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ORIGINAL ARTICLE

Matrine induces caspase-dependent apoptosis in human osteosarcoma cells in vitro and in vivo through the upregulation of Bax and Fas/FasL and downregulation of Bcl-2

Cheng Zhen Liang · Jia Kai Zhang · Zhongli Shi · Bing Liu · Chengchun Q. Shen · Hui Min Tao

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Abstract
Purpose Matrine, one of the main active components of extracts from the dry roots of *Sophora flavescens*, has potent anti-tumor activity in various cancer cell lines. However, the activity of matrine against osteosarcoma remains unclear. In the present study, we examined the effects of matrine on human osteosarcoma cells and explored the underlying mechanism.
Methods Four human osteosarcoma cell lines: MG-63, U-2OS, Saos-2, and MNNG/HOS were treated by matrine and subjected to MTT assay, annexin V-FITC/PI double staining, and TUNEL assay. The activation of caspases and the expression of pro-apoptotic and anti-apoptotic factors were examined by qRT-PCR and Western blot. In addition, MNNG/HOS xenograft tumors were established in female nude BALB/c mice, and matrine was intraperitoneally (i.p.) administered to evaluate the anti-cancer capacity of matrine in vivo.
Results We found that matrine inhibited the proliferation and induced apoptosis of the four osteosarcoma cell lines in vitro and induced the activation of caspase-3, -8, and -9 in a dose-dependent manner. Furthermore, the pro-apoptotic factors Bax and Fas/FasL were upregulated, and the anti-apoptotic Bcl-2 was downregulated. More importantly our in vivo, studies showed that administration of matrine decreased tumor growth in a dose-dependent manner. Immunohistochemistry analysis demonstrated the downregulation of Bcl-2 and upregulation of Bax and Fas/FasL in MNNG/HOS tumor tissues following matrine treatment, consistent with the in vitro results.
Conclusion Our results demonstrate that matrine inhibits the proliferation and induces apoptosis of human osteosarcoma cells in vitro and in vivo. The induction of apoptosis appears to occur through the upregulation of Fas/FasL and Bax, downregulation of Bcl-2, and activation of caspase-3, -8, and -9, which then trigger major apoptotic cascades.

Keywords Matrine · Apoptosis · Caspase · Bcl-2 family · Fas/FasL · Osteosarcoma

Introduction

Osteosarcoma, the most common type of malignant bone tumor, occurs predominantly in adolescents and young adults. The therapeutic modality that is currently favored involves neoadjuvant chemotherapy, followed by surgical resection, and the 5-year survival of patients with osteosarcoma has increased to 60% through the use of such protocols. Induction of apoptosis is one goal in treating neoplasms, and recent studies have focused on the testing of novel drugs for their ability to induce and enhance apoptosis [1–3].

Apoptosis is a form of cell death coordinated by a network of genes and is a key target in the development of new anti-cancer therapies. Apoptosis can be activated through at least two signaling pathways: caspase dependent and caspase independent [4]. Both pathways involve mitochondria

C. Z. Liang · J. K. Zhang · B. Liu · C. Q. Shen · H. M. Tao (✉)
Department of Orthopedics, 2nd Affiliated Hospital,
School of Medicine, Zhejiang University, #88 Jie Fang Road,
Hangzhou 310009, Zhejiang, People's Republic of China
e-mail: huimintao_zrgk@163.com

Z. Shi
Institute of Orthopedic Research, 2nd Affiliated Hospital,
School of Medicine, Zhejiang University,
Hangzhou, People's Republic of China

and Bcl-2 family proteins. The Bcl-2 family comprises both pro-survival and pro-apoptotic proteins. Pro-survival Bcl-2 family members include the following: Bcl-2, Bcl-xL, Mcl-1, A1, and Bcl-W, while pro-apoptotic members include the following: Bad, Bid, Bim, Puma, Bmf, Bax, Bak, and Bok [5].

Caspase activation is regulated by various cellular factors, including "inhibitor of apoptosis proteins" (IAPs), pro-apoptotic Bcl-2 family members, and/or the Fas/Fas ligand (FasL) system. The Fas/FasL system is an important apoptosis signal transduction pathway in which a ligand–receptor interaction activates the cell death pathway [6]. Fas, a member of the TNF family, is a 45 kDa type I transmembrane protein that induces apoptosis in susceptible cells by cross-linking its ligand. After trimerization of Fas on the cell membrane by extracellular FasL, Fas-associated death domain (FADD) and caspase-8 form the death-inducing signal complex that mediates Fas-induced cell death [7]. Once activated, caspase-8 activates effector caspases, including caspase-3, -6, and -7, ultimately leading to the hydrolysis of cytosolic and nuclear substrates.

Matrine, the molecular formula of which is $C_{15}H_{24}N_2O$, is one of the key components extracted from *Sophora flavescens*, a leguminous plant grown in China, Japan, and some European countries [8]. Matrine has been shown to produce a wide range of pharmacological effects and has been used in the treatment of viral hepatitis and isoproterenol-induced cardiotoxicity [9, 10]. It has not shown obvious toxicity or side effects.

Preliminary studies have shown that matrine has antitumor effects by inducing apoptosis and inhibiting proliferation of many different cancer cells including cervical, stomach, breast, and lung cancers, as well as hepatocellular carcinoma, leukemia, and multiple myeloma [11–15]. Additionally, it is considered a useful agent in the treatment of oesophageal and laryngeal cancers and murine hepatocellular carcinoma [16, 17]. The ability of matrine to inhibit tumor growth has been proposed to be through the modulation of apoptosis- and/or proliferation-related genes and proteins, including N-ras, p53, c-myc, E2F-1, Apaf-1, Rb, Bcl-2 family members, and caspases [12, 13]. Moreover, a recent study reported that matrine inhibited pancreatic cancer growth through the induction of Fas/FasL and suppression of Bcl-2/Bax ratio [18].

However, it remains unclear whether matrine also induces apoptosis in osteosarcoma. Therefore, in the present study, we examined the effects of matrine on human osteosarcoma cells and explored the underlying mechanism. Our results showed that matrine inhibited proliferation and induced apoptosis in human osteosarcoma cells in vitro and in vivo, and these effects were mediated through the induction of the pro-apoptotic molecules Bax and Fas/FasL and downregulation of Bcl-2.

Materials and methods

Reagents and antibodies

Matrine was purchased from Sigma–Aldrich, and its purity was >99% confirmed by high-performance liquid chromatography (HPLC). The molecular formula of matrine is $C_{15}H_{24}N_2O$, and its molecular weight is 248.36 (Fig. 1) [19]. It was dissolved in cell culture medium at a stock concentration of 20 mg/ml and stored at -20°C. Matrine stock solution was freshly diluted in the medium just before the use in each experiment. Mouse monoclonal antibodies specific for caspase-3, caspase-8, and caspase-9 were purchased from Cell Signaling Technology; antibodies specific for Bcl-2, Bax, Fas, FasL, and β-actin were from Santa Cruz Biotechnology. Horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies were obtained from Santa Cruz Biotechnology. Cisplatin, Hoechst 33258, and MTT were from Sigma–Aldrich. Female BALB/c mice weighing 16–18 g (age 30–40 days) were obtained from the Shanghai Pharmaceutical Institute of Academy of Science (Shanghai, China).

Cells and cell culture

The human osteosarcoma cell lines MG-63 (CRL-1427TM, ATCC), U-2OS (HTB-96TM, ATCC), Saos-2 (HTB-85TM, ATCC), and MNNG/HOS (CRL-1547TM, ATCC) were obtained from Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China), where they were tested and authenticated. These procedures include cross-species checks, DNA authentication, and quarantine. Cell lines used in the present study were in culture for less than 6 months. MG-63 and MNNG/HOS were cultured in Eagle's Minimum Essential Medium, supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin mixture. U-2OS was

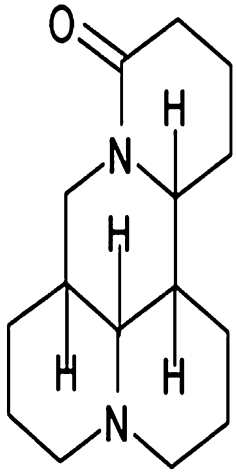


Fig. 1 The chemical structure of matrine. The molecular formula of matrine is $C_{15}H_{24}N_2O$, and its molecular weight is 248.36

cultured in McCoy's 5a Medium, supplemented with 10% FCS and 1% penicillin/streptomycin mixture. Saos-2 was cultured in McCoy's 5a Medium, supplemented with 15% FCS and 1% penicillin/streptomycin mixture. All the cells were cultured at 37°C in a humidified atmosphere (5% CO2, 95% air).

