

Fangchinoline suppresses the proliferation, invasion and tumorigenesis of human osteosarcoma cells through the inhibition of PI3K and downstream signaling pathways

XIUCHENG LI^{1*}, ZHIFAN YANG^{1,2*}, WEIQI HAN¹, XUANYUAN LU¹,
SONGTAO JIN¹, WANLEI YANG¹, JIANLEI LI¹, WEI HE¹ and YU QIAN¹

¹Department of Orthopaedics, Shaoxing People's Hospital, Shaoxing Hospital of Zhejiang University, Shaoxing, Zhejiang 312000; ²The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, Wenzhou, Zhejiang 325027, P.R. China

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Abstract. Osteosarcoma is the most common malignant bone tumor. Most patients diagnosed with osteosarcoma are less than 20 years of age. Osteosarcoma cells proliferate rapidly and invade other tissues. At present, neoadjuvant chemotherapy is the primary pharmacodynamic strategy to prevent the progression of osteosarcoma. However, adverse effects of this strategy limit its long-term application. Previous research has shown that fangchinoline exerts antitumor effects on several types of tumor cells; however, its effect on osteosarcoma cells remains unknown. The present study evaluated the effects of fangchinoline on the proliferation, apoptosis, migration and invasion of osteosarcoma cells *in vitro* and on their tumorigenesis *in vivo* and determined the possible underlying mechanism of action. Fangchinoline-treated MG63 and U2OS cells showed significantly decreased proliferation and significantly increased apoptosis. Fangchinoline markedly suppressed the migration and invasion of the MG63 cells. Fangchinoline-treated MG63 cells showed significantly decreased expression of phosphoinositide 3-kinase (PI3K) and Akt^{p-Thr308}. Moreover, fangchinoline-treated MG63 cells showed downregulated expression of cyclin D1 and matrix metalloproteinase 2 and 9, which act downstream of PI3K, and upregulated expression of caspase-3 and caspase-8. Furthermore, fangchinoline suppressed the growth of subcutaneous osteosarcoma tumors in Balb/c mice subcutaneously injected with osteosarcoma cells. These findings suggest that fangchinoline inhibits the progression of osteosarcoma by suppressing the proliferation, migration and invasion and by

accelerating the apoptosis of osteosarcoma cells. In addition, our results suggest that the mechanism underlying the antitumor effects of fangchinoline involve the inhibition of PI3K and its downstream signaling pathways.

Introduction

Osteosarcoma is the most common primary malignant bone tumor (1). It is derived from primitive mesenchymal cells and commonly develops in the distal femur and proximal tibia (2). Osteosarcoma predominantly affects young people, in particular, adolescents aged below 20 years (3,4). The prognosis of osteosarcoma patients is poor with an overall 5-year survival rate below 30% in cases showing relapse and metastasis (5).

At present, neoadjuvant chemotherapy is the predominant treatment for osteosarcoma. Neoadjuvant chemotherapy combines surgery with pre- or post-operative multi-agent chemotherapy. Most patients require intensive chemotherapy with a combination of drugs such as methotrexate, cisplatin, doxorubicin, ifosfamide and etoposide (6). This leads to chemotherapy toxicity in normal tissues and cellular chemoresistance, which prevent the long-term application of this treatment (7,8). Introduction of several targeted therapeutic agents has markedly improved the results of preclinical and clinical trials on osteosarcoma (9). However, the high cost of these therapeutic agents limit their widespread application. Therefore, safe and effective therapeutic approaches are urgently needed for treating osteosarcoma.

Fangchinoline is a bisbenzylisoquinoline alkaloid with a complex structure (Fig. 1A) and is extracted from an alkaloid tetrandrine, found in a traditional Chinese medicinal plant belonging to the Menispermaceae family. Increasing evidence suggests that fangchinoline possesses various pharmacological activities (10,11). Current research has focused on the antitumor effects of fangchinoline and has shown that it exerts antitumor effects on lung cancer (12), breast cancer (13,14), hepatocellular carcinoma (15), chronic myelogenous leukemia (16), glioblastoma (17), gastric cancer (18), and prostate cancer cells (19). However, the antitumor effects of fangchinoline on osteosarcoma cells are unclear.

Correspondence to: Professor Yu Qian, Department of Orthopaedics, Shaoxing People's Hospital, Shaoxing Hospital of Zhejiang University, 568 Zhongxing North Road, Shaoxing, Zhejiang 312000, P.R. China
E-mail: doctor120@hotmail.com

*Contributed equally

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Therefore, the present study was designed to evaluate the effects of fangchinoline on osteosarcoma cell lines MG63 and U2OS. The study evaluated the effects of fangchinoline on the proliferation, apoptosis, migration and invasion of osteosarcoma cells *in vitro* and on their tumorigenesis *in vivo* and determined the possible underlying mechanism of action.

