

L-mimosine induces caspase-9-mediated apoptosis in human osteosarcoma cells

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Abstract. L-mimosine is a rare plant amino acid extracted from *Mimosa* or *Leucaena* spp., and it has been reported to exhibit antitumor activity in a number of types of cancer. However, the underlying mechanisms remain to be clarified. In the present study, the effect of L-mimosine was investigated in human osteosarcoma cells. A Cell Counting Kit-8 assay and flow cytometry were used for toxicity detection. Hoechst staining and transmission electron microscopy (TEM), in addition to western blot analysis, were used for the examination of the associated mechanisms. The results of the present study indicated that L-mimosine significantly inhibited cell proliferation by inducing cellular apoptosis in osteosarcoma cells. The Hoechst staining results and TEM revealed that nuclear damage increased with the concentration increase in L-mimosine, as did the formation of apoptotic bodies. Additionally, the results of the western blot analysis confirmed that the treatment of cells with L-mimosine was accompanied by increasing expression of cleaved caspase-9. L-mimosine-induced apoptosis was inhibited by the caspase-9 inhibitor Z-LEHD-FMK. In addition, the extracellular signal-regulated kinase (ERK) signaling pathway was suppressed following treatment with L-mimosine. In conclusion, the results of the present study suggested that L-mimosine induced apoptosis via the mitochondrial apoptotic pathway. The ERK signaling pathway was indicated to be an additional mechanism underlying apoptosis induction. The results provided evidence for the use of L-mimosine as a promising candidate for osteosarcoma therapy.

Introduction

Osteosarcoma, the most common primary malignant bone tumor in children and adolescents, is considered to be a significant potential threat to the health of teenagers (1). Osteosarcoma

originates from mesenchymal tissue, and frequently occurs in the metaphyseal region of growing bones. With its characteristics of rapid growth and marked invasiveness, osteosarcoma is highly malignant and prone to lung metastases. Pulmonary micrometastasis may be observed in ~80% of patients at the time of diagnosis, which may be the cause of the low survival rate of patients with osteosarcoma (2,3). At present, the treatment for osteosarcoma is primarily surgery combined with preoperative chemotherapy (2). Although progress has been made in the treatment of osteosarcoma, the 5-year survival rate of patients with osteosarcoma is only ~30% (2). It is very important to identify novel therapeutic targets and develop effective drugs for the treatment of osteosarcoma.

L-mimosine, a plant amino acid which is extracted from *Leucaena leucocephala* or *Mimosa pudica*, is a type of iron chelator and prolyl hydroxylase inhibitor (4,5). L-mimosine has been reported to exhibit anti-tumor activity in a number of types of tumor, including pancreatic cancer, prostate cancer, breast cancer and cervical cancer (4-6); however, the effect of L-mimosine in osteosarcoma has not been reported, and the underlying mechanisms remain to be clarified. In the present study, two osteosarcoma cell lines, MG63 and U2OS, were used to examine the antitumor activity of L-mimosine in osteosarcoma. In addition, the associated mechanisms were further investigated.

Materials and methods

Reagents. The following reagents were used in the present study: L-mimosine and Z-LEHD-FMK (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany); SCH772984 [specific inhibitor of extracellular signal-regulated kinase (ERK)] (MedChem Express, Monmouth Junction, NJ, USA); fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA); RPMI-1640 medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA); Dulbecco's modified Eagle's medium (DMEM; HyClone; GE Healthcare Life Sciences); DMEM/F-12 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA); Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies Inc., Kumamoto, Japan); Annexin V/propidium iodide (PI) apoptosis kit [Hangzhou Multi Sciences (Lianke) Biotech Co., Ltd., Hangzhou, China]; Hoechst staining kit (Beyotime Institute of Biotechnology, Haimen, China); cleaved caspase-9 (cat. no. 9929), cleaved caspase-3 (cat. no. 9929), cleaved poly(ADP-ribose)

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polymerase (PARP) (cat. no. 9929), apoptosis regulator Bcl-2 (Bcl-2) (cat. no. 9941), apoptosis regulator BAX (BAX) (cat. no. 9942), ERK (cat. no. 9902), phosphorylated (p)-ERK (cat. no. 9910) and GAPDH (cat. no. 5174) antibodies (Cell Signaling Technology, Inc., Danvers, MA, USA); and cleaved caspase-8 antibody (Novus Biologicals, LLC, Littleton, CO, USA). The secondary antibody was a donkey anti-rabbit IgG (cat. no. 925-32213; LI-COR Biosciences, Lincoln, NE, USA).