Cell proliferation assay by MTT assay and BrdU incorporation

MTT assay was employed to examine the viability of human osteosarcoma cells treated with matrine. Briefly, cells were seeded in 96-well plates at 4–8 $\times$ 10^3 cells/well in 200 μl medium and cultured for 12 h to allow attachment. They were treated with various concentrations of matrine (0–1.5 mg/ml) for different periods of time (0–72 h). Four hours before the end of each incubation period, MTT solution (5 mg/ml in 20 μl PBS) was added to each well and incubated for 4 h at 37°C. The growth medium was then removed and replaced with formazan dissolved in dimethyl sulfoxide (DMSO; 150 μl/well). A MR7000 microplate reader (Dynatech) was used to measure the absorbance of each well at 570 nm, and IC_{50} values were calculated using the probit model.

Moreover, cell proliferation was measured using a cell proliferation enzyme-linked immunosorbent assay (ELISA) kit (Roche Applied Science, USA).After treated as above description, cells were incubated with 20 μl BrdU labeling solution per well for 4 h, then dried, fixed, and detected using anti-BrdU mAb according to the manufacturer's instructions. Finally, the absorbance of the samples was measured by a microplate reader at 450 nm.

The inhibitory rate of cellular proliferation was calculated as $[1 - A_{570}\,(\text{test})/A_{570}\,(\text{control})] \times 100\%$. Data represented the mean of six replicates, each performed in triplicate.

Hoechst staining

Hoechst staining was employed to evaluate the apoptosis of human osteosarcoma cells treated with matrine. Briefly, the cells were exposed to matrine for 24 h and then stained with Hoechst 33258 (5–10 μg/ml) for 10 min. After being washed with PBS, they were observed using a fluorescence BX51 microscope (Olympus, Japan). Hoechst 33258 freely permeates cell membranes and stains nuclear DNA as blue. Apoptotic cells were identified by the presence of condensed or fragmented nuclei stained either blue or red, depending on apoptotic stage.

Annexin V-FITC/PI double staining

Annexin V-FITC/PI double staining was employed to quantify the apoptosis of human osteosarcoma cells treated with matrine. Briefly, cells were seeded in 6-well plates (2×10^5 cells/ml) and exposed to matrine (0.0–1.25 mg/ml) for 24 h. The cells were then stained using annexin V-FITC/PI double-fluorescence apoptosis detection kit (Biouniquer Technology) following the manufacturer's instruction. Samples were analyzed using a FACSCalibur flow cytometer within 1 h after the staining.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

TUNEL assay was employed to quantify the apoptosis of human osteosarcoma cells treated with matrine. Briefly, 5 $\times$ 105 cells were exposed to matrine (0.0–1.25 mg/ml) for 24 h. Then, apoptosis was detected using an in situ cell death detection reagent (Roche) following the manufacturer's instruction. Stained cells were analyzed using a flow cytometer (FACSCalibur, BD Biosciences).

Real-time quantitative RT–PCR analysis

For gene expression analysis, osteosarcoma cells (2×10^5 cells/well in a 6-well plate) were grown for 24 h and subsequently treated with required amount of matrine. RNA was isolated using Trizol (Invitrogen) according to the manufacturer's instructions. cDNA was generated from total denatured RNA (2 μg) using 1 μl of oligo$(dT)_{18}$ primer, 25 units of RNase inhibitor, 2 μl of dNTPs (10 mM), and a Moloney Murine Leukemia Virus reverse transcriptase cDNA synthesis kit (Promega, Madison, WI, USA). Quantitative real-time PCR was performed using Applied Biosystems 7500 Real-Time PCR System and SYBR® Premix Ex Taq™ kit Perfect Real Time) TaKaRa, Dalian, China. Human 18 s rRNA was used as internal control. The cycle threshold (CT) values for each gene were corrected using the mean CT value. Real-time PCR data were quantified using the ΔCT method with the formula: $n = 100 \times 2^{-(\Delta CT\ \text{targeted gene} - \Delta CT\ \text{GAPDH})}$. The primers used were 18 s rRNA forward: 5′GACTCAACACGGGAAACC TCAC3′ and reverse: 5′CCAGACAAATCGCTCCACC AAC3′, Bcl-2 forward: 5′ CGCCCTGTGGATGACTGA GTA 3′ and reverse: 5′ GGGCCGTACAGTTCCA CAAAG 3′, Bax forward: 5′ CCCTTTTGCTTCAGGGTT TCATCCA 3′ and reverse: 5′ CTTGAGACACTCGCTCA GCTTCTTG 3′, Fas forward: 5′GTTGGTGGACCCGCT CAGTA3′ and reverse: 5′AATCTAGCAACAGACGTAA GAACCAG3′, FasL forward: 5′GCAGCAGCCCTTCAA TTACCCAT3′ and reverse: 5′CACAGAGGTTGGACAG GGAAGAA3′, caspase 3 forward: 5′ GACAGACAGTGG TGTTGATGATGAC 3′ and reverse: 5′ GCATGGCACA AAGCGACTGGAT 3′, caspase 8 forward: 5′ GCTGGA GTGCAGTGGCGTGAT 3′ and reverse: 5′ GGGAGGCT GAGGCAGGAGAA 3′, caspase 9 forward: 5′ GCGAACT

AACAGGCAAGCAGCAA 3′ and reverse: 5′ CTCAAG AGCACCGACATCACCAAA 3′.

Immunoblotting

Briefly, cells were washed twice with ice-cold PBS, resuspended in 200 µl ice-cold solubilizing buffer (300 mM NaCl, 50 mM Tris–HCl, pH 7.6, 0.5% Triton X-100, 2 mM phenyl-methanesulfonyl fluoride (PMSF), 2 µl/ml aprotinin, 2 µl/ml leupeptin), and incubated at 4°C for 60 min. Lysates were collected after centrifuging at 13,000 rpm for 20 min at 4°C. Protein levels were quantified using a BCA protein assay kit (Pierce, Rockford, IL) according to the manufacturer's instruction. Equivalent amounts of protein were separated by 8–12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to PVDF membranes. The membranes were blocked in PBS containing 5% non-fat dry milk (w/v) and then incubated overnight at 4°C with antibodies against caspase-3, caspase-8, caspase-9, Bcl-2, Bax, Fas, and anti-FasL (Santa Cruz) at the recommended dilutions. The membranes were then incubated with HRP-conjugated goat anti-mouse and anti-rabbit secondary antibodies (Cell Signaling Technology) at room temperature for 1 h and developed using the enhanced chemiluminescence reagents (Cell Signaling Technology) and exposed to X-ray film.

Xenograft model

Female BALB/c mice (weight 16–18 g) were maintained under specific pathogen-free conditions and supplied with sterile food and water ad libitum. Approximately 5×10^6 MNNG/HOS cells (suspended in 0.2 ml PBS) were injected subcutaneously into the right axillary fossa of each nude mouse under aseptic conditions. At 48 h after the injection (day 1), mice were randomly assigned to one of four groups ($n = 10$). From day 1, one group received cisplatin (2 mg/kg) on alternate days and two other groups received five intraperitoneal injections of matrine at 50 mg/kg (M_{low}) or 100 mg/kg (M_{high}) per week (on days 1–5). Control group received an equal volume of normal saline. The diameter method was used every other day to observe dynamic changes in tumor growth. After 3 weeks of drug administration, mice were sacrificed on day 20 and tumors were dissected and weighed. The tumor volume was calculated by the following formula $0.5 \times a \times b^2$, where "a" was the largest dimension and "b" the perpendicular diameter. Lethal toxicity was defined as any death in treated animals occurring before the first death in the control group. Mortality was monitored daily. The animal studies were approved by the Zhejiang University Medical College Ethics Committee, and the principles of laboratory animal care were followed in all animal experiments.

Immunohistochemistry analysis of Bcl-2, Bax, Fas, and FasL expression

Formaldehyde-fixed, paraffin-embedded tissue blocks were prepared from xenograft tissue and cut into serial sections (3 µm), which were then mounted on glass slides and dried at 60°C for 2 h. Tissue sections were deparaffinized in xylene, rehydrated, and then immersed in PBS. To block endogenous peroxidase activity, the slides were treated with 3% H_2O_2 for 15 min, then washed with PBS three times (3 min each), and immersed in boiling 0.01 M sodium citrate buffer (pH 6.0) for 10 min. Next sections were incubated with goat serum for 30 min at room temperature, then with mouse monoclonal antibodies against Bcl-2, Bax, Fas, and FasL (diluted at 1:1,000) at 4°C overnight. After washing in PBS for 5 min, sections were incubated in biotin-labeled goat anti-rabbit IgG (diluted 1:200) at 37°C for 30 min. Bound secondary antibody was detected by the SABC method, according to the manufacturer's protocol. Digital images of positively stained fields were assessed by measuring the optical density (OD) of stained regions of tumor tissue. Brown granules in the cytoplasm or/and on the cell membrane represented positive staining, and staining intensity indicated the expression level of Bcl-2, Bax, Fas, and FasL. Photographs were taken at high magnification (400×) for analysis.