Materials and methods

Cell culture. Human osteosarcoma cell lines MG63 and U2OS were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in an incubator with a humidified atmosphere of 5% CO₂. Cell medium was replaced with fresh medium containing dimethyl sulfoxide (DMSO) or 10, 20 or 30 μM fangchinoline for the following experiments. Changes in the morphology of the cells were determined using an inverted phase-contrast microscope.

Cell proliferation assay. Cell viability was evaluated by performing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described previously (20). Briefly, MG63 or U2OS cells were seeded in 96-well plates (cell density, 1x10⁴ cells/well) containing 0.1 ml medium supplemented with the indicated concentration of fangchinoline and were incubated for 24, 48 and 96 h. At each time point, 0.01 ml MTT solution (5 mg/ml in PBS) was added to each well, and the cells were incubated for 4 h at 37°C. The medium was replaced with DMSO for 10 min to solubilize crystals, and optical densities (ODs) were measured at 570 nm.

Hoechst 33258 staining. MG63 cells were treated with DMSO or 10, 20 or 30 μM fangchinoline for 24 h. Next, the cells were fixed with 4% polyoxymethylene, washed twice with PBS, and then incubated with 10 μg/ml Hoechst 33258 solution in the dark for 5 min at room temperature. Finally, the cells were washed three times with PBS and were observed under a fluorescence microscope.

Flow cytometry. Cell apoptosis was assessed using Annexin V-fluorescein isothiocyanate or Annexin V-propidium iodide (Annexin V-FITC/Annexin V-PI) apoptosis detection kit (Thermo Fisher Scientific, Waltham, MA, USA). MG63 cells were harvested, stained with Annexin V-FITC or Annexin V-PI, and analyzed using a FACScan flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA). Cells showing Annexin V(+)/PI(-) staining were considered as early apoptotic cells and those showing Annexin V(+)/PI(+) staining were considered as late apoptotic cells.

Scratch wound healing assay. MG63 cells were cultured in 6-well plates until confluency and were incubated in serum-free DMEM overnight before wounding. Scratches were made using a pipette tip, and the cells were treated with a medium containing DMSO or 10, 20 or 30 μM fangchinoline at 37°C for 24 and 48 h. Images of the wounded area were obtained and quantified using an optical microscope.

Transwell assay. Transwell assay was performed to evaluate cell invasion. The upper part of each Transwell chamber was coated with 1x10⁵ cells cultured in 0.1 ml serum-free DMEM supplemented with DMSO or 10, 20 or 30 μM fangchinoline, and the lower part of the chamber was filled with 0.6 ml DMEM containing 10% FBS. After incubation for 24 h at 37°C, the cells in the upper part of chamber were removed. Cells invading the lower part of the chamber were fixed, stained, and counted using a high-power microscope.

Western blot analysis. Total cellular proteins were extracted from 1x10⁶ cultured cells using 100 μl RIPA lysis buffer. Next, 60 μg of the protein samples were resolved by performing SDS-PAGE and were transferred onto nitrocellulose membranes through electroblotting. The membranes were blocked using 5% non-fat dry milk for 1 h and were probed by incubating overnight with the primary antibodies at 4°C. Next, the membranes were washed and incubated with HRP-conjugated secondary antibodies (Sigma-Aldrich, St. Louis, MO, USA) for 1 h. Immunoreactivity was detected using Western Lighting Ultra (ECL; Pierce Technology, Rockford, IL, USA). The following primary antibodies were used: i) rabbit polyclonal antibody against caspase-9 (ab32068) and monoclonal antibody against caspase-3 (ab32351) (Abcam, Cambridge, UK) at a dilution of 1:5,000; ii) rabbit polyclonal antibody against GAPDH (sc-25778) and monoclonal antibodies against phosphoinositide 3-kinase (PI3K; sc-7175), Akt (sc-135829), and Akt^{p-Thr308} (sc-135650) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at a dilution of 1:1,000; and iii) rabbit monoclonal antibodies against cyclin D1 (60186-1-1g), MMP-2 (10373-2-AP), and MMP-9 (10375-2-AP) (ProteinTech Group, Inc., Rosemont, IL, USA) at a dilution of 1:1,000.

Animal model of osteosarcoma. Six-week-old female Balb/c-nu-nu mice were purchased from Guangxi Medical University (Guangxi, China). All animal experimental procedures were approved by the Ethics Committee (2016/025) of the Shaoxing People's Hospital (Zhejiang, China) and were performed as described previously (12). The mice were randomly divided into fangchinoline treatment and control groups, with six mice in each group. All the mice were maintained under sterile conditions at 20-25°C room temperature, 50-60% relative humidity, and a 12 h light/dark cycle and were fed a sterilized diet. After habituation for 7 days, the mice were anesthetized by intraperitoneally injecting 12 mg/kg chloral hydrate. Next, 0.2 ml of the U2OS cell suspension (cell density, 1x10⁷ cells/ml) was subcutaneously injected into the left armpit of each mouse. Tumors became palpable 1 week after xenografting. Next, 0.1 ml fangchinoline solution (0.5 mg/ml) or 0.1 ml DMSO solution was locally injected into the tumors of each mouse three times per week. The mice were sacrificed 4 weeks after xenografting, and their tumors were harvested and weighed. The length and width of the tumors were measured to calculate tumor volume (tumor volume = length x width²/2).