Cell culture. Human osteosarcoma cell lines MG63 and U2OS, which were originally purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), were conserved in the laboratory, and were respectively cultured in DMEM and RPMI-1640, supplemented with 10% fetal bovine serum and 20 $\mu\text{g}/\text{ml}$ antibiotics (ampicillin and kanamycin), at 37°C and 5% CO_2 . Human normal osteoblast cells hFOB 1.19 were obtained from the Type Culture Collection of the Chinese Academy of Sciences, and was cultured in DMEM/F-12, supplemented with 10% fetal bovine serum and 20 $\mu\text{g}/\text{ml}$ antibiotics (ampicillin and kanamycin), at 33.5°C and 5% CO_2 .

Cell proliferation assay. Cells were harvested and adjusted to 2×10^4 cells/ml, and seeded in 96-well plates. A total of three replicates were performed in every group, and a blank control was additionally set up. A concentration gradient of L-mimosine (0, 200, 400 and 800 μM) was used for treatment. Following 24, 48 and 72 h of culture, 10 μl CCK-8 was added to each well, and the plate was incubated at 37°C for 1 h, and the absorbance value was measured at 450 nm. The experiment was repeated three times independently.

Flow cytometry. A concentration gradient of L-mimosine (0, 200, 400 and 800 μM) was used for treatment for 24 h. Cells in 6-well plate at a density of $1 \times 10^4/\text{well}$ were collected and washed twice with cold PBS. An Annexin V/PI apoptosis kit was used for detection. The cells were resuspended in Annexin-V binding buffer, and stained with 5 μl Annexin-V-fluorescein isothiocyanate (FITC) and 10 μl PI in the dark for 15 min at room temperature. Fluorescence was analyzed on a FACSCanto™ II spectrometer (BD Biosciences, Franklin Lakes, NJ, USA), and the software used for the analysis was CellQuest Pro (BD Biosciences). Cells stained with FITC/PI were counted as apoptotic cells. The experiment was repeated three times independently.

Hoechst staining. Cells were harvested and seeded into 6-well plates at a density of 1×10^4 cells/well, a concentration gradient of L-mimosine (0, 200, 400 and 800 μM) was used for treatment for 24 h. Cells were washed with PBS once, 1 ml/well Hoechst was added, and the plate was placed in the dark for a 30 min incubation. Subsequently, the Hoechst was removed and the cells were washed with PBS twice, and observed with a fluorescence microscope at a magnification of $\times 40$. The staining results were quantified using Image Studio v3.1 software (LI-COR Biosciences) and the experiment was repeated three times independently.

Transmission electron microscopy (TEM). Cells were harvested and seeded into 6-well plates at a density of 1×10^4 cells/well,

and a concentration gradient of L-mimosine (0, 200, 400 and 800 μM) was used for treatment for 24 h. Cells were placed in 4°C pre-cooled 2.5% glutaraldehyde and fixed for 2 h. Cells were washed 3 times with PBS buffer, fixed in 1% osmium tetroxide for 2 h at 4°C, and washed with buffer three times. The cells were soaked with gradient ethanol, acetone dehydrated and embedded with Epon 812 at 60°C for 24 h. Double staining was performed with uranyl acetate and lead citrate at room temperature for 20 min. Sections were observed under TEM and images were captured.

Western blotting. A concentration gradient of L-mimosine (0, 200, 400 and 800 μM) was used for treatment for 24 h. Z-LEHD-FMK (40 μM) was used for caspase-9 inhibition; and 5 nM SCH772984 was used for ERK inhibition. Cells were collected and seeded into 6-well plates, and a concentration gradient of L-mimosine (0, 200, 400 and 800 μM) was used for treatment for 24 h. Cells were harvested and lysed in a radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology) containing a protease inhibitor cocktail and 2 mM dithiothreitol. A biconchonic acid assay (Thermo Fisher Scientific, Inc.) was used to determine the protein concentration in each sample. The loading quantity of samples per lane was 30 μg . Lysates were resolved by SDS-PAGE on a 10% gel, transferred to polyvinylidene fluoride (PVDF) membranes. The PVDF membrane was blocked with the blocking solution (5% milk) at room temperature for 2 h. And then immunoblotted with primary antibodies (1:1,000). Following immunoblotting with secondary antibodies (1:10,000), the membranes were scanned with the Odyssey CLx Infrared Imaging System (LI-COR Biosciences). The western blot bands were quantified using Image Studio v3.1 software, and the experiment was repeated three times independently.

