

# Tetramethylpyrazine inhibits osteosarcoma cell proliferation via downregulation of NF- $\kappa$ B *in vitro* and *in vivo*

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**Abstract.** Tetramethylpyrazine (TMP) is an effective component of the traditional Chinese medicine Chuanxiong, which has been reported to have beneficial effects in various types of cancer. However, the activity and mechanism of action of TMP in osteosarcoma (OS) have not been elucidated to date. The aim of the present study was to investigate the inhibitory effect of TMP on OS and its underlying mechanism of action. OS cells were treated with various concentrations of TMP for 48 h. BALB/c nude mice with OS were treated with an intraperitoneal injection of TMP at a dose of 100 mg/kg every other day for 28 days. Cell proliferation was evaluated using an MTT assay. Cell cycle and apoptosis were measured using flow cytometry. The protein expression of nuclear and cytosolic nuclear factor- $\kappa$ B (NF- $\kappa$ B) p65, BCL-2 and cyclin D1 was measured using western blot analysis. TMP inhibited the proliferation of OS cells (MG-63, SAOS-2 and U2OS) in a dose-dependent manner. Additionally, TMP significantly induced apoptosis and G0/G1 arrest in MG-63 OS cells ( $P < 0.05$ ). TMP upregulated the protein expression of cytosolic NF- $\kappa$ B p65, while downregulating the protein expression of nuclear NF- $\kappa$ B p65, BCL-2 and cyclin D1. Furthermore, TMP exerted a significant antitumor effect against OS in a xenograft tumor mouse model and exhibited a low toxicity. The present study provided fundamental evidence for the application of TMP in chemotherapy against OS.

## Introduction

Osteosarcoma (OS) is the most common nonhematological malignant bone tumor in children and adults, and its peak incidence is during adolescence (1-4). Currently, chemotherapeutic regimens for human OS treatment involve the

combination of multiple chemotherapeutic agents, including high-dose methotrexate with leucovorin rescue, doxorubicin, cisplatin (DDP) and ifosfamide either with or without etoposide (5). Although these regimens have remained the mainstay of OS chemotherapy for decades, none have provided any major improvement in survival compared with the original combination reported by Rosen *et al* (6,7). Furthermore, these regimens have only been demonstrated to be efficient in the treatment of localized OS, while they were shown to perform poorly in the treatment of metastatic and recurrent OS (5).

The process of carcinogenesis is driven by several growth factors and pro-inflammatory cytokines, which are released by the tumor microenvironment and neighboring cells of the innate immune system. These cytokines, including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), trigger the activation of the transcription factor nuclear factor- $\kappa$ B (NF- $\kappa$ B), which in turn has been shown to induce the expression of pro-inflammatory cytokine genes, to include interleukin-6 (IL-6), IL-8 and TNF- $\alpha$ , and affect tumor cell proliferation, apoptosis, angiogenesis and tumor invasion (8).

Among the novel antineoplastic drugs that are currently under investigation, bioactive natural products have emerged and gained considerable research attention. Tetramethylpyrazine (TMP), an effective component of the traditional Chinese medicine Chuanxiong, has been used in the treatment of neurovascular and cardiovascular diseases. TMP has been reported to have a beneficial effect in various types of cancer, including glioma (9-10), lung cancer (11), breast cancer (12), ovarian cancer (13), hepatocellular cancer (14), leukemia (15) and melanoma (16). Additionally, TMP was found to have anti-inflammatory actions in a rat model of spinal cord ischemia-reperfusion injury (17). However, the effects and underlying mechanism of action of TMP in OS have not been elucidated to date.

The aim of the present study was to assess the antitumor effects of TMP against OS and to investigate its underlying biological mechanisms.

## Materials and methods

**Cell culture and reagents.** Human OS cell lines MG-63, SAOS-2 and U2OS were obtained from the Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences, Shanghai,

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China). The OS cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco) in a humidified incubator at 37°C supplied with 5% CO<sub>2</sub>. Only cells in the exponential growth phase were used in this study. TMP and DDP were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies against p65, Bcl-2 and cyclin D1 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The study was approved by the ethics committee of China Medical University (Shenyang, China).