The expression of Bcl-2, Bax, Fas, and FasL was analyzed using the Image Pro-Plus software (ver. 6.0; Media Cybernetics, Silver Spring, MD), according to the method developed by Xavier et al. [20]. Briefly, a DP 70 CCD camera (Olympus) coupled to an AX-70 microscope (Olympus) was used to capture, at 400× magnification, 10 digital images (resolution 2,560 × 1,920 pixels) per slide. Measurement parameters included the following: IOD, total area, and mean density. OD was calibrated, and the area of interest assigned values for hue (0–30), saturation (0–255), and intensity (0–220). Images were then converted to gray-scale, and values were counted.

Electron microscopy

Matrine-treated and matrine-untreated tumor tissues were examined by transmission electronic microscopy (TEM). Briefly, tissue was fixed in 4% (w/v) glutaraldehyde and then 1% (w/v) osmium tetroxide and embedded in epoxy resin. Serial sections (0.5 µm) were stained with uranyl acetate and lead citrate and then examined under a TECNAI 10 transmission electron microscope (Philips).

Statistical analysis

Results were expressed as the mean ± standard deviation (SD). Mean values were calculated from data obtained from

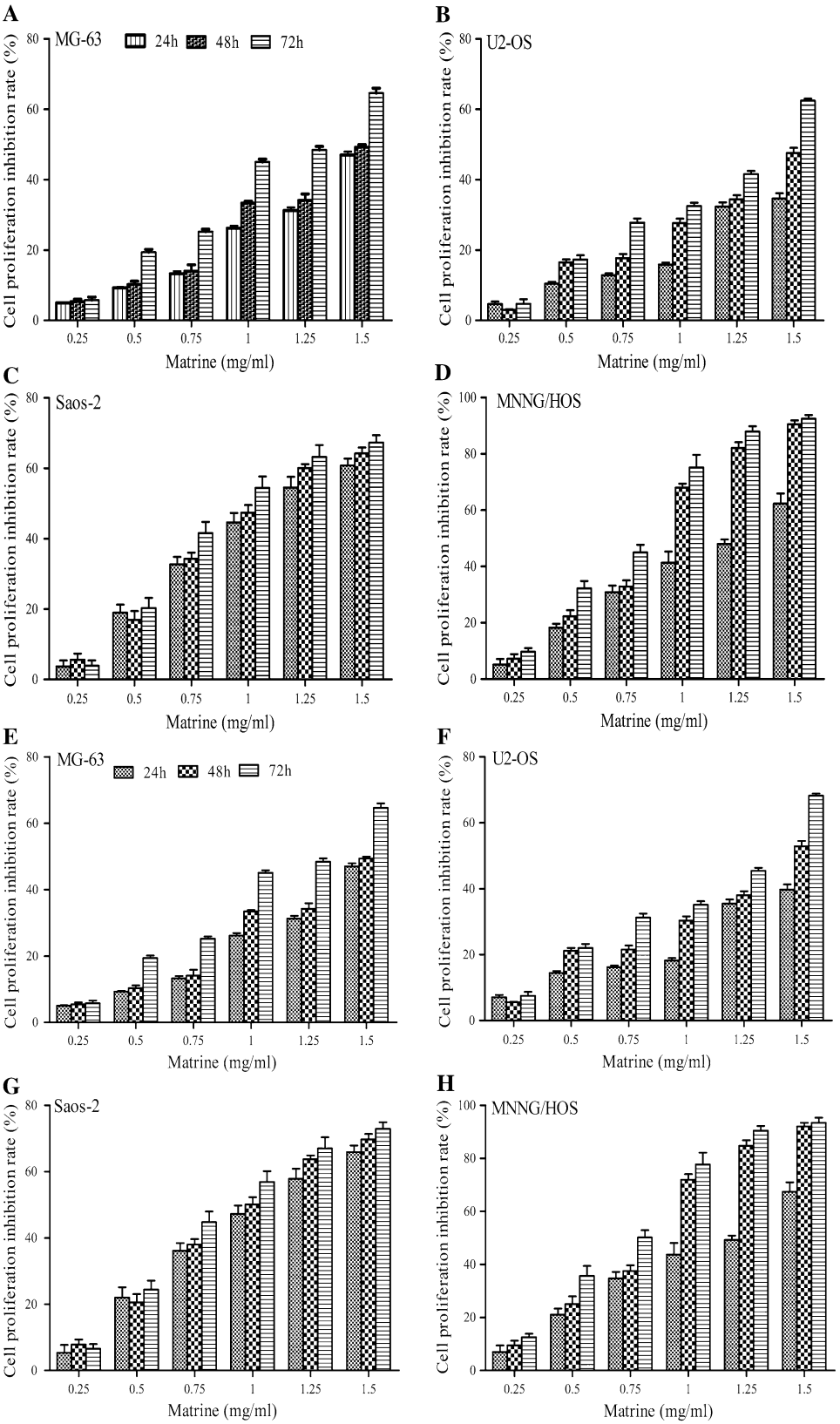


Fig. 2 Matrine inhibits the proliferation of human osteosarcoma cells in a dose- and time-dependent manner. **a**–**d** Cells were exposed to matrine with different concentrations for various time, and the OD values was obtained through reading plate at 570 nm with 96-well micro test spectrophotometer by MTT assay. The inhibition rate was expressed as the percentage of cell inhibition rate compared with the control. The data were expressed as mean ± SD obtained from triplicate samples. **e**–**h** After exposing to matrine with different concentrations for various time, we analyzed cell proliferation using BrdU incorporation assay and the percentage is represented graphically. Values are mean 6 standard deviation (SD) from three different experiments