Statistical analysis. All values are expressed as the mean \pm standard deviation. Statistical analyses were performed using one-way analysis of variance followed by Tukey's post hoc test with SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effect of L-mimosine on the proliferation of the osteosarcoma cell lines MG63 and U2OS. In order to evaluate the *in vitro* effect of L-mimosine on the proliferation of the osteosarcoma cell lines MG63 and U2OS, the CCK-8 method was used to assess cell viability (Fig. 1). A concentration gradient of L-mimosine (0, 200, 400 and 800 μM) was used to treat the osteosarcoma cell lines MG63 and U2OS for 24, 48 and 72 h. The results demonstrated that L-mimosine exhibited anti-proliferative effects in human osteosarcoma cells, and the effects were observed to be concentration-dependent (Fig. 1A and B). The results indicated that the cell line MG63 was more sensitive to L-mimosine compared with U2OS. In addition, the toxicity of L-mimosine on human normal osteoblasts was assessed in the present study. The human normal osteoblast cell line hFOB 1.19 was chosen as the normal control in the CCK-8 assay to evaluate the toxicity of L-mimosine. The results demonstrated that L-mimosine was less toxic to

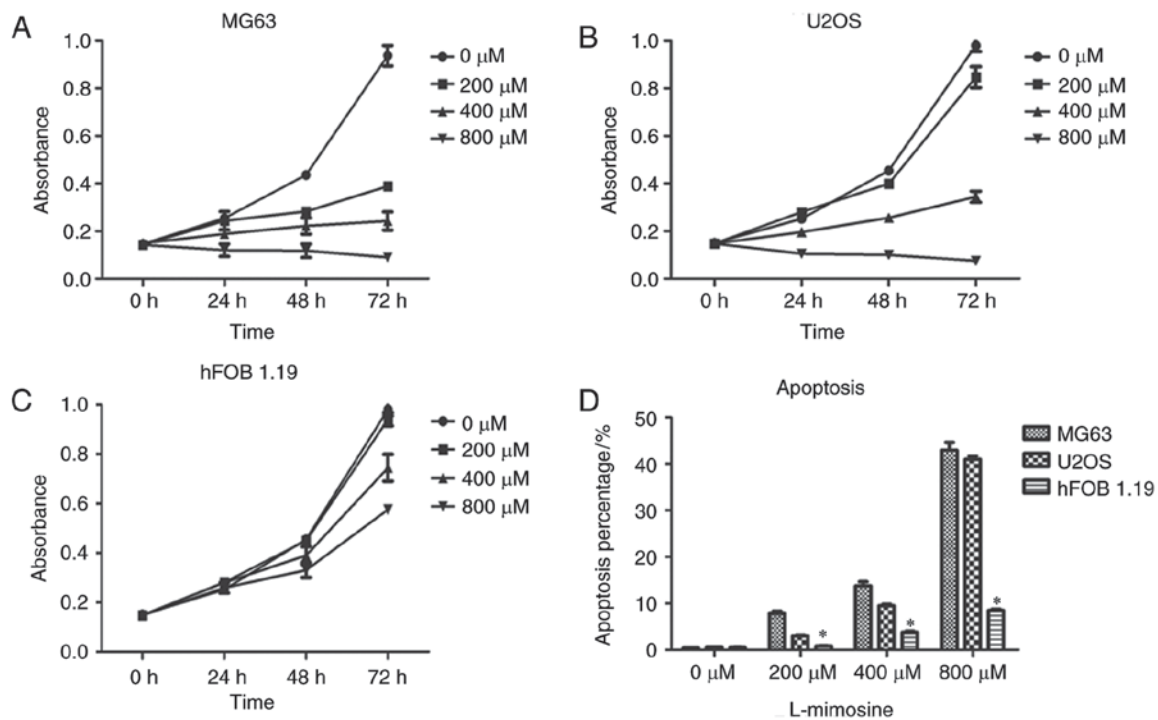


Figure 1. Effect of L-mimosine on the proliferation and apoptosis of the osteosarcoma cell lines MG63 and U2OS. The CCK-8 results demonstrated that L-mimosine exerted anti-proliferative effects on human osteosarcoma cells (A) MG63 and (B) U2OS in a concentration-dependent manner. (C) Human normal osteoblasts hFOB 1.19 were chosen as the normal control in the evaluation of the toxicity of L-mimosine. The results of the CCK-8 assay demonstrated that L-mimosine was less toxic to normal human osteoblasts. (D) The flow cytometry results demonstrated the apoptosis rate of MG63 and U2OS treated with gradient concentrations of L-mimosine (0, 200, 400 and 800 μ M). The apoptosis rate of the cells increased with the increase in the concentration of L-mimosine, and the effect was concentration dependent. CCK-8, Cell Counting Kit-8. * $P < 0.05$ vs. MG63 and U2OS.

normal human osteoblasts, exerting a weak inhibitory effect on proliferation (Fig. 1C).

Effect of L-mimosine on the apoptosis of osteosarcoma cell lines MG63 and U2OS. To test the *in vitro* effect of L-mimosine on the apoptosis of osteosarcoma cell lines MG63 and U2OS, a flow cytometry experiment was performed with gradient concentrations of L-mimosine (0, 200, 400 and 800 μ M) incubated for 24 h. The results demonstrated that the apoptosis rate of the cells increased with the increase in the concentration