15-hydroxy-6α,12-epoxy-7β,10αH,11βH-spiroax-4-ene-12-one exerts anti-tumor effects against osteosarcoma through apoptosis induction

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Abstract. Osteosarcoma is the most common type of malignant bone tumor, which has an overall survival rate of only 15-30%. The present study aimed to investigate the effects of 15-hydroxy-6α,12-epoxy-7β,10αH,11βH-spiroax-4-ene-12-one (HESEO), a compound extracted from the endophytic fungus *Penicillium* sp. FJ-1 isolated from *Avicennia marina*, on the proliferation of osteosarcoma cells and to explore its underlying mechanisms of action. Cell number was counted to measure the cell proliferation. JC-1 reagent was used to measure mitochondrial membrane potential. ELISA was used to measure the cytochrome *c* level and caspase activities. Apoptosis was detected by Annexin V-Propidium Iodide staining. Gene and protein expression were measured by reverse-transcription-PCR and western blot analysis, respectively. Additionally, the anti-tumor effects of HESEO were explored within a syngeneic osteosarcoma tumor model. The results suggested that HESEO significantly inhibited the proliferation of osteosarcoma cells and induced apoptosis of MG-63 cells, evidenced by their decreased mitochondrial membrane potential, and increased cytochrome *c* release, caspase activities and percentage of apoptotic cells. In addition, HESEO increased the expression of pro-apoptotic genes and proteins compared with control cells. The results indicated that HESEO may act through increasing p53 upregulated modulator of apoptosis expression. Furthermore, HESEO treatment significantly increased the survival time and decreased the tumor burden of osteosarcoma tumor-bearing mice compared with vehicle treatment. Furthermore, combined treatment with HESEO enhanced the effects of the chemotherapeutic agent methotrexate on a lung metastasis osteosarcoma model. These data suggested that HESEO could be developed as a potential anti-tumor agent against osteosarcoma.

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Introduction

Osteosarcoma is a malignant bone tumor originating in the skeletal system, which often occurs in adolescents (1,2), and is characterized by a high metastasis rate, poor prognosis and a high recurrence rate (3,4). Although adjuvant and neoadjuvant chemotherapeutic strategies have been widely applied in the treatment of osteosarcoma and improve the rate of long-term survival (5,6), the 5-year overall survival rate of patients with distant metastasis and a high degree of malignancy remains low (approximately 30%) (7). It is therefore of great importance to search for novel agents to treat this disease.

The nuclear factor-κB (NF-κB) transcription factor family includes NF-κB1, NF-κB2, RelA, RelB and c-Rel, which have diverse biological activities (8). The mechanisms underlying NF-κB-driven regulation of apoptosis remain controversial and are poorly understood. Growing evidence suggests that NF-κB can promote apoptosis by increasing the expression of pro-apoptotic proteins, such as p53, Fas, Fas ligand and death receptors (9-11). As a downstream target of p53 and part of the BH3-only subset of Bcl-2 family proteins, p53 upregulated modulator of apoptosis (PUMA), a potent apoptosis inducer in various cancer cells, is induced by p53 following exposure to DNA-damaging agents, such as chemotherapeutic drugs (12,13). It has been demonstrated that NF-κB is induced early after tumor necrosis factor-α (TNF-α) exposure and upregulates PUMA expression (14). Mutations in the NF-κB binding site of the PUMA promoter have been suggested to

abolish the response of the luciferase reporter containing the NF-κB binding site to cytokines, indicating that this NF-κB binding site may be crucial for PUMA gene transcription (15).

Natural products may have biological and pharmaceutical activities. A novel compound named 15-hydroxy-6α, 12-epoxy-7β,10αH,11βH-spiroax-4-ene-12-one (HESEO), isolated from the endophytic fungus *Penicillium* sp. FJ-1 of the mangrove plant *Avicennia marina*, has been suggested to inhibit the tumor growth of human xenograft osteosarcoma in nude mice (16). In the present study, the effects of HESEO on osteosarcoma were further investigated and the results suggested that HESEO may have anti-tumor activities, as evidenced by inhibition of cell proliferation through the induction of apoptosis and an increased survival time, as well as a decreased tumor burden in osteosarcoma tumor-bearing mice when compared with control treatments. These results suggest a scientific rationale to develop HESEO as a novel potential agent against osteosarcoma.