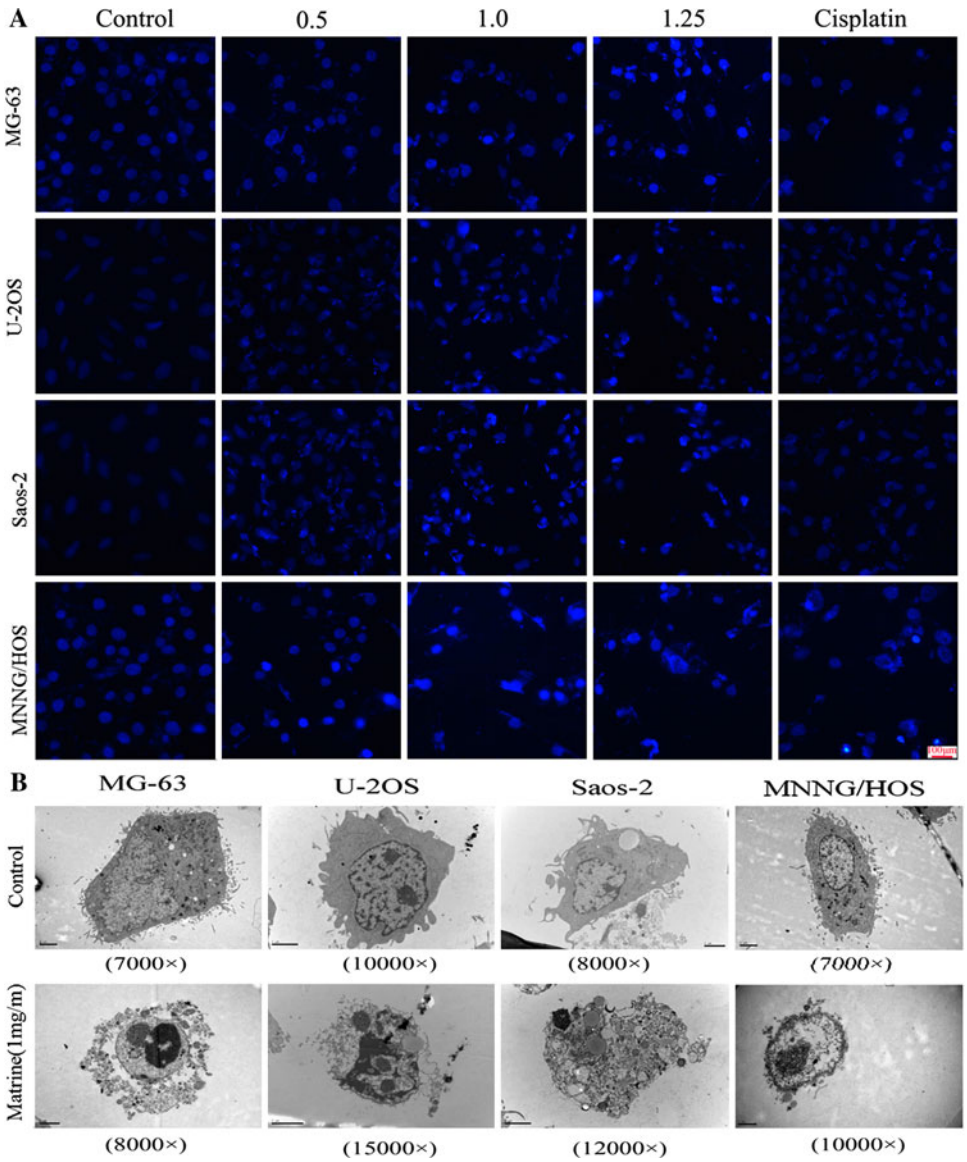


Fig. 3 Matrine induces apoptosis of human osteosarcoma cells. **a** Hoechst staining of osteosarcoma cells treated with 0.5, 1.0, 1.25 mg/ml matrine and 10 μg/ml cisplatin for 24 h, respectively. The nuclei was stained by Hoechst 33258 and visualized under fluorescence microscope (original magnification, 200×, *bar* = 100 μm). **b** The apoptosis ultrastructure of osteosarcoma cells treated by matrine. The cells were examined under a transmission electron microscope (×3,700 power). In the 1.0 mg/ml matrine group, typical apoptotic cells were observed in MG-63 and U-2OS; Cellular swelling and cytoplasmic vacuoles were observed in U-2OS and Saos-2, even karyotin was concentrated and congregated into small pieces in MNNG/HOS, *bar* = 2 μm. **c** AnnexinV-FITC/PI staining of osteosarcoma cells treated by matrine. *a* blank control group; *b* 0.5 mg/ml matrine; *c* 1.0 mg/ml matrine; *d* 1.25 mg/ml matrine; *e* 10 μg/ml cisplatin. The data were representative of three independent experiments. **d** TUNEL assay of osteosarcoma cells treated by matrine. *Data* represented the mean of three measurements ± SD. **P* < 0.001 versus control group; ***P* < 0.01 versus control group; #*P* < 0.05 versus control group

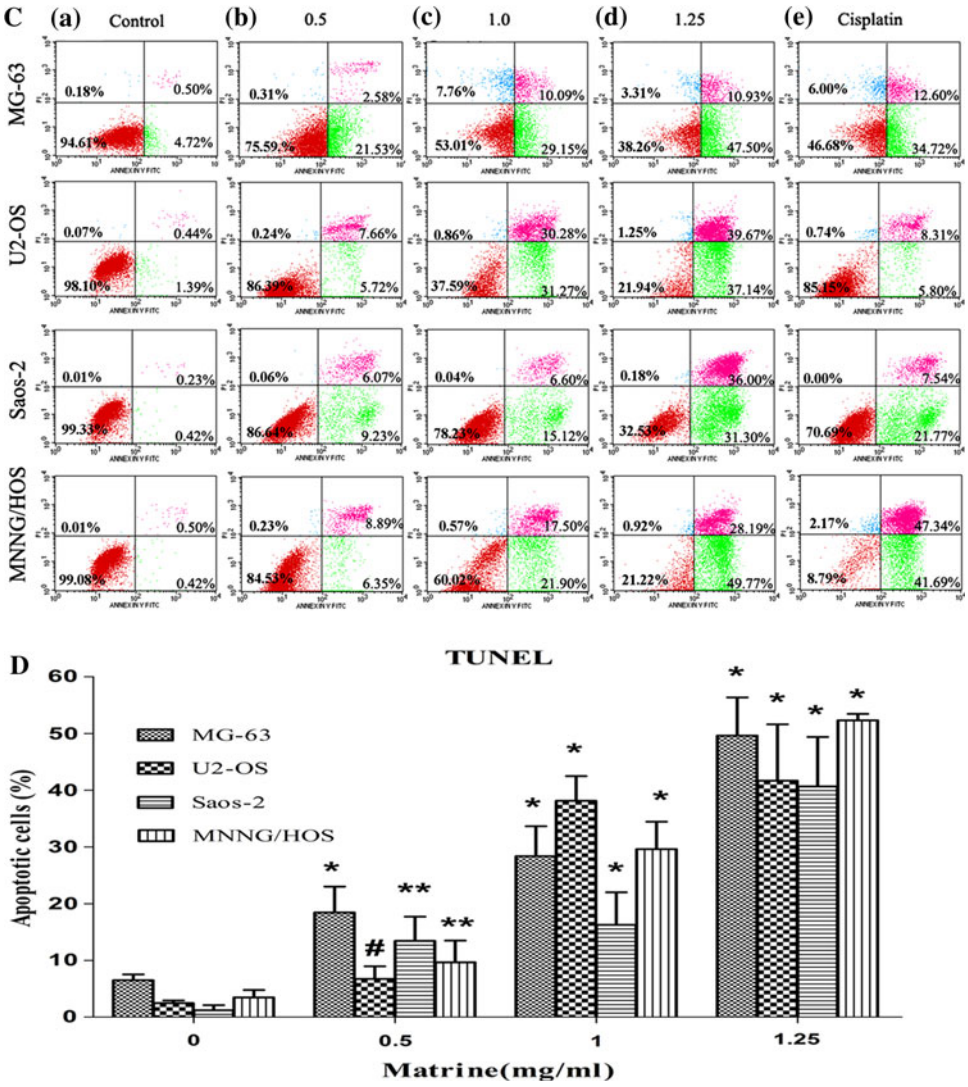
experiments performed in triplicate. One-way ANOVA was used to identify statistically significant differences between the experimental and control groups. IC_{50} values (and 95% confidence intervals) were calculated from MTT assay data by probit regression. Values of the ratio of mean Bcl-2 to Bax density, and of the mean intensities of Bax Bax, Fas, and FasL, were $\log_{10}$-transformed. The Kruskal–Wallis and Mann–Whitney *U* tests were used to identify differences in Bcl-2 and Bax expression between different treatment groups.

All statistical analyses were performed using the SPSS software (ver. 16.0; SPSS, Inc., Chicago, IL). *P* values were two-tailed, and a value <0.05 was considered statistical significance.

Results

Matrine inhibits the proliferation of human osteosarcoma cells in a dose- and time-dependent manner

To investigate the effects of matrine on the proliferation of osteosarcoma cells, we measured the growth of four human osteosarcoma cell lines, MG-63, U-2OS, Saos-2, and MNNG/HOS, using the MTT and BrdU incorporation assay. We found that matrine inhibited cell proliferation in a dose- and time-dependent manner (Fig. 2a–h). After 48 h, IC_{50} values for matrine were 1.75 mg/ml in MG-63, 1.80 mg/ml in U-2OS, 1.06 mg/ml in Saos-2, and 0.77 mg/mL in MNNG/HOS cells based on MTT assay. Furthermore,



the similar IC_{50} values were calculated based on Brdu assay (data not shown). Inhibition was not saturated in any of the four osteosarcoma cell lines at the concentrations of matrine used.

Matrine induces the apoptosis of human osteosarcoma cells

Hoechst 33258 staining showed that osteosarcoma cells treated with matrine exhibited morphological features of early apoptotic cells, such as bright nuclear condensation or fragments. At higher matrine concentrations, apoptotic bodies began to appear (Fig. 3a). We also found that the number of late apoptotic cells increased at higher matrine concentration in all osteosarcoma cell lines. Maximal cell death occurred within 48 h of drug exposure (data not shown). Thus, 24 h exposure to matrine was selected as an appropriate time point for subsequent experiments.

TEM revealed typical apoptotic morphological features induced by matrine: denser cytoplasm, concentrated and aggregated karyotin, and the formation of dense, round apoptotic bodies (Fig. 3b). These observations were consistent with the morphological alterations detected by light and fluorescence microscopy.

Next, we quantified matrine-induced apoptosis of human osteosarcoma cells. As shown in Fig. 3c, cells treated with matrine displayed much higher rates of apoptosis than control cells. Most apoptotic cells were in the early stages. As a positive control, MG-63, Saos-2, and MNNG/HOS cells treated with cisplatin (10 μg/ml) also showed high rates of. However, unlike matrine, cisplatin treatment primarily yielded late apoptotic cells and necrotic cells. U-2OS cells treated with cisplatin displayed only a low rate of apoptosis (15.23%), consistent with the findings of other researchers [21].