of L-mimosine, and that the effect was therefore concentration dependent (Fig. 1). The results demonstrated that L-mimosine exerted a pro-apoptotic effect on human osteosarcoma cells, and that MG63 cells were more sensitive to L-mimosine (Fig. 1). In addition, human normal osteoblast hFOB 1.19 cells were selected as the normal control in the flow cytometry assay to evaluate the toxicity of L-mimosine. The results demonstrated that L-mimosine was less toxic to normal human osteoblasts, exerting a weak effect on apoptosis (Fig. 1D).

Nuclear damage in MG63 cells increases with the increase in L-mimosine concentration. The flow cytometry assay illustrated marked apoptosis following treatment with gradient concentrations of L-mimosine; the effect was most notable in the MG63 cell line. In order to further confirm this result, the nuclear damage induced by L-mimosine was examined in the more sensitive MG63 cell line using Hoechst staining (Fig. 2). The cells in the control group exhibited weak blue fluorescence, and the apoptotic cells exhibited

membrane permeability, which was observed as bright blue fluorescence. The experimental results demonstrated that with the increased concentration of L-mimosine (0, 200, 400, 800 μ M), the number of nuclei appearing with bright blue fluorescence increased, indicating that the number of apoptotic cells increased.

Ultrastructural alterations in MG63 cells treated with L-mimosine. Hoechst staining in the previous experiment demonstrated that the apoptosis of MG63 cells was markedly induced by L-mimosine. In order to understand the alterations in the cell during apoptosis, TEM was used for the observation of the ultrastructural alterations in MG63 treated with L-mimosine. Under TEM observation (Fig. 3), the control group of MG63 displayed varied forms, a large nucleus and an imbalance in the nucleus-cytoplasm ratio, with an intact nuclear membrane, prominent nucleoli and evenly distributed nuclear chromatin (Fig. 3A). By contrast, the L-mimosine-treated group appeared with typical apoptosis morphological features, including cell shrinkage, cytoplasm condensation, pyknotic nuclei and a lack of nucleoli (Fig. 3B), and apoptotic bodies were observed (Fig. 3C; white arrow).

L-mimosine regulated apoptosis related proteins in MG63 cells. To investigate the apoptotic effect of L-mimosine, western blotting was performed (Fig. 4). As presented in Fig. 4A, cleaved PARP, cleaved caspase-9 and cleaved caspase-3 exhibited increased expression as the concentration of L-mimosine increased, while the expression of cleaved caspase-8 did not notably alter. Attenuated expression of Bcl-2 and increased