Histological staining. The kidneys and livers of the mice were harvested, fixed, and embedded in paraffin. Histological sections were prepared for hematoxylin and eosin (H&E) staining, and images were obtained using a light microscope.

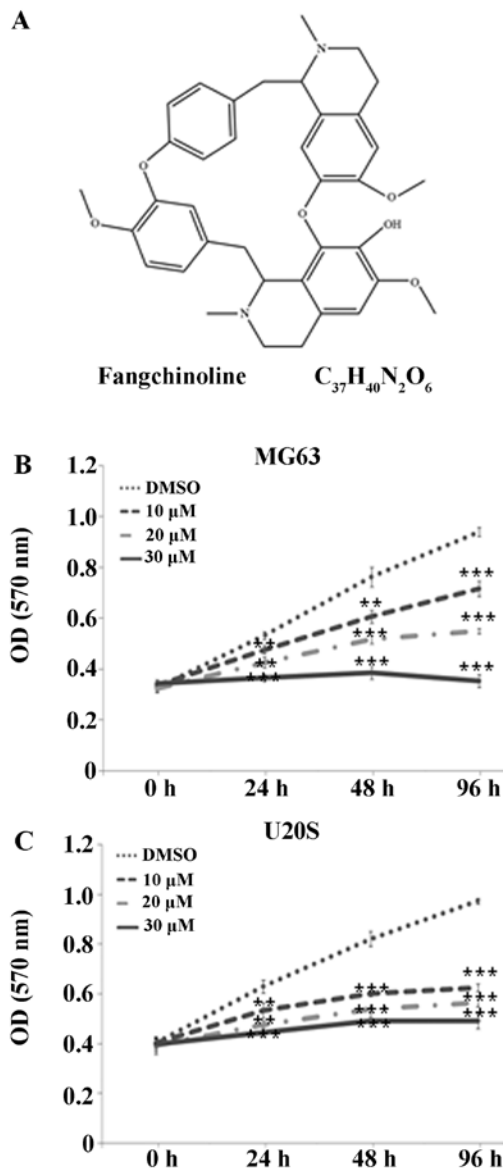


Figure 1. Fangchinoline suppresses the proliferation of osteosarcoma cells. (A) Molecular structure of fangchinoline. MG63 (B) and U2OS cells (C) were cultured with the indicated concentrations of fangchinoline for the indicated times in 96-well plates. Cell viability was determined by performing the MTT assay, and results are expressed as mean \pm SD of three experiments performed in triplicate. ** $P < 0.01$ and *** $P < 0.001$.

Statistical analysis. Data for all groups are presented as mean \pm SD. Differences between the groups were evaluated using one-way analysis of variance (ANOVA) with LSD test, and $P < 0.05$ was considered statistically significant. Statistical analysis was performed using SPSS software version 19.0.

Results

Fangchinoline decreases the viability of osteosarcoma cells. Fangchinoline markedly suppressed the proliferation of human osteosarcoma cell lines MG63 and U2OS in a dose-dependent manner. The MTT assay showed that the ODs of the MG63 cells treated with 10, 20 and 30 μ M fangchinoline significantly decreased to 0.72 ± 0.03 ($P < 0.001$), 0.55 ± 0.01 ($P < 0.001$), and 0.35 ± 0.03 ($P < 0.001$), respectively, after 96 h compared with the OD of the control cells (0.94 ± 0.02 ; Fig. 1B). Similar trends

were observed at the 24 and 48 h time points. The experiment was repeated using U2OS cells, which responded similarly to the inhibitory effects of fangchinoline (Fig. 1C).

Fangchinoline increases the apoptosis of osteosarcoma cells. Fangchinoline treatment significantly accelerated the apoptosis of MG63 and U2OS cells in a dose-dependent manner. Fangchinoline-untreated MG63 and U2OS cells were rhombus-like and angular, as determined by performing inverted phase-contrast microscopy, and were attached to the culture plates along with few dead cells that were round and suspended (Fig. 2A). Treatment with 10, 20 and 30 μ M fangchinoline markedly increased the number of apoptotic cells to 177 ± 19.1 ($P < 0.001$), 322 ± 21.5 ($P < 0.001$), and 418 ± 8.9 ($P < 0.001$), respectively, compared with that in the control cells (8.7 ± 5.7 ; Fig. 2B). Similar trends were observed for U2OS cells (Fig. 2C and D). Hoechst 33258 staining showed that fangchinoline treatment of MG63 cells induced the formation of apoptotic nuclei and condensed their chromatin, which appeared bright blue (Fig. 2E). Results of the flow cytometry showed that the percentages of late apoptotic cells increased to 10.35 ± 0.55 ($P = 0.001$), 12.77 ± 0.69 ($P < 0.001$) and $30.56 \pm 0.80\%$ ($P < 0.001$) in the 10, 20 and 30 μ M fangchinoline-treated MG63 cells, respectively, compared with $7.23\% \pm 0.15\%$ in the control cells (Fig. 2F). A similar trend was observed for the percentages of early apoptotic MG63 cells.