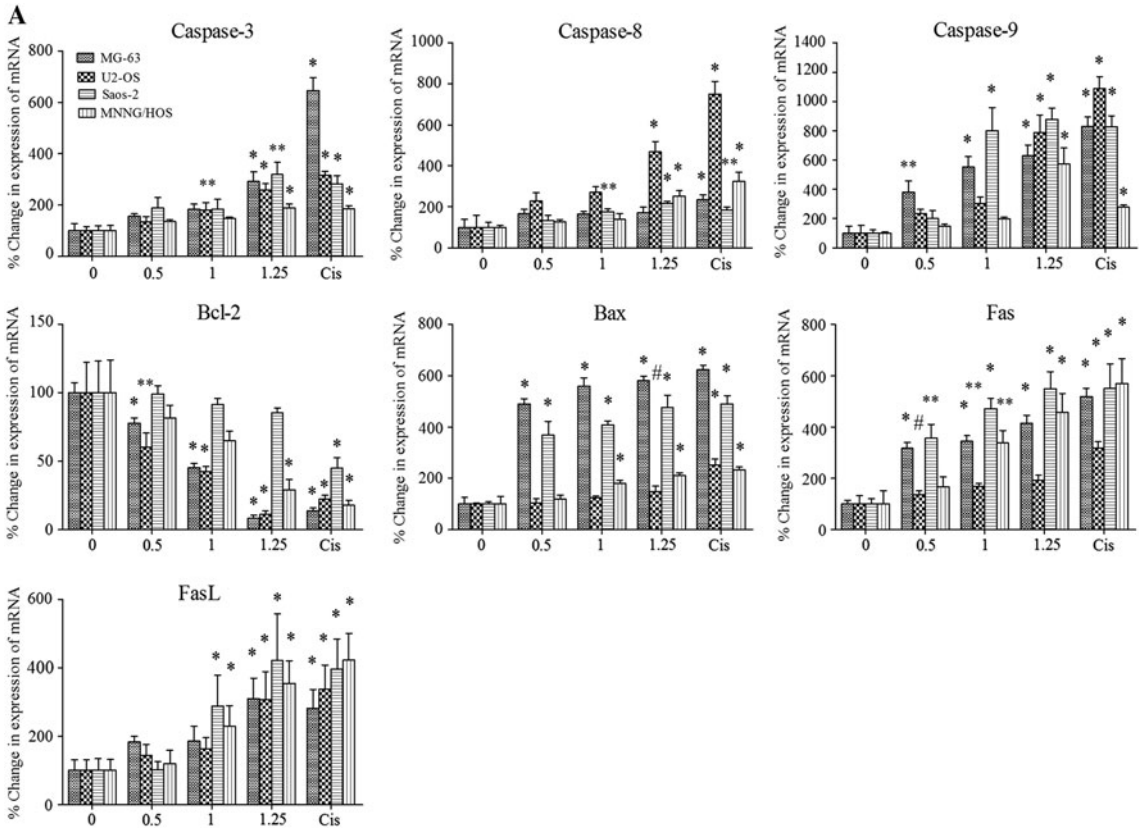


Fig. 4 Matrine induces the activation of caspase-3, -8, -9, upregulates Fas/FasL and downregulates Bax in human osteosarcoma cells in a dose-dependent manner. **a** Real-time RT–PCR analysis for quantitative evaluation of the mRNA expression of target genes. The data are mean ± SD of three samples. $*P < 0.001$ versus control group; $**P < 0.01$ versus control group; $\#P < 0.05$ versus control group. **b–e** Western blotting analysis of caspase-3, -8, -9, Fas, FasL, Bcl-2 and Bax in human osteosarcoma cells treated with 0.5, 1.0, 1.25 mg/ml of matrine and 10 μg/ml cisplatin for 24 h, respectively. Moreover, the relative ratio of colorimetric density of cleaved caspase-3, -8, -9, Fas, FasL, Bcl-2 and Bax was analyzed by Gel-Pro-analyzer (Media Cybernetics, USA). *β*-actin served as loading control. *Data* represented the mean of three measurements ± SD. $*P < 0.001$ versus control group; $**P < 0.01$ versus control group; $\#P < 0.05$ versus control group

To distinguish the apoptotic and necrotic cells, we performed the TUNEL assay. The result revealed that cells treated with matrine displayed much higher rates of apoptosis than control cells (Fig. 3d). These findings combined with the data from annexin V-FITC/PI assay show that matrine inhibited the proliferation of human osteosarcoma cells mainly through inducing cells apoptosis rather than inducing cell necrosis.

Matrine regulates the expression of apoptosis-related genes in human osteosarcoma cell lines

qRT–PCR analysis showed that mRNA expression levels of caspase 3, -8, -9 were increased after exposure to matrine in four cell lines. Furthermore, the mRNA expression level of Fas/FasL was obviously increased. Most importantly, we found that matrine decreased Bcl-2 mRNA expression and increased Bax mRNA expression in MG-63, U2-OS, and MNNG/HOS cells in a dose-dependent manner (Fig. 4a). Taken together, these data suggest that matrine induces apoptosis of osteosarcoma cells through regulating the expression of apoptosis-related genes.

Next, we analyzed the levels of cleaved effector caspases (caspase-3, -8, and -9) by immunoblotting. We found that incubation of human osteosarcoma cells with matrine triggered the activation of caspase-3, -8, and -9, and matrine significantly increased the levels of cleavage caspase-3, -8, and -9 at 1.25 mg/ml (Fig. 4b, c), indicating the involvement of the mitochondrial and extrinsic death pathways in matrine-induced apoptosis.

To confirm that matrine regulates the expression of apoptosis-related genes in human osteosarcoma cells, we performed Western blot analysis and found that matrine slightly increased the expression of Fas and FasL but decreased Bcl-2 expression and increased Bax expression in MG-63, U2-OS, and MNNG/HOS cells in a dose-dependent manner (Fig. 4d, e). However, increased Bax expression but no change in Bcl-2 expression was observed in

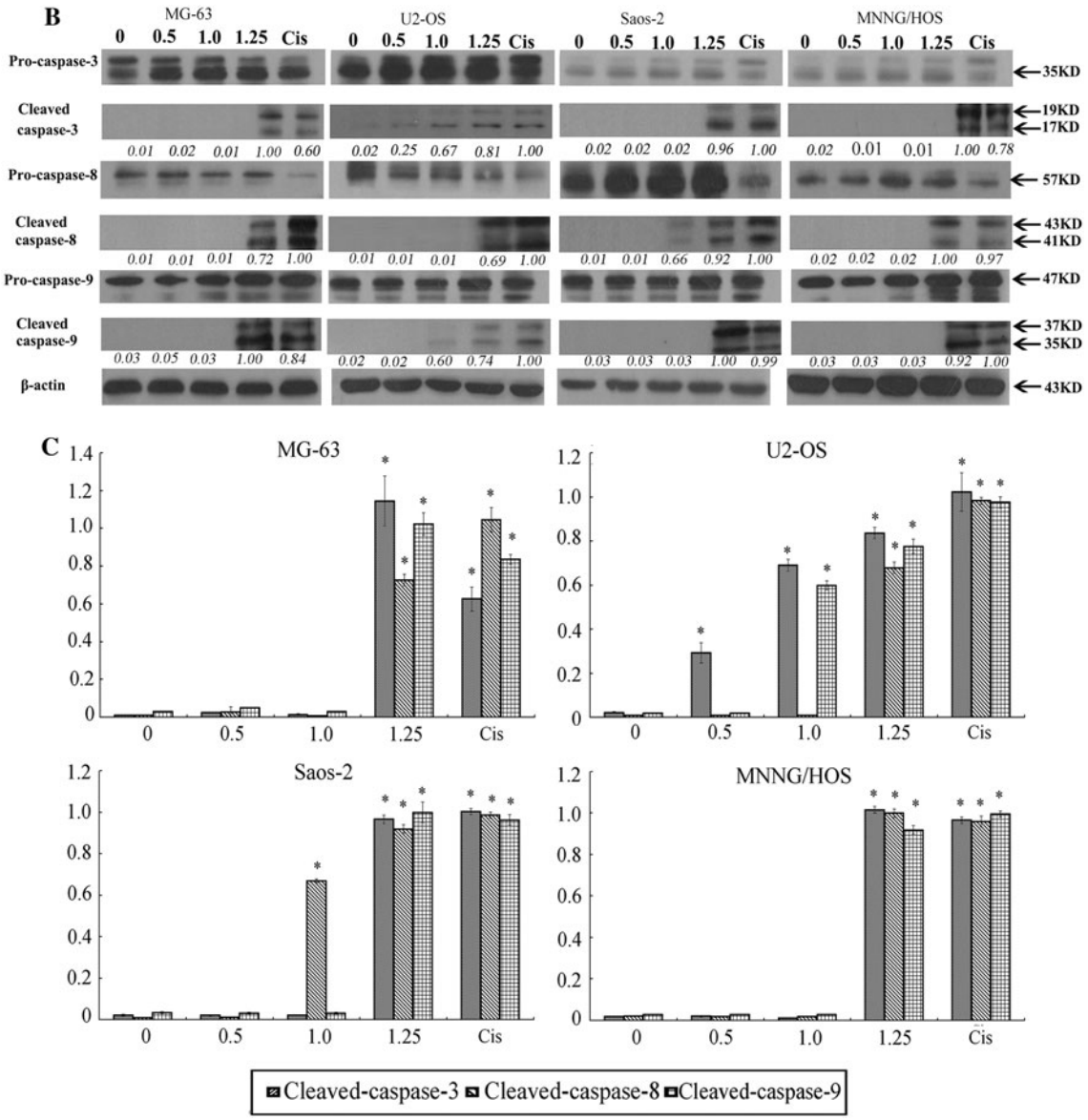


Fig. 4 continued

Saos-2 cells treated with matrine, suggesting that matrine induced apoptosis of Saos-2 cells mainly through increasing Bax expression. These results indicate that matrine triggers apoptosis in human osteosarcoma cells in vitro by activating caspase-3, -8, and -9 and decreasing the rate of Bcl-2/Bax.