Materials and methods

Cell lines, reagents and chemicals. MG-63, SaOS2, HOS and U2OS human bone osteosarcoma and K7M2-WT murine osteosarcoma cell lines were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Gibco; Thermo Fisher Scientific, Inc. Caspase activity assay kits (cat. no. K106-100 for caspase-3; cat. no. K119-110 for caspase-9) were purchased from R&D Systems, Inc. JC-1 was obtained from Molecular Probes; Thermo Fisher Scientific, Inc. Cytochrome *c* immunoassay kit (cat. no. DCTC0) was purchased from R&D Systems, Inc. The Annexin V-FITC/PI apoptosis detection kit and PCR reagents were obtained from Thermo Fisher Scientific, Inc. Methotrexate (MTX) was purchased from Shanxi Pude Pharmaceutical Co., Ltd. All solvents used in this study were purchased from Sinopharm Chemical Reagent Co., Ltd. as analytical grade. HESEO was provided by Dr. Xiao-Ming Shi from Linyi People's Hospital, Shandong, China. For the *in vitro* studies, HESEO was dissolved in DMSO. For the *in vivo* experiment, HESEO was prepared in 0.5% carboxymethylated cellulose freshly before use. MTX was dissolved in saline.

Laboratory animals. Female Balb/c mice (age, 6-weeks; weight, 18-20 g) were purchased from Charles River Laboratories, Inc. and housed in an animal facility at 23±2˚C and 40-70% humidity under a 12-h light/dark cycle. Mice had free access to food and water throughout the experimental period. All animal experimental procedures were performed in strict accordance with the Chinese legislation on the use and care of laboratory animals, and were approved by the Ethical Committee on Animal Care and Use of Guizhou Medical University. Animal health and behavior were monitored three times every day. Mice were euthanized immediately when they showed signs of distress, including palpable hypothermia, hunching, lethargy or body weight loss of >20%, or when they reached the humane endpoints of tumor volume reaching 1,500 mm^3 or the maximum diameter exhibited by a single subcutaneous tumor reaching 20 mm, in order to minimize their suffering. Mice were euthanized by inhalation of CO_2 at a flow rate of 1.6 l/min in an 8 l chamber for 5 min; death was confirmed by cervical dislocation.

Cell proliferation. Cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 100 μg/ml streptomycin/penicillin at 37˚C and 5% CO_2. Cells were treated with HESEO at 1, 3 and 10 nM for 72 h and cell number was measured using a cell counter (Vi-Cell; Beckman Coulter, Inc.).

Mitochondrial membrane potential (MMP) measurement. MMP was measured using the staining reagent JC-1. After treatment with HESEO at 1, 3 and 10 nM or DMSO as a negative control for 48 h at 37˚C, MG-63 cells were suspended at $5x10^5$ cells/ml and incubated with JC-1 at a final concentration of 2 μM for 15 min at 37˚C in the dark. After washing twice, the red/green fluorescence intensity was measured using a fluorescence microplate reader at an excitation of 490 nm and emission of 530/590 nm.

Cytochrome c assay. After treatment, MG-63 cells were collected and fractionated with a subcellular protein fractionation kit for cultured cells (cat. no. 78840; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The cytoplasmic fraction was used to measure cytochrome *c* levels with the quantikine ELISA kit (cat. no. DCTC0; R&D Systems, Inc.). Briefly, 100 μl calibrator diluent was added to each well of a 96-well microplate before 100 μl standard, control or sample was added to each well. The wells were covered with a plate seal and incubated at room temperature for 2 h. Each well was then aspirated and washed three times before 200 μl conjugate was added to each well. The wells were, once again, covered with a plate seal and incubated at room temperature for 2 h. Wells were aspirated and washed four times prior to the addition of 200 μl substrate solution to each well. The wells were incubated at room temperature for 30 min before 50 μl stop solution was added to each well. The absorbance was read at 450 nm within 30 min.