Fangchinoline suppresses the migration and invasion of MG63 cells. Fangchinoline significantly inhibited the migration and invasion of MG63 cells in a dose-dependent manner. Distances traveled by 10, 20 and 30 μ M fangchinoline-treated MG63 cells decreased to 0.86 ± 0.03 ($P < 0.001$), 0.50 ± 0.03 ($P < 0.001$), and 0.43 ± 0.03 mm ($P < 0.001$), respectively, compared with the distance travelled by the control cells (1.18 ± 0.02 mm) at the 48 h time point (Fig. 3A and B). Similar trends were observed at the 24 h time point. The Transwell assay showed that the number of 10, 20 and 30 μ M fangchinoline-treated MG63 cells traversing through the polycarbonate membrane decreased to 79 ± 4.6 ($P < 0.001$), 44 ± 7.0 ($P < 0.001$) and 30 ± 3.5 ($P < 0.001$), respectively, compared with the number of control cells (115 ± 4.5 ; Fig. 3C and D).

Fangchinoline inhibits the PI3K/Akt signaling pathway. Fangchinoline treatment markedly decreased PI3K and Akt^{p-Thr308} expression in a dose-dependent manner but did not alter the total Akt level. Moreover, fangchinoline treatment markedly decreased the level of cell proliferation regulator cyclin D1 but obviously increased the expression of caspase-9 and caspase-3. In contrast, fangchinoline treatment significantly decreased MMP-2 and MMP-9 expression in the MG63 cells (Fig. 4A-C).

Fangchinoline inhibits the tumorigenesis of MG63 cells in vivo. The average size (178.23 ± 23.28 mm³, $P = 0.043$) and weight (0.19 ± 0.03 g, $P = 0.034$) of tumors in the fangchinoline-treated mice were significantly decreased compared with these parameters in the control mice (307.31 ± 43.29 mm³ and 0.33 ± 0.04 g, respectively; Fig. 5A-C). In addition, none of the mice died during the experimental period. Moreover, no histological impact was observed on the kidneys and livers of mice in both the fangchinoline and control groups (Fig. 5D).