Matrine inhibits osteosarcoma in vivo

We next examined the effects of matrine on tumor cell growth in vivo. While palpable tumors (average volume: 13–19 mm^3) developed in vehicle-treated mice within 5 days, cisplatin (as positive treatment control) and matrine both delayed palpable tumor formation by approximately 4 days. Moreover, tumors in matrine-treated mice were smaller than those in vehicle-treated animals (Fig. 5a). While matrine and cisplatin led to similar reduction in tumor size before day 15, cisplatin exhibited stronger anti-tumor activity in the final week of the study period (Fig. 5a). Both matrine and cisplatin significantly inhibited tumor growth (Fig. 5a). At day 20, the weight of tumor was significantly lower in matrine and cisplatin groups than in control group ($^*P < 0.001$; Fig. 5b).

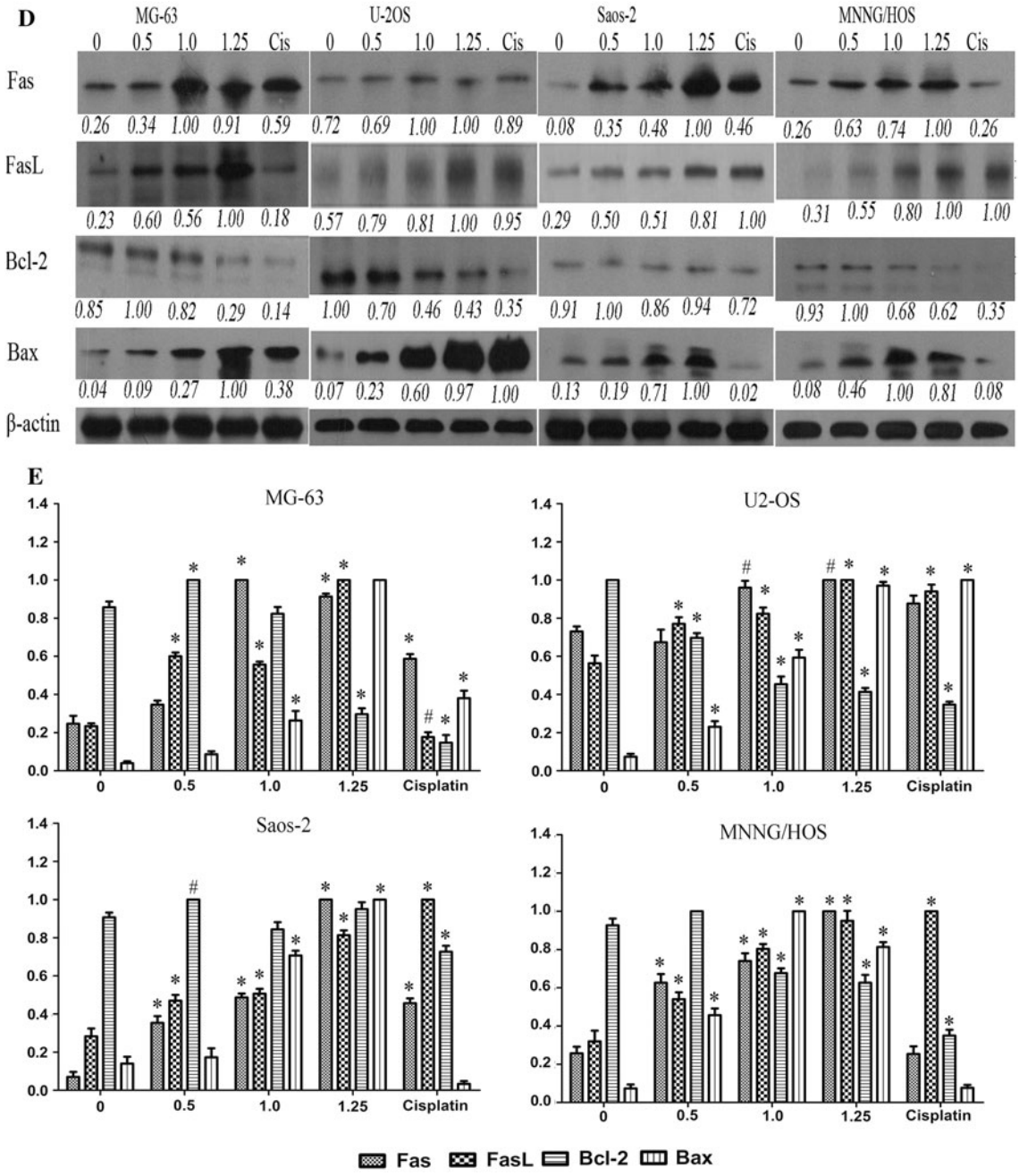


Fig. 4 continued

TEM showed that the tumor cells in control mice were round and had irregular large nuclei. Amounts of karyotin were high, and they were rich in endoplasmic reticulum and mitochondria, signs of active metabolism (Fig. 6a). In contrast, cancer cells in matrine- and cisplatin-treated mice contained few or no organelles and displayed increased electron density. Moreover, the chromatin was concentrated and aggregated, or even fragmented and condensed into an irregular mass, and karyopyknosis and apoptotic bodies were detected. These observations were consistent with the morphological changes that characterize apoptosis (Fig. 6b–d) and with those that occurred in human osteosarcoma cells treated with matrine or cisplatin in vitro (Figs. 2, 3).

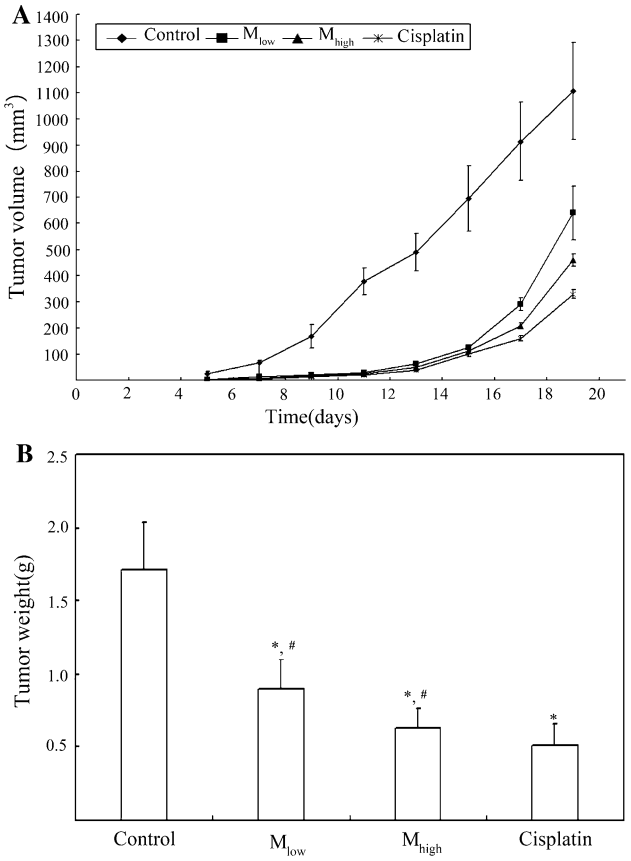


Fig. 5 Matrine inhibits MNNG/HOS xenograft growth. **a** Forty mice were inoculated s.c. with tumor cells and were divided into four groups: control, cisplatin (2 mg/kg), and matrine (50 and 100 mg/kg) treatment groups. Tumor volume was measured every other day, and data were expressed as mean ± SD ($n = 10$). **b** The weight of tumor. *$P < 0.001$ versus control; #$P < 0.05$ versus cispaltin group. M_{low} indicated 50 mg/kg matrine group, and M_{high} indicated 100 mg/kg matrine group

Further histological evaluation of tumor tissue from control mice showed disorganized arrangement of sarcoma cells and a high cell density. The sarcoma cells were ovoid or round and displayed increased ratios of nucleus to cytoplasm. Nuclei were dark and showed clear signs of heteromorphism. In contrast, in tumor tissues from animals treated with matrine or cisplatin, the ratio of nucleus to cytoplasm was reduced and the nuclei were polygonal and lightly stained. Nucleoli were small and inconspicuous. The sarcoma cells were loosely arranged, and there were marked signs of widespread tumor destruction; coagulation and necrotic and apoptotic cells were observed. Additionally, infiltrating lymphocytes and macrophages were present (Fig. 7a).