Caspase activity measurement. After treatment, MG-63 cells were collected and lysed with cell lysis buffer (cat. no. #9803, Cell Signaling Technology, Inc.). Caspase activity was measured using assay kits (cat. no. K106-100 for caspase-3; cat. no. K119-110 for caspase-9; R&D Systems, Inc.), according to the manufacturer's instructions.

Apoptosis analysis. After treatment, MG-63 cells were harvested and washed with pre-chilled PBS, and then resuspended in binding buffer to adjust the cell concentration to $5x10^5$ cells/ml. Subsequently, 5 μl Annexin V-FITC was added to 195 μl cell suspension and incubated for 10 min at room temperature. Cells were then washed and resuspended in 190 μl binding buffer, and 10 μl PI was added to each sample for 1 min in the dark at room temperature. Analysis was performed using a FACScan flow cytometer (BD Biosciences). The data were analysed with FlowJo version 10 (FlowJo LLC).

Reverse transcription-quantitative PCR. After treatment, total RNA was extracted from MG-63 cells using a TRIzol® extraction kit (Invitrogen; Thermo Fisher, Scientific, Inc.).

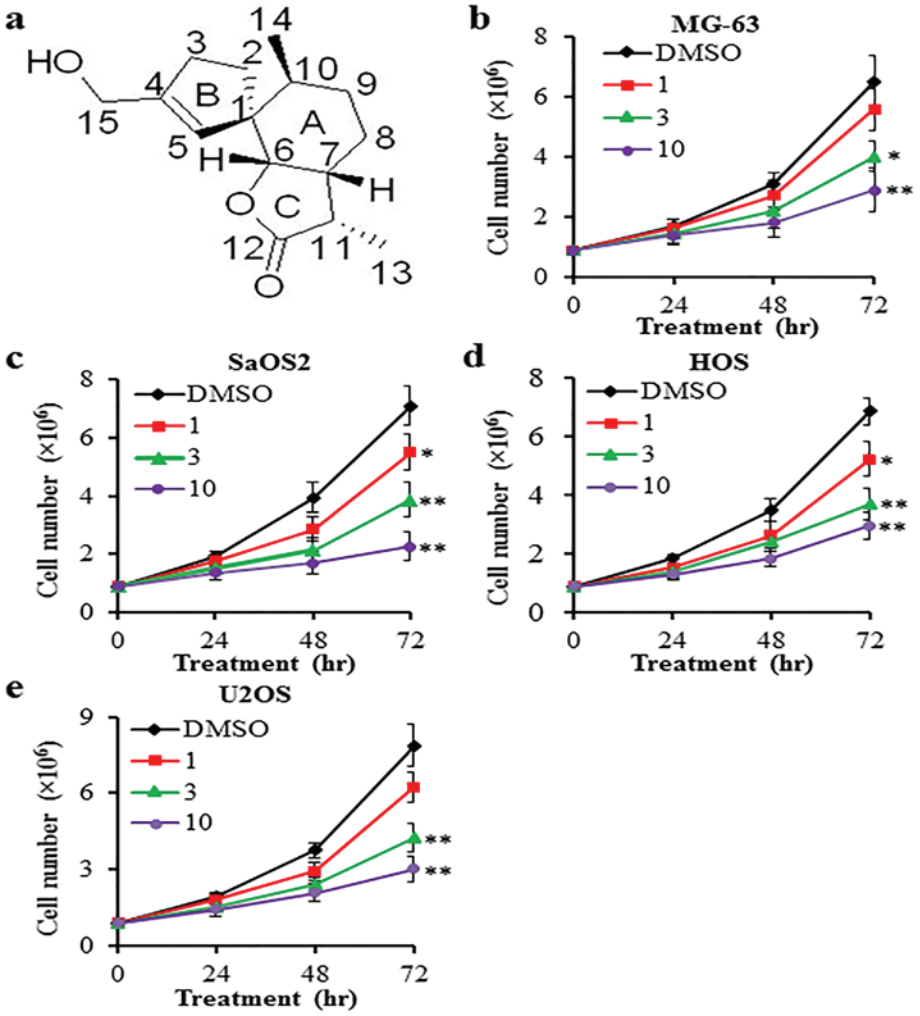

Figure 1. HESEO inhibits osteosarcoma cell proliferation. (A) HESEO structure. Osteosarcoma cells were treated with HESEO at 1, 3 and 10 nM for 72 h. Cell numbers were counted. HESEO inhibited the proliferation of (B) MG-63, (C) SaOS2, (D) HOS and (E) U2OS cells. Data are presented as the mean ± SD (n=3). *P<0.05, **P<0.01 vs. control group. HESEO, 15-hydroxy-6α,12-epoxy-7β,10αH,11βH-spiroax-4-ene-12-one.

The RNA concentration was measured using a spectrophotometer. mRNA was transcribed into cDNA (25˚C for 5 min, 46˚C for 20 min, 95˚C for 1 min) using SuperScript master mix (Bio-Rad Laboratories, Inc.). RT-qPCR was run on a StepOne system using SYBR green Supermix (Thermo Fisher Scientific, Inc.). The thermocycling conditions were: 95˚C for 10 min, and 40 cycles of 95˚C for 15 sec and 60˚C for 60 