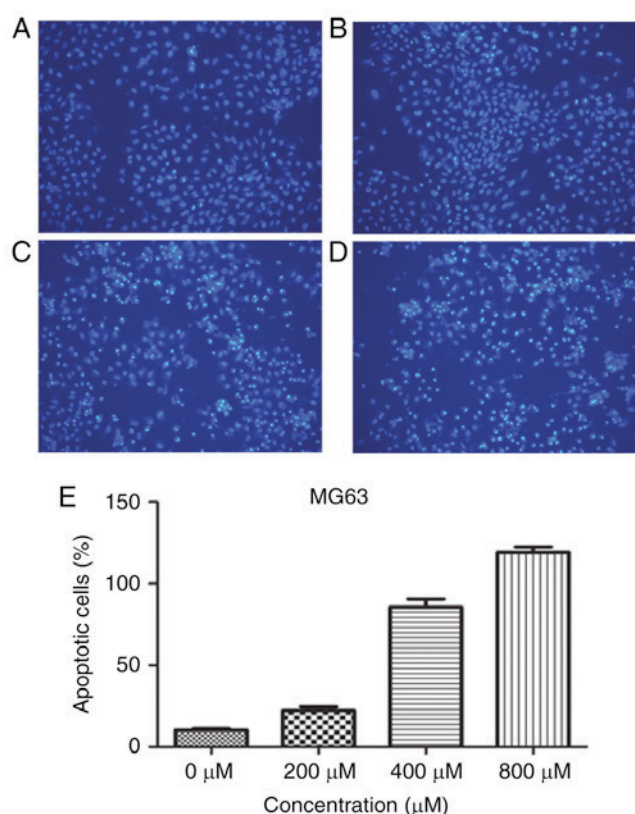


Figure 2. Nuclear damage to MG63 increases with the increase in L-mimosine concentration. Hoechst staining was used to examine the alterations in nuclear morphology induced by L-mimosine. The experimental results demonstrated that with the concentration gradient of L-mimosine (0, 200, 400 and 800 μM) (A-D), the number of nuclei exhibiting bright blue fluorescence increased. (A) The cells in the control group exhibited weak blue fluorescence, the number of apoptotic cells was $\sim 0.5\%$. (B) Cells were treated with 200 μM L-mimosine, and the apoptotic cells exhibited membrane permeability, which was observed as bright blue fluorescence. The number of apoptotic cells was $\sim 7.8\%$. (C) Cells were treated with 400 μM L-mimosine, and the number of apoptotic cells was $\sim 13.8\%$. (D) Cells were treated with 800 μM L-mimosine and the number of apoptotic cells was $\sim 42.9\%$. Magnification, $\times 40$. (E) Quantification of apoptotic cells.

expression of BAX were observed in MG63 cells treated with gradient concentrations of L-mimosine. Additionally, it is known that xenobiotics may alter cellular functions, including proliferation, the cell cycle and apoptosis, by affecting cell survival pathways; consequently, the present study further examined the signaling pathways associated with L-mimosine. As presented in Fig. 4A, L-mimosine reduced the levels of ERK and p-ERK in a concentration-dependent manner. The role of L-mimosine in this signaling pathway was further confirmed by the ERK signaling specific inhibitor SCH772984. As presented in Fig. 4C, the suppressed ERK signaling pathway following treatment with L-mimosine suggested this pathway be an additional mechanism for apoptosis induction.

According to the results of the western blot analysis, it was hypothesized that the treatment of cells with L-mimosine was accompanied by an increase in cleaved caspase-9 expression. The present study assessed whether L-mimosine-induced apoptosis was inhibited by the caspase-9 inhibitor Z-LEHD-FMK. As presented in Fig. 4B, the results suggested that L-mimosine induced apoptosis through the mitochondrial apoptotic pathway.