In addition, the expression of Bcl-2, Bax, Fas, and FasL in matrine-treated tumors was examined by immunohistochemistry. As shown in Fig. 7b–j, the mean areas that stained positively for Bcl-2 were lower in matrine-treated tumor tissue sections than in sections from control, and the mean areas that stained positively for Bax, Fas, and FasL were higher ($P < 0.001$). The ratio of mean Bcl-2 to Bax density was approximately 1.11 in the M_{low} (50 mg/kg matrine) group, 0.05 in the M_{high} (100 mg/kg matrine), 0.09 in the cisplatin groups, and 204.02 in the control group ($P < 0.001$). These data confirm the down-regulation of Bcl-2 and upregulation of Bax, Fas, and FasL following 3 weeks of treatment with matrine or cisplatin.

Discussion

Osteosarcoma is the most important primary malignant tumor of bone, especially in children. Until recently, a 5-year survival rate of 20% for treatment through surgical intervention alone was considered acceptable (because approximately 80% of patients had pulmonary metastasis at time of presentation) [22, 23]. In the past decade, the survival of patients with osteosarcoma has increased, due to rapid advances in neoadjuvant chemotherapy. However, the effectiveness of cytotoxic drugs often declines, due to acquired chemoresistance. Finding new therapeutic agents to target the malignant behavior of osteosarcoma cells is, therefore, important for improving the prognosis.

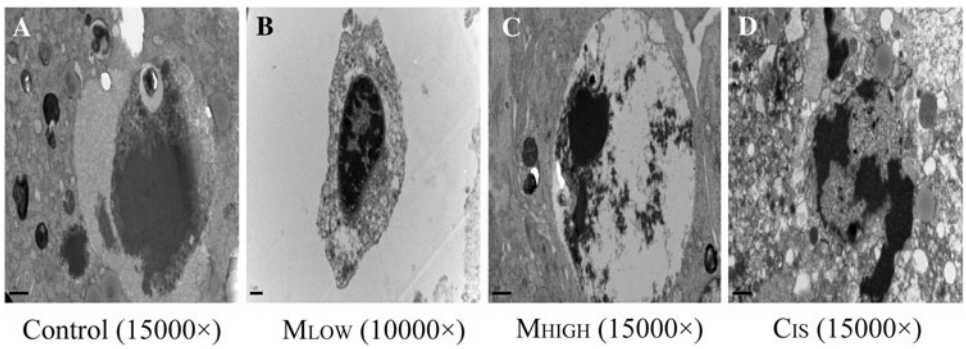


Fig. 6 The apoptosis ultrastructure in human MNNG/HOS xenograft examined under a transmission electron microscope. M_{low} indicated 50 mg/kg matrine group, and M_{high} indicated 100 mg/kg matrine group, *bar* = 1 μm

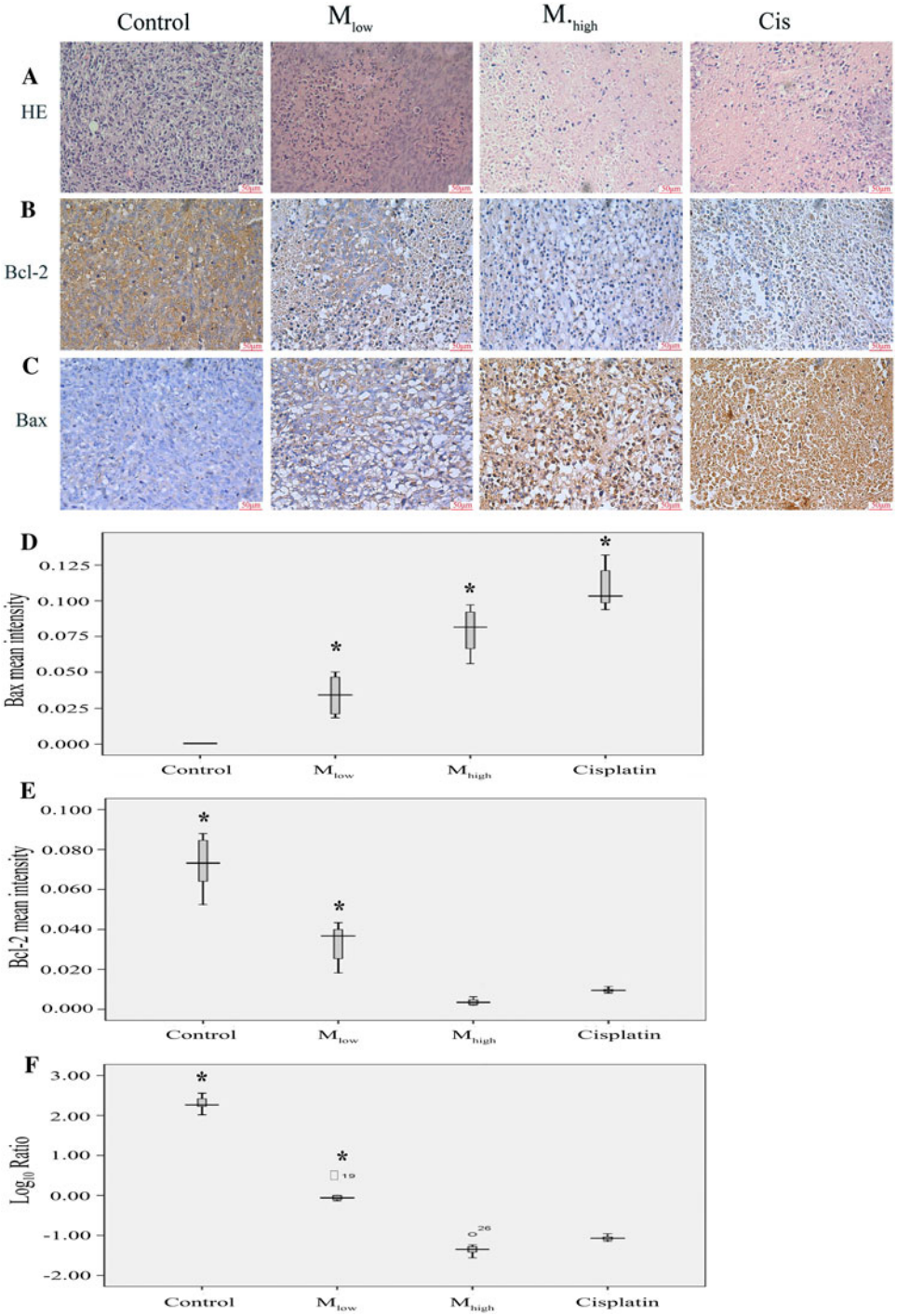
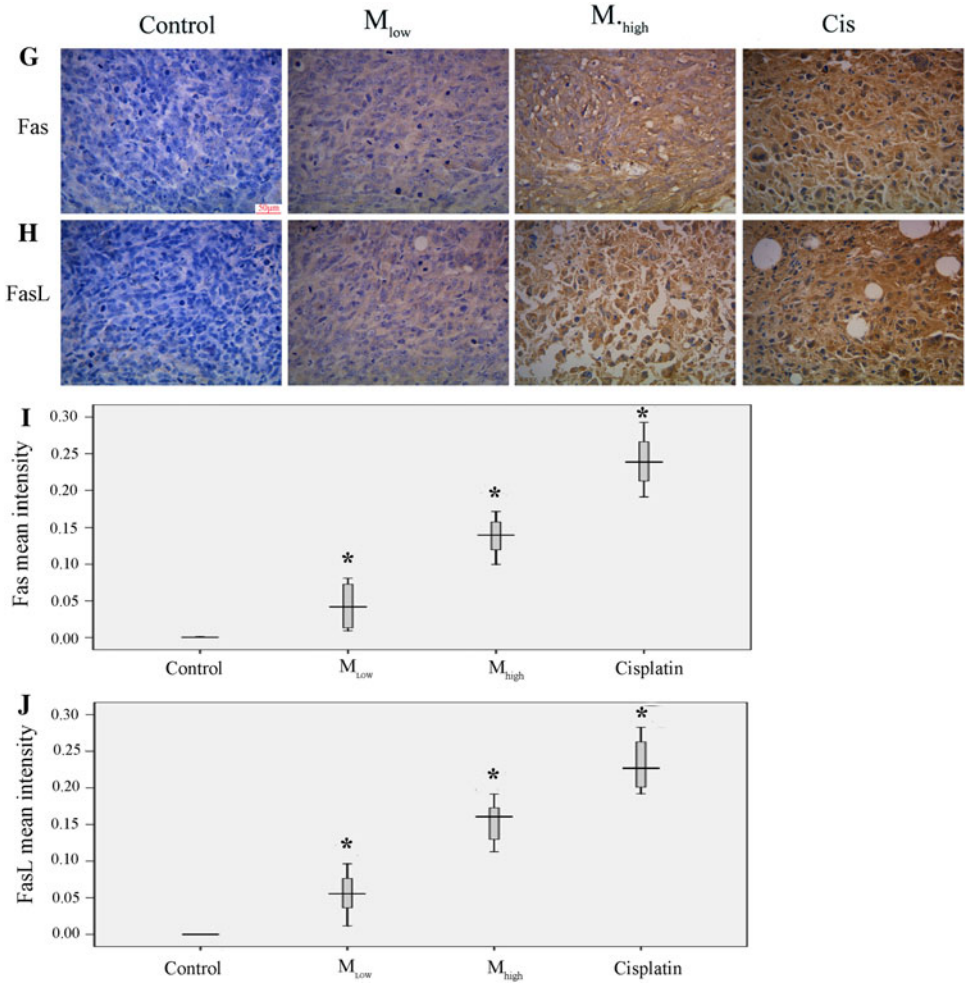