**Cell viability assay.** TMP cytotoxicity was assessed using an MTT assay (Sigma Chemical Co.), which measured the metabolic activity of viable cells. The cells were dislodged and suspended in medium, and an appropriate number of cells were added to 96-well plates prior to treatment. Following treatment with TMP for 48 h, MTT was freshly prepared and added to each well at a final concentration of 0.5 mg/ml. After incubation for 4 h, formazan crystals were dissolved in 100 µl DMSO, and the optical density was determined using an ELISA reader (BioTek Instruments, Inc., Winooski, VT, USA) at 570 nm. All the measurements were repeated in triplicate. The inhibitory ratio was calculated using the following equation: Inhibitory ratio (%) = (OD<sub>control group</sub> - OD<sub>drug-treated group</sub>)/OD<sub>control group</sub>.

**Cell cycle analysis and apoptosis assay.** The cells were harvested following treatment with TMP for 48 h. For cell cycle analysis, the cells were washed twice with PBS, resuspended in 100 µl propidium iodide (PI) solution and incubated for 30 min in the dark. The distribution of cells with differing DNA content was analyzed on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences, San Jose, CA, USA) at an excitation wavelength of 530 nm. Fluorescence emission was measured using a 620-nm bandpass filter. Apoptosis was assessed by labeling cells with annexin V-FITC and PI. Briefly, the cells were harvested following treatment with TMP for 48 h, then washed twice with cold PBS, resuspended in binding buffer and stained with 5 µl annexin V-FITC and 5 µl PI solution (BD Pharmingen, San Diego, CA, USA) for 15 min at room temperature in the dark. Following incubation, 400 µl binding buffer was added and the cells were analyzed by flow cytometry (BD Biosciences). All the measurements were repeated in triplicate.

**Xenograft tumor model.** A xenograft tumor model was established as previously described (18). Female BALB/c nude mice (4 to 6 weeks old) were obtained from China Medical University and all the procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals (19). Cells (5x10<sup>6</sup>) resuspended in 0.2 ml PBS were subcutaneously inoculated into the lower right flank of nude mice. When the developing tumors reached 100 mm<sup>3</sup> in size, treatment was initiated by intraperitoneal injection of vehicle (0.9% saline), TMP (100 mg/kg) or DDP (1 mg/kg) every other day for 28 days. Tumor size and body weight were measured every 3 days with calipers, and the tumor volume (V) was calculated using the following formula: V = length x width x height x 0.5236 (20). Following the last dose of TMP or DDP, all the mice were sacrificed and the tumors were weighed. One section of the tissue was fixed in formalin and another section was frozen using liquid nitrogen.

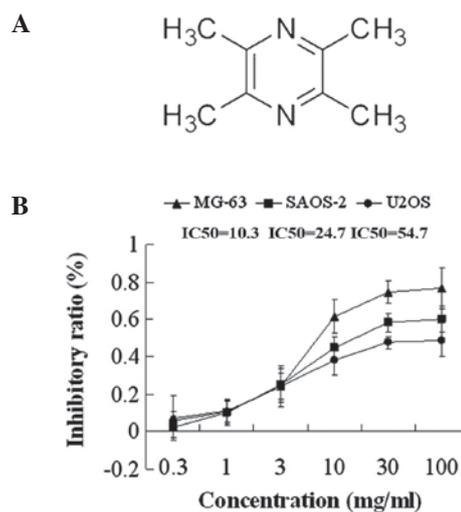


Figure 1. Effect of tetramethylpyrazine (TMP) on the proliferation of osteosarcoma (OS) cells *in vitro*. Cell viability was assessed using an MTT assay. (A) Chemical structure of TMP. (B) OS cells (MG-63, SAOS-2 and U2OS) were treated with TMP at various concentrations (0.3, 1, 3, 10, 30 and 100 mg/ml) for 48 h. Measurements were repeated in triplicate. Data are expressed as the mean ± SD.

**Western blot analysis.** Nuclear and cytoplasmic proteins were extracted using the Nuclear-Cytosol Extraction kit (Applygen Technologies, Inc., Beijing, China) according to the manufacturer's instructions. Protein concentration was quantified using the BCA protein assay kit (Santa Cruz Biotechnology, Inc.). For western blot analysis, equal amounts of total protein were boiled and then separated by SDS-PAGE. Following electrophoresis, proteins were blotted onto PVDF membranes and blocked for 1 h at room temperature. Each membrane was incubated with appropriate primary antibodies at 4°C overnight. The blots were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Protein bands were detected on X-ray films using an enhanced chemiluminescence detection system.

**Statistical analysis.** The Statistical Package for the Social Sciences (SPSS) version 13.0 was used to analyze the data. The data are expressed as the mean ± SD, and the means of different groups were compared using a one-way analysis of variance (ANOVA) test. P<0.05 was considered to indicate a statistically significant difference.