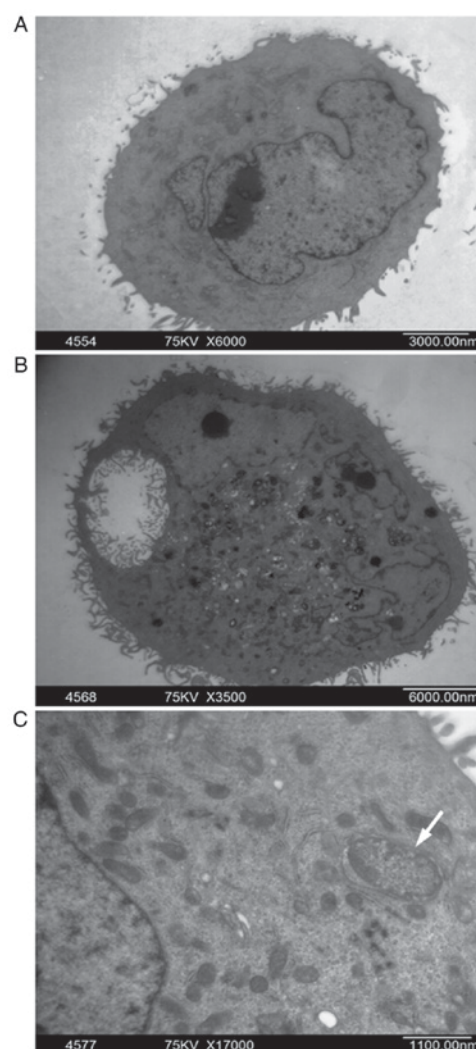


Figure 3. Ultrastructural alterations in MG63 cells treated with L-mimosine. Transmission electron microscopy was used for the observation. (A) The control group of MG63 cells displayed a large nucleus, intact nuclear membrane, prominent nucleoli and evenly distributed nuclear chromatin. (B) L-mimosine-treated cells exhibited typical apoptosis morphological features, including cell shrinkage, cytoplasm condensation, pyknotic nuclei and a lack of nucleoli disappeared, in addition to (C) apoptotic body formation (white arrow).

Discussion

L-mimosine is a rare plant amino acid extracted from *Mimosa* or *Leucaena* spp. The molecular formula of L-mimosine is α -amino- β -N-[3-hydroxy-4-pyridone]-propionic acid, $\text{C}_8\text{H}_{10}\text{N}_2\text{O}_4$. The molecular weight is 198.2. L-mimosine is structurally different from other commonly used anti-cancer drugs, and has a high degree of similarity to the structure of thymine (7,8). Due to the particular chemical structure of L-mimosine and its inhibitory effects on mammalian DNA replication, L-mimosine is used as a type of cell cycle synchronization drug in experiments, in addition to the study of the induction of tumor cell death. Previous studies have demonstrated the cytotoxicity of L-mimosine against a number of types of tumor cell line. The sensitivity of L-mimosine in numerous human tumor cell lines was detected and the possible mechanisms were examined. Studies have reported that L-mimosine is a reversible cell cycle inhibitor in mammalian