Fig. 7 Matrine upregulates Bax, Fas, and FasL and downregulates Bcl-2 in MNNG/HOS xenografts. **a** H and E analyses of the pathological features of the tumors from the four groups (original magnification, 400×). **b** and **c** The expression of Bax and Bcl-2 in xenografts analyzed by immunohistochemistry (original magnification, 400×). The number of Bax positive cells and the mean optical density in the matrine and cisplatin groups were higher than in the control group, but the number of Bcl-2 positive cells and the mean optical density were significantly reduced compared with the control group, bar = 50 μm. **d** Mean density of Bax determined by Image Pro-Plus. $^*P < 0.001$ versus control group. **e** Mean density of Bcl-2 determined by Image Pro-Plus. $^*P < 0.001$ versus control group. **f** The ratio of Bcl-2/Bax density. $^*P < 0.001$ versus control group. **g** and **h** Compared with the control group, the expression of Fas and FasL increased in the matrine and cisplatin groups. **i** and **j** Mean density of Fas and FasL determined by Image Pro-Plus. $^*P < 0.001$ versus control group

In this study, we proved the anti-cancer effects of matrine on four human osteosarcoma cell lines. We found that matrine-induced apoptosis dose and time dependently in human osteosarcoma cells, with the IC_{50} values for the osteosarcoma cell lines tested ranging from 0.77 to 1.80 mg/ml. These values differ from those reported for human stomach cancer, melanoma, hepatoma, and glioma cell lines, probably due to the differences in the cell types studied and the genetic alterations they carry [24, 25].

Apoptosis plays a crucial role in protecting organisms against tumorigenesis. Many anti-cancer drugs act to induce apoptosis, eliminating cells that harbor genetic damage or divide inappropriately [26]. To determine the mechanisms of the anti-cancer actions of matrine, we first studied its pro-apoptotic properties. Results from annexin V-FITC/PI double staining and TUNEL assay suggested that matrine-induced apoptosis in a dose-dependent manner in the range of 0.5–1.25 mg/ml. Apoptotic cells were further

Fig. 7 continued



characterized using fluorescence and electron microscopy. Cytoplasmic vacuoles were observed in cells treated with matrine at 1.0 and 1.25 mg/ml. Lipid droplets were also detected, perhaps resulting from drug-induced defects in lipid metabolism. A similar response has been reported in A375 cells [24].

The caspase signaling cascades are central to the process of apoptosis, and the extrinsic and intrinsic cascades are triggered by caspase-8 and caspase-9, respectively. The pro-forms of the initiator caspases are activated in different ways. While procaspase-8 is activated by membrane-associated protein complexes, the activation of procaspase-9 is mitochondria dependent [27]. In addition, caspase-3 acts a key executor of apoptosis and plays a vital role in programmed cell death. In our study, matrine dose dependently increased the cleavage of caspase-3, -8, and -9 in four human osteosarcoma cell lines and concomitantly reduced the levels of procaspase-3, -8, and -9, in parallel with the observed increase in the rate of apoptosis. These findings indicate that matrine induces apoptosis in human osteosarcoma cells by activating both death receptor and mitochondrial apoptotic pathways.

The intrinsic apoptosis pathway involves the Bcl-2 family, the members of which play important roles in apoptosis. Bcl-2 promotes cell survival by limiting the pro-apoptotic effects of Bax and blocking the release of cytochrome C from mitochondria [28]. The induction of apoptosis is influenced by the Bcl-2/Bax ratio. Both chemotherapeutic agents and cell type influence the participation of Bcl-2 family members in the induction of apoptosis [29]. By immunoblotting analysis, we found that matrine upregulated Bax and downregulated Bcl-2. Our results suggest that matrine may inhibit the growth of human osteosarcoma cells in vitro by inducing Bax and suppressing Bcl-2 expression. Matrine-induced human osteosarcoma cells apoptosis via negatively regulate Bcl-2 family protein by two possible ways: first, Puma releases Bim from Bcl-2-like proteins, so that Bax (or Bak) can be directly engaged by Bim to induce cell death [30]. Second, Bim and Puma may activate Bax and Bak by releasing them from the Bcl-2 pro-survival proteins that sequester them [31]. Our study showed that matrine downregulated pro-survival member Bcl-2 in MG-63, U2-OS, MNNG/HOS cells while upregulated pro-apoptotic proteins Bax in all four osteosarcoma cell lines.

Activation of the extrinsic pathway triggers the activation of death receptors, such as Fas, TNF receptors, and TNF-α-related apoptosis-inducing ligand receptors. Activation of these death receptors is triggered by cellular stress or death signals and leads to the recruitment of Fas-associated death domain (FADD) and death domain (DD), which in turn induce the activation of caspase-8 and -10. This leads to the activation of Bax, which then interacts with Bak to induce the release of cytochrome C from the mitochondria. The release of cytochrome C leads to cleavage of caspase-9, which contributes to the activation of caspase-3, -6, and -7 [32]. In the present study, levels of Fas and FasL were slightly increased in matrine-treated osteosarcoma cells, which were also previously found in gastric carcinoma cells [19].

In our in vivo study, we found that matrine (100 mg/kg) and cisplatin significantly inhibited tumor growth during the 3-week treatment period. Histological and ultrastructural analyses of the tumors from mice treated with matrine or cisplatin revealed morphological features characteristic of apoptotic cells, in agreement with our findings in vitro. Furthermore, immunohistochemical analysis confirmed the downregulation of Bcl-2 and upregulation of Bax, Fas, and FasL following treatment with matrine or cisplatin in vivo. Although subcutaneous grafting is easy to administrate and measure the tumor, the perfect in vivo model for bone tumor is the orthotopic transplantation. Therefore, further study employing this model will be more valuable to assess the therapeutic effects of matrine on human osteosarcoma.

Interestingly, matrine also modulates the expression of pro-inflammatory transcription factors nuclear factor-kappa gene binding (NF-kB), STAT(Signal Transducer and Activator of Transcription), and Matrix metalloproteinase-1 (MMP-1). Matrine could induce the activation of NF-kB in hepatocellular carcinoma HepG2 cells [33]. Matrine inhibited the proliferation of human hepatocellular carcinoma SMMC-7721 cells via downregulating the expression of STAT-3 and STAT-5 [34]. Matrine also suppressed phorbol myristate acetate (PMA)-induced MMP-1 expression through inhibition of the activation of activator protein-1 (AP-1) signaling pathway and was beneficial for treatment of some inflammatory skin disorders [35].

In conclusion, in this study, we demonstrate that matrine as a tumor inhibitor suppressed the growth of human osteosarcoma cells in vitro and in vivo. It appears that matrine induces apoptosis in human osteosarcoma cells by decreasing the Bcl-2/Bax ratio and increasing the activation of the Fas/FasL and cleavage of caspase-3, -8, and -9. Our data suggest the potential clinical use of matrine in the treatment of patients with osteosarcoma, alone or in combination with conventional agents or monoclonal antibodies.

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Conflict of interest None.

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