding and translocation of newly synthesized proteins, and serving as a signaling molecule (26). In colon and lung cancers, Hsp70 expression is correlated with metastasis and poor prognosis (27,28). Since Hsp70 is crucially involved in multiple steps of cancer developments (29), the present study investigated the potential of triptolide to be used as a treatment for osteosarcoma, possibly via a mechanism associated with the regulation of MKP-1 and Hsp70.

In the present results indicated that triptolide effectively reduced the viability of osteosarcoma cells. Treatments of triptolide concentrations of 25 nM reduced the cell viability to 42 and 39% in the U-2 OS and MG-63 cells, respectively. Furthermore, treatments with triptolide at a concentration of 50 nM led to cell viabilities of 25 and 19%, respectively. These findings suggest that a dose of triptolide as low as 25 nM is be effective for reducing cell viability of osteosarcoma cells.

The present western blot analyses indicated that triptolide effectively reduces MKP-1 and Hsp70 protein expression levels. In addition, RT-qPCR assays showed that triptolide reduced MKP-1 and Hsp70 mRNA expression in the U-2 OS and MG-63 cells. Although the mechanisms underlying this downregulation of MKP-1 and Hsp70 mRNA was not clear, the effects of triptolide were confirmed.

Overexpression of Hsp70 may lead to increased tumor growth, cancer cell migration and metastatic potential (29). Generally, increased levels of Hsp70 are frequently observed in cancer cells, where Hsp70 improves resistance to stress-induced apoptosis, contributing to suppression of senescence, and is also associated with metastatic development and drug resistance (30-32). Triptolide is known to induce apoptosis in gastric cancer cells via the inhibition of murine double minute 2 overexpression (33). In animal model experiments, triptolide reduced neuropathology in a mouse model of Alzheimer's disease by upregulating the level of an

insulin-degrading enzyme, a major A β -degrading enzyme in the brain (34). The results of the present study improve our understanding of triptolide as a potential therapy for cancer.

The results of the present study indicated that triptolide treatment significantly decreases the viability of osteosarcoma cells. This effect may be associated with the decreased expression levels of MKP-1 and Hsp70 following treatment with triptolide. Future studies investigating the manipulation of the molecular structure of triptolide are required in order to obtain a series of derivatives and to evaluate the effects on OS.

Acknowledgements

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