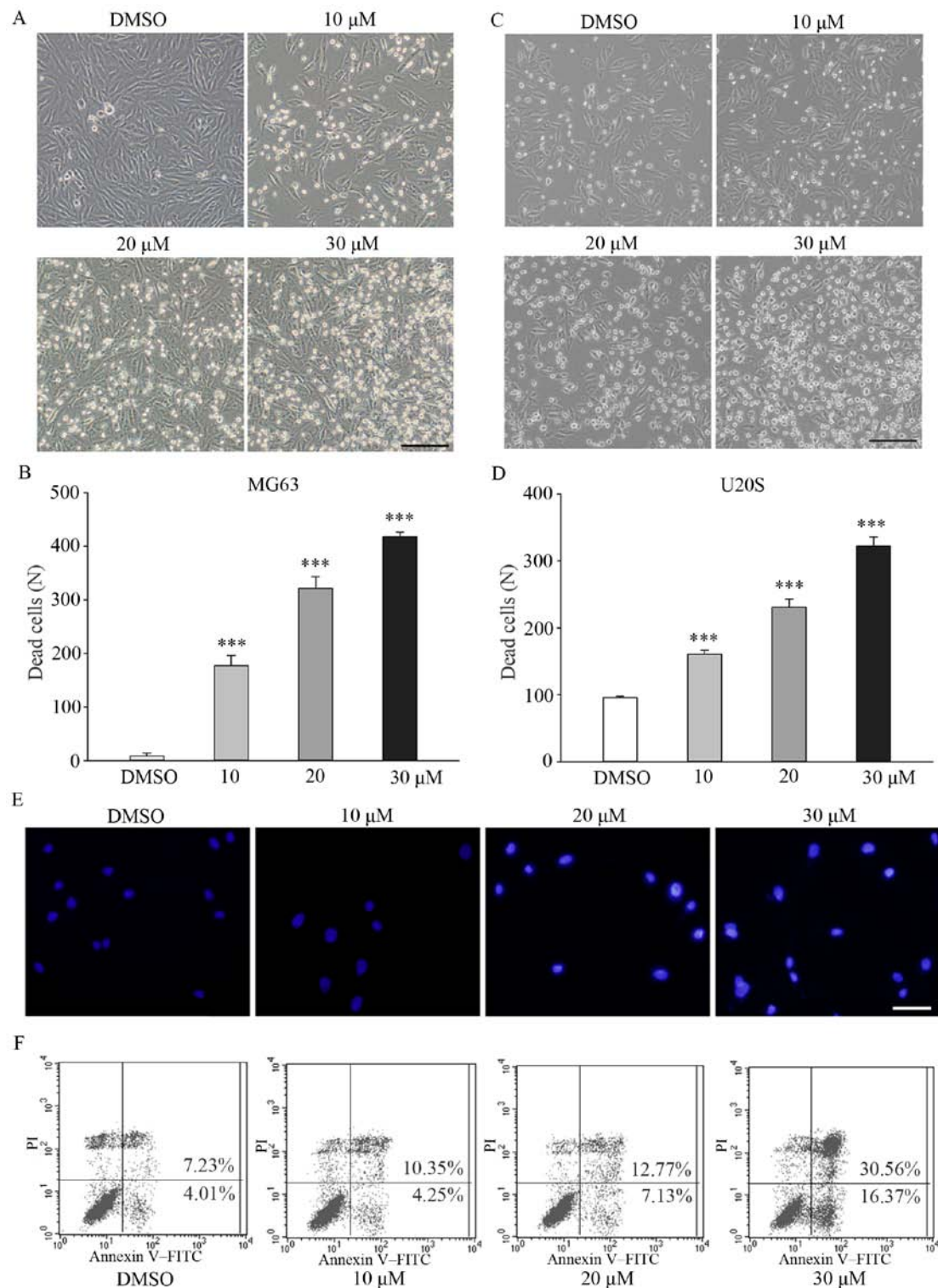


Figure 2. Fangchinoline increases the apoptosis of osteosarcoma cells. (A) Changes in the morphology of MG63 cells treated with DMSO or 10, 20 or 30 μM fangchinoline for 48 h were determined using an inverted phase-contrast microscope; scale bar, 100 μm . (B) Dead MG63 cells were counted, and results are presented as mean \pm SD. (C) Changes in the morphology of U2OS cell treated with DMSO or 10, 20 or 30 μM fangchinoline for 48 h were determined using an inverted phase-contrast microscope; scale bar, 100 μm . (D) Dead U2OS cells were counted, and results are presented as mean \pm SD; *** P <0.001. (E) MG63 cells were preincubated with DMSO or 10, 20 or 30 μM fangchinoline for 48 h. Next, the cells were stained with Hoechst 33258 solution and were observed under a fluorescence microscope; scale bar, 50 μm . (F) Flow cytometric analysis of MG63 cells treated with 0, 10, 25 or 30 μM fangchinoline for 24 h. Annexin V(+)/PI(-) cells were considered as early apoptotic cells.

Discussion

Previous studies have reported that fangchinoline exerts antitumor effects on several tumor cell lines by inhibiting

proliferation and by inducing apoptosis. However, its effects on osteosarcoma cells have not been elucidated to date. In the present study, we observed that fangchinoline suppressed the proliferation, migration and invasion of osteosarcoma