## Results

**TMP inhibits the proliferation of OS cells *in vitro*.** To investigate the effects of TMP, a cell proliferation assay using MTT was performed on OS cells (MG-63, SAOS-2 and U2OS). All the OS cell lines were treated with various concentrations of TMP (0.3, 1, 3, 10, 30 and 100 mg/ml) for 48 h. The MTT assay demonstrated that treatment with TMP inhibited the proliferation of OS cells in a dose-dependent manner; the IC<sub>50</sub> for 48 h was 10.3, 24.7 and 54.7 mg/ml in MG-63, SAOS-2 and U2OS cells, respectively (Fig. 1).

**TMP induces apoptosis and G1/G0 arrest in MG-63 OS cells *in vitro*.** As shown in Fig. 2, MG-63 OS cells were treated

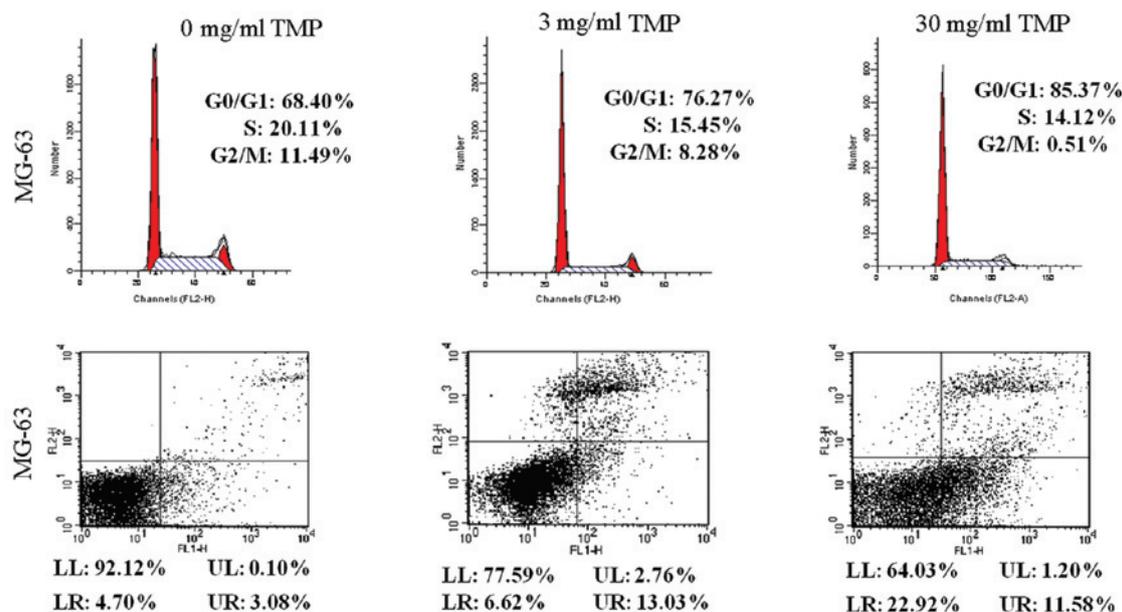


Figure 2. Tetramethylpyrazine (TMP) induced apoptosis and G1/G0 arrest in MG-63 osteosarcoma (OS) cells *in vitro*. MG-63 OS cells were exposed to TMP at a final concentration of 3 or 30 mg/ml for 48 h. G1/G0 phase cell cycle arrest after 48 h of exposure to TMP was visualized using propidium iodide (PI) staining and analyzed by FACS. Apoptosis following 48 h of exposure to TMP was measured using PI and annexin V-FITC labeling and FACS analysis. Measurements were repeated in triplicate. Data are expressed as the mean  $\pm$  SD. DDP, cisplatin; LL, lower left; UL, upper left; LR, lower right; UR, upper right.

with TMP (3 and 30 mg/ml) for 48 h. G1/G0 arrest and apoptosis induced by TMP were evaluated by flow cytometry. Results showed that treatment with TMP (3 and 30 mg/ml) significantly increased G1/G0 arrest (76.27 and 85.37%, respectively, vs. 68.40%) and apoptosis (19.65 and 34.50%, respectively, vs. 7.78%) in MG-63 cells, when compared with control cells ( $P < 0.05$ ).