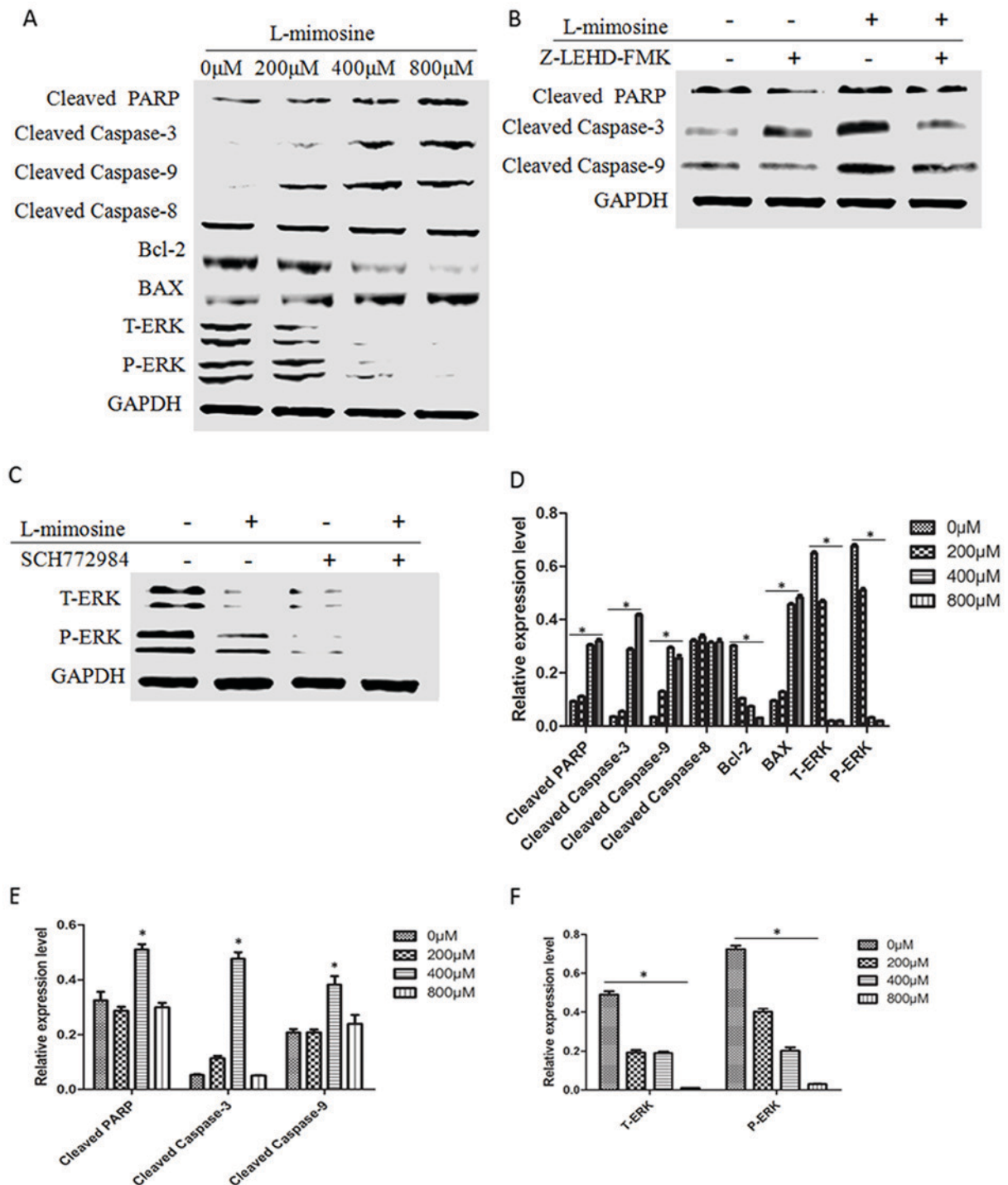


Figure 4. Effect of L-mimosine on the expression of apoptosis-associated proteins and the ERK signaling pathway in MG63 cells. The results of the western blot analysis demonstrated that (A) as L-mimosine concentration increases, cleaved PARP, cleaved caspase-9 and cleaved caspase-3 exhibited increased expression levels, whereas the expression of cleaved caspase-8 did not change significantly. Attenuated expression of Bcl-2 and increased expression of BAX was observed as the L-mimosine concentration increased. L-mimosine reduced the levels of t-ERK and p-ERK in a concentration-dependent manner. (B) L-mimosine-induced apoptosis was inhibited by the caspase-9 inhibitor Z-LEHD-FMK. (C) The role of L-mimosine in the ERK signaling pathway was confirmed by the ERK signaling specific inhibitor SCH772984. (D-F) The statistical graphs of the data shown in parts A-C, respectively. *P<0.05. PARP, poly(ADP ribose) polymerase; Bcl-2, apoptosis regulator Bcl-2; BAX, apoptosis regulator BAX; ERK, extracellular signal-regulated kinase; p, phosphorylated; t, total.

cells, which acts on the G1/S phase of the cell cycle (9,10). In addition, L-mimosine may interfere with the initiation of DNA replication and the extension of the replication chain (7,11,12). However, the exact mechanism of action of L-mimosine remains unclear. Mechanisms which have previously been reported include: Effectively preventing DNA synthesis by

blocking the late G1 phase of the cell cycle (13); interfering with the synthesis of histone H1 kinase (14,15); and upregulating cyclin-dependent kinase inhibitor p27 protein expression (8,16-18).

The question of whether L-mimosine is a reversible cell cycle inhibitor has remained controversial due to a number of reasons, including different research methods or experimental

conditions, and differences among different species and different cells in previous studies. Cell type is one of the factors which determines whether cells are prone to apoptosis (19); for example, cell lines which are sensitive to chemotherapeutic agents are prone to apoptosis (20), while apoptosis is difficult to induce in certain cell lines following treatment with chemotherapeutic agents (21). Previously, researchers have reported the pro-apoptotic effect of L-mimosine in a number of types of cancer (4,6,17,22), including pancreatic cancer, prostate cancer, breast cancer and cervical cancer. However, when induced by L-mimosine, the effects on these tumors are different. Therefore, the present study sought to investigate whether L-mimosine may have a pro-apoptotic effect on osteosarcoma cells. The present study used two different types of osteosarcoma cell line, MG63 and U2OS.