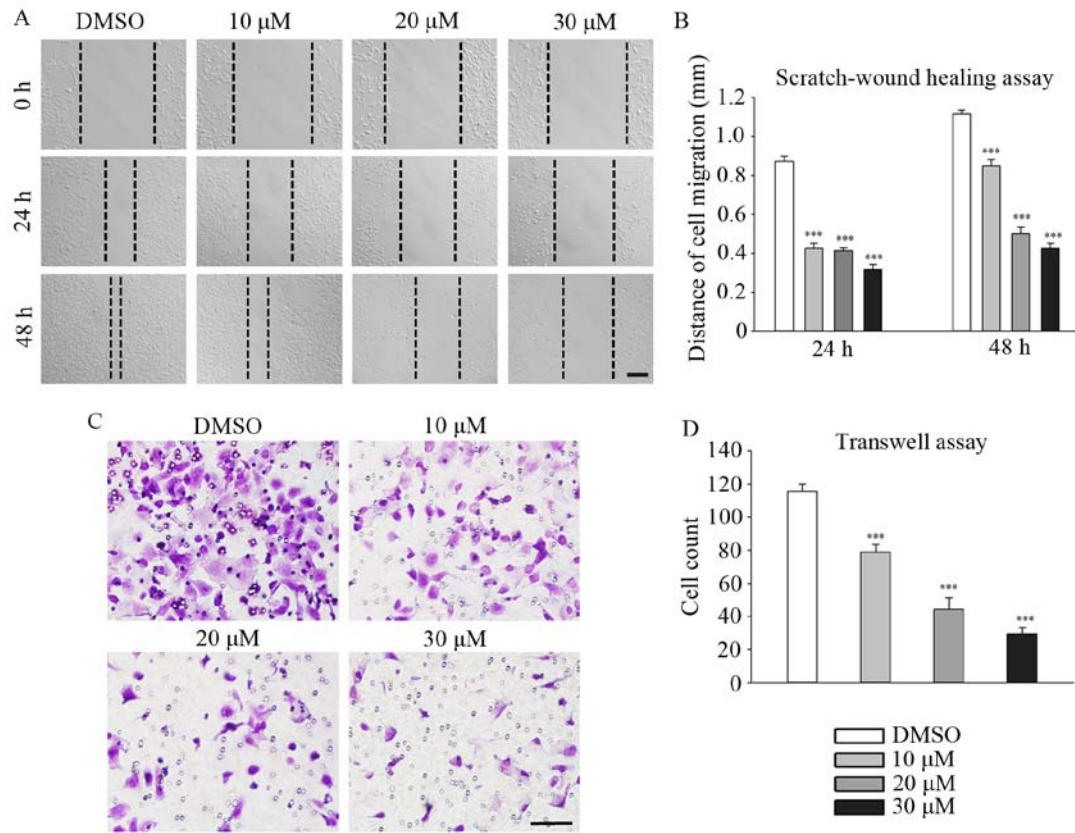


Figure 3. Fangchinoline inhibits the migration and invasion of osteosarcoma cells. (A) Representative microscopic images of the scratch wound healing assay showing the migration of MG63 cells treated with DMSO alone or with the indicated concentrations of fangchinoline for 24 and 48 h; scale bar, 100 μ m. (B) Measurement of cell migration distance. (C) Invasion of MG63 cells treated with DMSO or 10, 20 or 30 μ M fangchinoline for 48 h was determined by performing the Transwell assay; scale bar, 100 μ m. (D) Cells that passed through the polycarbonate membrane were counted, and results are presented as mean \pm SD. ***P<0.001.

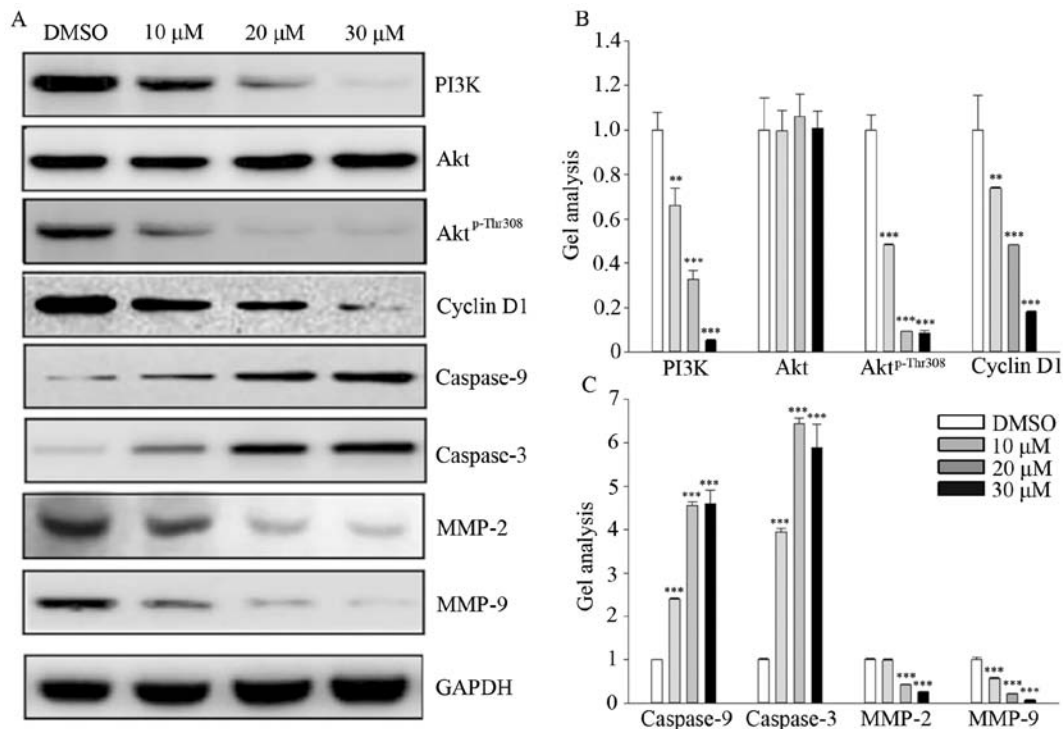


Figure 4. Fangchinoline inhibits the function of osteosarcoma cells by inhibiting the PI3K/Akt signaling pathway. (A) MG63 cells were treated with DMSO alone or with the indicated concentrations of fangchinoline for 48 h. Expression of PI3K, Akt, Akt^{p-Thr308}, cyclin D1, caspase-9, caspase-3, MMP-2, MMP-9, and GAPDH was detected by western blot analysis. (B and C) Results of quantitative western blot analysis were normalized using the protein expression profile of DMSO-treated cells. Data are presented as mean \pm SD. **P<0.01 and ***P<0.001. PI3K, phosphoinositide 3-kinase.