*TMP inhibits protein expression of cytosolic and nuclear NF- $\kappa$ B p65, BCL-2 and cyclin D1 in MG-63 OS cells in vitro.* To investigate the mechanism by which TMP acts on MG-63 OS cells, the protein expression of cytosolic and nuclear NF- $\kappa$ B p65, BCL-2 and cyclin D1 was determined by western blot analysis. Results showed that TMP treatment (3 and 30 mg/ml) upregulated the protein expression of cytosolic NF- $\kappa$ B p65, while downregulating the protein expression of nuclear NF- $\kappa$ B p65, cyclin D1 and BCL-2 in MG-63 OS cells ( $P < 0.05$ ; Fig. 3).

*TMP exerts antitumor effects against OS in a xenograft nude mouse model.* The inhibitory potential of TMP on the growth of subcutaneously implanted MG-63 OS cells in nude mice was investigated. The experimental protocol is shown in Fig. 4A. TMP administered at a dose of 100 mg/kg every other day for 28 days was found to significantly inhibit the growth of tumors compared with that in the vehicle group ( $P < 0.05$ ). DDP administered at a dose of 1 mg/kg every other day for 28 days also effectively inhibited tumor growth compared with that of vehicle-treated tumors ( $P < 0.05$ ; Fig. 4B and C). However, DDP significantly reduced the body weight of mice ( $P < 0.05$ ), while TMP had a smaller effect on the body weight of mice (Fig. 4D).

*TMP inhibits protein expression of cytosolic and nuclear NF- $\kappa$ B p65, BCL-2 and cyclin D1 in OS tissues.* We also evalu-

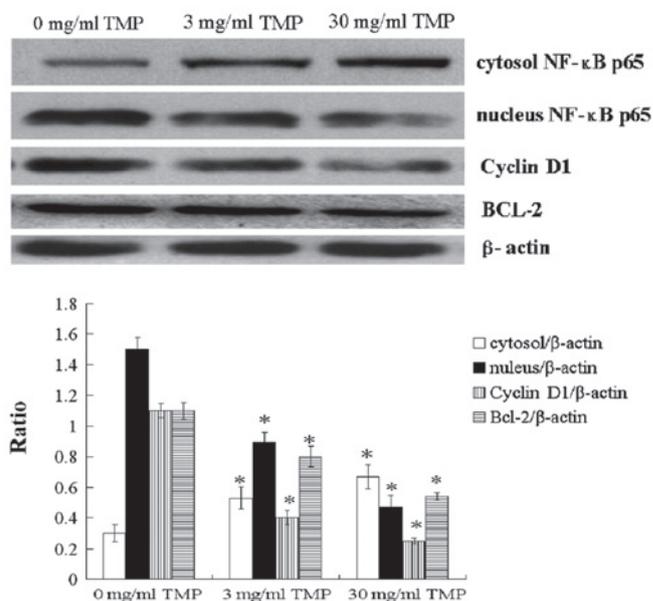


Figure 3. Tetramethylpyrazine (TMP) inhibited protein expression of cytosolic and nuclear NF- $\kappa$ B p65, BCL-2 and cyclin D1 in MG-63 osteosarcoma (OS) cells *in vitro*. MG-63 OS cells were exposed to TMP at a final concentration of 3 or 30 mg/ml for 48 h. Cytoplasmic and nuclear proteins were prepared and western blot analysis was conducted as described in Materials and methods to detect the protein expression of cytosolic and nuclear NF- $\kappa$ B p65, BCL-2 and cyclin D1 ( $n = 5$ ). Measurements were repeated in triplicate. Data were expressed as the mean  $\pm$  SD. \* $P < 0.05$  compared with the control cells. NF- $\kappa$ B p65, nuclear factor- $\kappa$ B p65.

ated the effect of TMP on the protein expression of cytosolic and nuclear NF- $\kappa$ B p65, BCL-2 and cyclin D1 in OS tissues. As shown in Fig. 5, TMP administered at a dose of 100 mg/kg every other day for 28 days was effective in suppressing the