The present study tested the effect of L-mimosine on osteosarcoma cell proliferation. The results of the CCK-8 assay indicated that L-mimosine inhibited osteosarcoma cell proliferation, and that the inhibitory effect was dose-dependent. Subsequently, apoptosis was assessed in osteosarcoma cells induced by L-mimosine. The Annexin V-FITC/PI double staining assay demonstrated that L-mimosine induced osteosarcoma cell apoptosis, and that the induction effect was dose-dependent. Previous studies reported that L-mimosine inhibited tumor cell proliferation by inducing tumor cell cycle arrest (17,23). In addition, it is reported L-mimosine inhibited tumor cell proliferation by altering the expression of proliferation-associated genes (4). In the present study, it was hypothesized that L-mimosine inhibited osteosarcoma cell proliferation through the induction of cellular apoptosis.

Apoptosis is characterized by membrane blebbing, cell shrinkage, chromatin condensation, DNA damage and fragmentation of the cell into membrane-bound apoptotic bodies (24). Subsequently, it was observed that the apoptosis of osteosarcoma cells was caused by DNA damage, via a Hoechst assay and TEM. In the Hoechst assay, the damaged nuclei were increased with the increase in L-mimosine concentration. When observed under TEM, the L-mimosine treated group exhibited typical morphological features of apoptosis: Cell shrinkage, cytoplasm condensation, nuclear pyknosis and a lack of nucleoli, in addition to apoptotic body formation.

Cellular apoptosis induced by L-mimosine was confirmed by western blot analysis of apoptosis-associated proteins. The caspases belong to a family of highly conserved aspartate-specific cysteine proteases, and they constitute important components of the apoptotic pathway (25,26). PARP, a type of DNA repair enzyme, is recognized to be the cleavage substrate of caspase. PARP is thought to be an important indicator of apoptosis, and is generally considered to be an indicator of caspase-3 activation. Bcl-2, encoded by the BCL2 gene, is the key member of the Bcl-2 protein family and negatively regulates cellular apoptosis (27). Bax protein, another apoptosis regulator belonging to the Bcl-2 protein family, promotes apoptosis by binding to the Bcl-2 protein (28). The expression of the proteins mentioned above was detected when cells were treated with gradient concentrations of L-mimosine. The expression of cleaved PARP and cleaved caspase-3 was increased as the concentration of L-mimosine increased. Attenuated expression of Bcl-2 and increased expression of BAX were observed as the concentration of L-mimosine increased.

It is known that cellular apoptosis is mediated through two principal pathways: The extrinsic (death receptor-mediated) and intrinsic (mitochondrial-mediated) pathways (29). Caspase-8 is important for the initiation of apoptosis via death receptors, as its recruitment to and activation at the death-inducing signaling complex is the decisive step for the initiation of the caspase cascade, leading to apoptosis (30). Caspase-9 is the apoptotic initiator protease of the intrinsic, or mitochondrial, apoptotic pathway (31). In the present study, with the increase in the concentration of L-mimosine, cleaved caspase-9 exhibited increased expression, while the expression alteration of cleaved caspase-8 was not apparent, indicating that the intrinsic (mitochondrial) apoptotic pathway was induced by L-mimosine in osteosarcoma cells. This hypothesis was additionally confirmed by treatment with the caspase-9 inhibitor Z-LEHD-FMK.

Xenobiotics may alter cell survival pathways and cause alterations in cell proliferation, the cell cycle and apoptosis (32), and the results of the present study further demonstrated that the ERK signaling pathway was associated with L-mimosine. L-mimosine reduced the levels of ERK and p-ERK in a concentration-dependent manner, and the ERK signaling specific inhibitor SCH772984 was used for verification. The results suggested ERK signaling to be an additional mechanism for apoptosis induction.

In conclusion, the present study confirmed that L-mimosine was able to effectively inhibit the proliferation of osteosarcoma cells, and concluded that L-mimosine induces caspase-9-mediated apoptosis in osteosarcoma cells. In the future, further studies are required to detect the inhibitory effects of L-mimosine in more types of tumor, or to compare the toxic effects of chemotherapeutic drugs with clear antitumor mechanisms and L-mimosine. The present study may provide the basis for a more comprehensive understanding and evaluation of this type of plant amino acid. Further research is required to assess the potential of L-mimosine as an antitumor drug. Different types of antitumor drugs act via different mechanisms, and the same type of drug may have different modes of action according to cell cycle specificity. Combinations of currently-used drugs and L-mimosine may provide a broader options for the treatment of cancer.

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