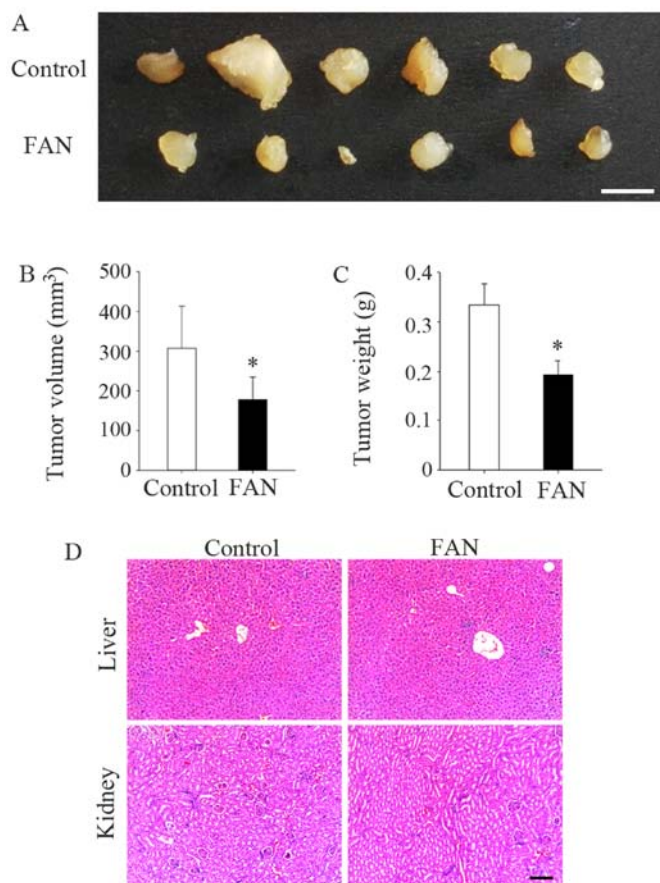


Figure 5. Fangchinoline inhibits the tumorigenesis of U2OS cells *in vivo*. (A) Representative images of tumors excised from the fangchinoline-treated and control nude mice at 4 weeks after xenografting; scale bar, 1 cm. (B) Tumor volumes and (C) tumor weights were measured after separation. Data are presented as mean \pm SD. * $P < 0.05$. (D) Representative images showing H&E-stained livers (upper) and kidneys (bottom) of the control and fangchinoline-treated mice. Scale bar, 100 μ m. FAN, fangchinoline.

cells lines MG63 and U2OS in a dose-dependent manner. We speculate that these effects are associated with the inhibition of PI3K and its downstream signaling pathways. Moreover, we observed that fangchinoline inhibited the growth of subcutaneous osteosarcoma tumors *in vivo*. These results suggest that fangchinoline exerts antitumor effects against osteosarcoma cell lines.

Chemotherapeutic drugs such as paclitaxel exert an obvious killing effect on osteosarcoma cells (21). However, drug resistance and toxic side-effects after long-term application remain the main obstacles for their clinical use. Fangchinoline is a well-known traditional Chinese medicine that is chemically extracted from the alkaloid tetrandrine. It is an effective agent for tumor treatment and prevention, with no significant toxicity or side-effects (18). Research on the functions of fangchinoline will provide a potential new treatment for osteosarcoma, with important clinical significance.

The PI3K/Akt signaling pathway mediates various cellular functions, including cell proliferation, apoptosis and invasion. The PI3K/Akt pathway plays a central role in regulating the functions of osteosarcoma cells (22) and is an important target for treating different tumors (23-25). Fangchinoline was found to effectively suppress the proliferation of SGC7901, MKN45, and human gastric cancer cell lines that highly express PI3K

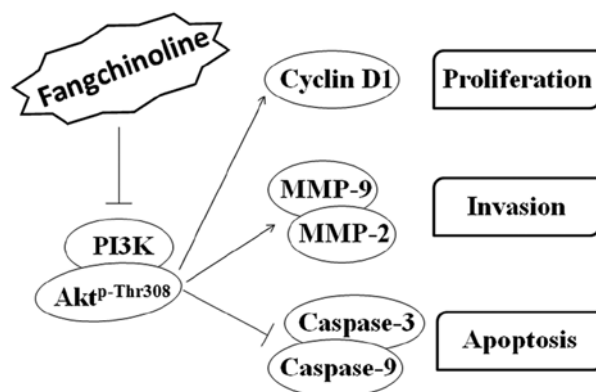


Figure 6. A schematic diagram showing that fangchinoline effectively suppresses proliferation and invasion and induces apoptosis by inhibiting PI3K and then suppressing PI3K-mediated signaling cascades. PI3K, phosphoinositide 3-kinase.