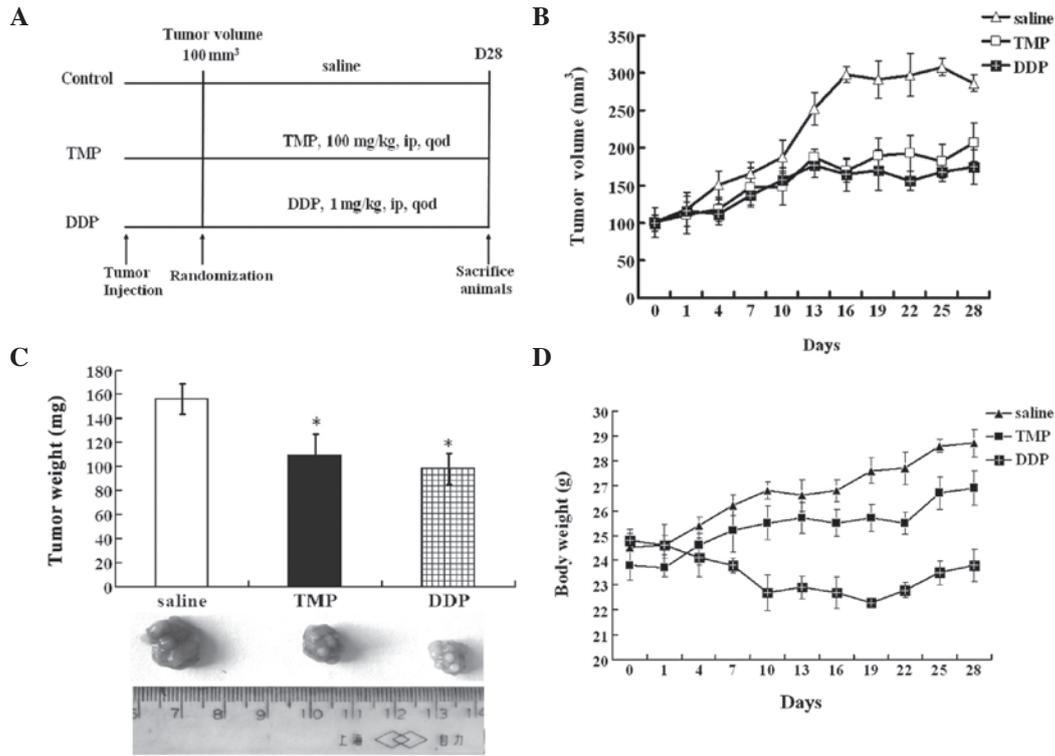


Figure 4. Tetramethylpyrazine (TMP) exerted antitumor effects against osteosarcoma (OS) in a xenograft nude mouse model. (A) Schematic representation of the experimental protocol described in Materials and methods. (B) Tumor volumes in mice were measured throughout the experiment and calculated using the following formula: length x width x height x 0.5236 (n=5). (C) On the last day of the experiment, the mice were sacrificed and tumor weights were measured (n=5). (D) The body weights of the nude mice were measured during the course of experiment (n=5). \*P<0.05 compared with the control tumors. DDP, cisplatin.

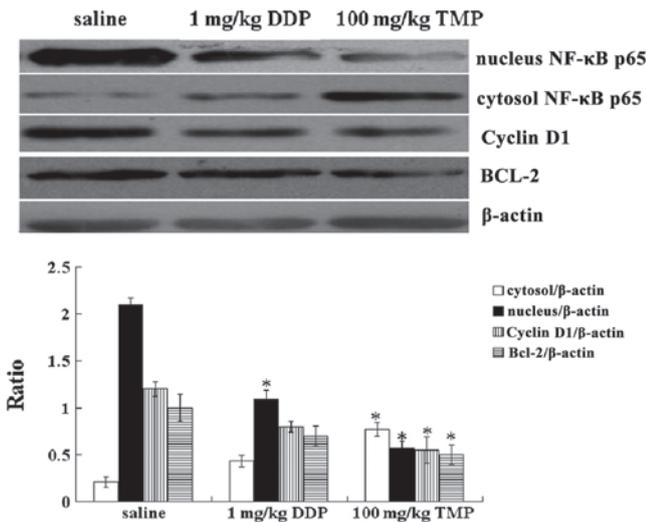


Figure 5. Tetramethylpyrazine (TMP) inhibited protein expression of cytosolic and nuclear NF-κB p65, BCL-2 and cyclin D1 in osteosarcoma (OS) tissues. BALB/c nude mice with OS were treated with 100 mg/kg TMP every other day for 28 days. The mice were sacrificed and the tumors were frozen in liquid nitrogen. Cytoplasmic and nuclear proteins were then prepared and western blot analysis was conducted as described in the Materials and methods to detect the expression of cytosolic and nuclear NF-κB p65, BCL-2 and cyclin D1 in OS tissues (n=5). Measurements were repeated in triplicate. Data are expressed as the mean ± SD. \*P<0.05 compared with the control cells. DDP, cisplatin; NF-κB p65, nuclear factor-κB p65.

protein expression of nuclear NF-κB p65, cyclin D1 and BCL-2, while increasing the protein expression of cytosolic NF-κB p65 in OS tissues (P<0.05).