but to exert a negligible effect on human embryonic kidney 293 cells and normal cells that express low levels of PI3K (18), suggesting that fangchinoline is a novel inhibitor of PI3K and exerts antitumor effects by suppressing PI3K. Consistently, we found that fangchinoline treatment significantly decreased the expression of PI3K and Akt^{p-Thr308} in a dose-independent manner. These findings may explain why traditional Chinese medicines show negligible toxicity against normal cells. Tumor cells grow rapidly because of their activated cell cycle progression. Cyclin D1, a downstream target of the PI3K/Akt signaling pathway, is a key regulator of the G1-S checkpoint (26,27). Previous studies have shown that fangchinoline inhibits cell cycle progression in breast cancer cells by inhibiting the Akt/GSK-3 β /cyclin D1 signaling pathway (13). Moreover, levels of cyclin D3 and cyclin E and cyclin-dependent kinase 2, 4 and 6 are decreased and levels of their endogenous inhibitors p21^{WAF1} and p27^{Kip1} are increased in fangchinoline-treated breast cancer cells (14). In the present study, cyclin D1 expression markedly decreased after fangchinoline treatment. These results suggest that fangchinoline prevents cell cycle progression in osteosarcoma cells by inhibiting the PI3K/Akt/cyclin D1 signaling pathway, thereby inhibiting the proliferation of these cells.

Enhancement of tumor cell apoptosis is another effective strategy for treating tumors. Fangchinoline was found to promote the early apoptosis of human glioblastoma cells by inhibiting Akt-mediated Bax and caspase-9 expression (17), suggesting that fangchinoline serves as a caspase agonist and can be used as an alternative treatment for tumors. Interestingly, another study showed that fangchinoline did not induce apoptotic cell death but induced autophagic cell death in hepatoma cell lines HepG2 and PLC/PRF-5 through the p53/sestrin2/AMPK signaling pathway (15). These results suggest that fangchinoline induces cell death through different mechanisms and exerts specific effects on different tumor cells. In the present study, we observed that fangchinoline significantly accelerated the apoptosis of MG63 cells and markedly decreased the expression of caspase-8 and caspase-3. However, further studies are needed to elucidate whether fangchinoline induces autophagic cell death in osteosarcoma cells. Therefore, it is reasonable to speculate

that fangchinoline enhances the apoptosis of osteosarcoma cells.

Besides accelerated proliferation, osteosarcoma cells show early metastasis. During metastasis, MMPs play a major role in degrading the extracellular matrix, thus allowing tumor cells to migrate and accelerate metastatic progression (28). A previous study showed that fangchinoline effectively inhibited the proliferation and invasion of gastric cancer cell lines BGC823 and SGC7901 by inhibiting PI3K-induced MMP-9 and MMP-2 expression (18). Similarly, we observed that fangchinoline significantly suppressed the migration and invasion of MG63 cells by downregulating MMP-9 and MMP-2 expression. This finding suggests that fangchinoline can be used as an antimetastatic agent. Moreover, fangchinoline suppressed the migratory and invasive potential of lung cancer cell line A549 by inhibiting the FAK/MMP-9/MMP-2 pathway (12). However, it is unclear whether fangchinoline targets PI3K in A549 cells. These findings suggest that fangchinoline inhibits MMPs by inhibiting different upstream signaling pathways. As fangchinoline downregulated the expression of MMPs and PI3K/Akt in the present study, we speculate that fangchinoline inhibits the migration and invasion of osteosarcoma cells by inhibiting the PI3K/Akt/MMP-9/MMP-2 signaling pathway.

An animal model of osteosarcoma was used to evaluate the antitumor effect of fangchinoline *in vivo*. Fangchinoline was previously found to inhibit the growth of lung tumors formed by A549 cells in a nude mouse model (12). Similarly, fangchinoline significantly suppressed the growth of subcutaneous osteosarcoma tumors in the present study. Moreover, fangchinoline treatment did not exert any toxic side-effects, as evidenced by the consistent histology of the kidneys and livers of mice in both the fangchinoline-treatment and control groups.

However, the present study has several limitations. First, a positive control group was required, such as cisplatin-treated osteosarcoma cells. Second, fangchinoline was injected locally into the tumors of the mouse models of osteosarcoma in the present study; however, it is unclear whether intravenous or oral administration of fangchinoline will exert the same effect. Third, the results of the H&E staining did not precisely show the effect of fangchinoline on the physiological functions of the livers and kidneys of treated mice. Moreover, further clinical trials are needed to assess whether systemic treatment with moderate doses of fangchinoline affects bone metabolism.

In conclusion, the present study showed that fangchinoline suppressed the proliferation, migration, and invasion and enhanced the apoptosis of osteosarcoma cells. In addition, fangchinoline inhibited the growth of osteosarcoma tumors *in vivo* by possibly inhibiting PI3K and Akt and their downstream signaling pathways (Fig. 6). Thus, fangchinoline may serve as an auxiliary therapeutic agent for treating osteosarcoma.

Acknowledgements

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