Discussion

OS is a heterogeneous group of malignancies characterized by varying degrees of mesenchymal differentiation. The unifying histological feature of OS is the presence of malignant osteoid produced by neoplastic cells (21). Successful clinical management of OS faces two major challenges. Firstly, the toxic and adverse effects associated with chemotherapy may significantly reduce patient quality of life, although preoperative and postoperative chemotherapy regimens have improved the 5-year survival rate (22). Secondly, OS has a high rate of recurrence and metastasis, which causes the majority of OS-related mortalities (22). Thus, there is an urgent requirement to identify less toxic and more efficacious treatment alternatives. As a result, increasing attention has been focused on the application of natural products in the treatment of OS.

TMP (molecular weight, 136.19), one of the major bioactive components purified from the Chinese herb *Ligusticum wallichii* Franch., has been widely used in the treatment of cardiovascular and cerebral diseases in China (23). However, a number of additional pharmacological effects of TMP have been identified; TMP has been found to act as an anti-inflammatory agent in a rat asthma model (24). Fu *et al* (9) also reported that TMP affects the growth and migration of a glioma cell line by inhibiting calcium influx. Similarly, Chen *et al* (15) showed that TMP inhibits melanoma metastasis *in vivo* partly through suppressing vascular endothelial growth factor (VEGF) activity. Therefore, TMP may constitute a potentially effective option for the treatment of inflammation and tumors. Furthermore, TMP has been reported to have a beneficial effect on various types

of cancer (9-16). However, the function and underlying mechanisms of TMP in OS have not been elucidated to date.

The aim of the present study was to investigate whether TMP had antitumor activity against human OS. TMP was found to suppress the proliferation of OS cell lines in a dose-dependent manner. These inhibitory effects of TMP were correlated with TMP-induced cell apoptosis and cell cycle arrest at the G1/G0 phase. It was also shown for the first time that the intraperitoneal administration of TMP effectively suppressed the growth of OS in a xenograft mouse model, and had a smaller effect on the body weight of mice compared with DDP treatment. These results indicate that TMP possesses more effective antitumor activities against OS and has a lower toxicity.

The NF- $\kappa$ B transcription factor is important in a number of cellular processes, particularly in inflammation and tumor development. NF- $\kappa$ B is normally sequestered in the cytoplasm by a family of inhibitory proteins, known as inhibitors of  $\kappa$ B (I $\kappa$ B). A wide variety of stimuli cause the phosphorylation of I $\kappa$ B $\alpha$ , which is followed by its ubiquitination and subsequent degradation. The loss of I $\kappa$ B $\alpha$  results in the release of free NF- $\kappa$ B unit p65, which translocates from the cytoplasm to the nucleus, where p65 activates the expression of target genes, including BCL-2, cyclin D1 and MMP-9. To investigate the underlying mechanisms of TMP against OS, the protein expression of cytosolic and nuclear NF- $\kappa$ B p65 and the NF- $\kappa$ B-regulated target genes, BCL-2 and cyclin D1, were detected. TMP was found to downregulate the expression of proteins associated with cell proliferation, including cyclin D1 and BCL-2, which may explain its potent antiproliferative effects on OS. Notably, TMP suppressed the expression of nuclear NF- $\kappa$ B p65 and increased the expression of cytosolic NF- $\kappa$ B p65. This may be associated with the inhibition of the NF- $\kappa$ B p65 translocation from the cytoplasm to the nucleus by TMP; however, additional evidence is required.

In conclusion, the present study showed that TMP exerted more effective antitumor activities against OS *in vitro* and *in vivo*, and that TMP had a lower toxicity. Furthermore, TMP induced cell apoptosis and cell cycle arrest at the G1/G0 phase and upregulated the protein expression of cytosolic NF- $\kappa$ B p65, while downregulating the protein expression of nuclear NF- $\kappa$ B p65, BCL-2 and cyclin D1; this may be the mechanism via which TMP exerts its effects in OS. However, further and more comprehensive studies are required to confirm this. These data strongly suggest for the first time that TMP may have a significant potential for application in the treatment of OS.

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