sec. Subsequently, relative expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method and were normalized to β-actin (17). The gene-specific primer sequences used were the following. Caspase-3: Forward-5'-ATTGTGGAATTGATGCGTGA-3', Reverse-3'-GGCAGGCCTGAATAATGAAA-5' (GenBank reference: AJ413269.1); Caspase-9: Forward-5'-agggaagaggga atggaaga-3', Reverse:-3'-GAGTCGTCACACTTCCAGCA-5' (GenBank reference: AY214168.1); β-actin: Forward-5'-GCT CTTTTCCAGCCTTCCTT-3', Reverse-3'-AGTACTTGCGCT CAGGAGGA-5' (GenBank reference: HQ154074.1).

Western blot analysis. After treatment, MG-63 cells or mouse tumor tissues were lysed with radioimmunoprecipitation assay lysis buffer (Thermo Fisher Scientific, Inc.) containing a cocktail of protease inhibitors. The lysates were centrifuged at 6,000 x g for 10 min at 4˚C, and protein concentrations were determined using the BCA method. After boiling for 5 min, proteins (40 μg/lane) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 4-12% gels and were then transferred to polyvinylidene fluoride membranes. After blocking with 1% BSA (Sigma-Aldrich; Merck KGaA) for 1 h at room temperature, proteins were incubated overnight at 4˚C with the appropriate rabbit primary antibodies: Anti-cleaved caspase-3 (cat. no. ab2302; 1:1,000), anti-cleaved caspase-9 (cat. no. ab2324; 1:1,000), anti-Bcl-2 (cat.no. ab196495; 1:1,000), anti-Bax (cat. no. ab104156; 1:500), anti-NF-κB phosphorylated (p)-p65 S536 (cat. no. ab86299; 1:3,000), anti-NF-κB p65 (cat. no. ab16502; 1:1,000); anti-PUMA (cat. no. ab9643; 1:500) and anti-β-actin (cat. no. ab227387; 1:3,000) and then further incubated with the secondary horseradish peroxidase-conjugated antibody (cat. no. ab205718; 1:3,000) for 2 h at room temperature. All antibodies were supplied by Abcam. The bands were detected using an ECL system (EMD Millipore).

Syngeneic tumor models. K7M2 cells ($6x10^5$ in 100 μl PBS) were injected into Balb/c mice through the tail vein. Mice

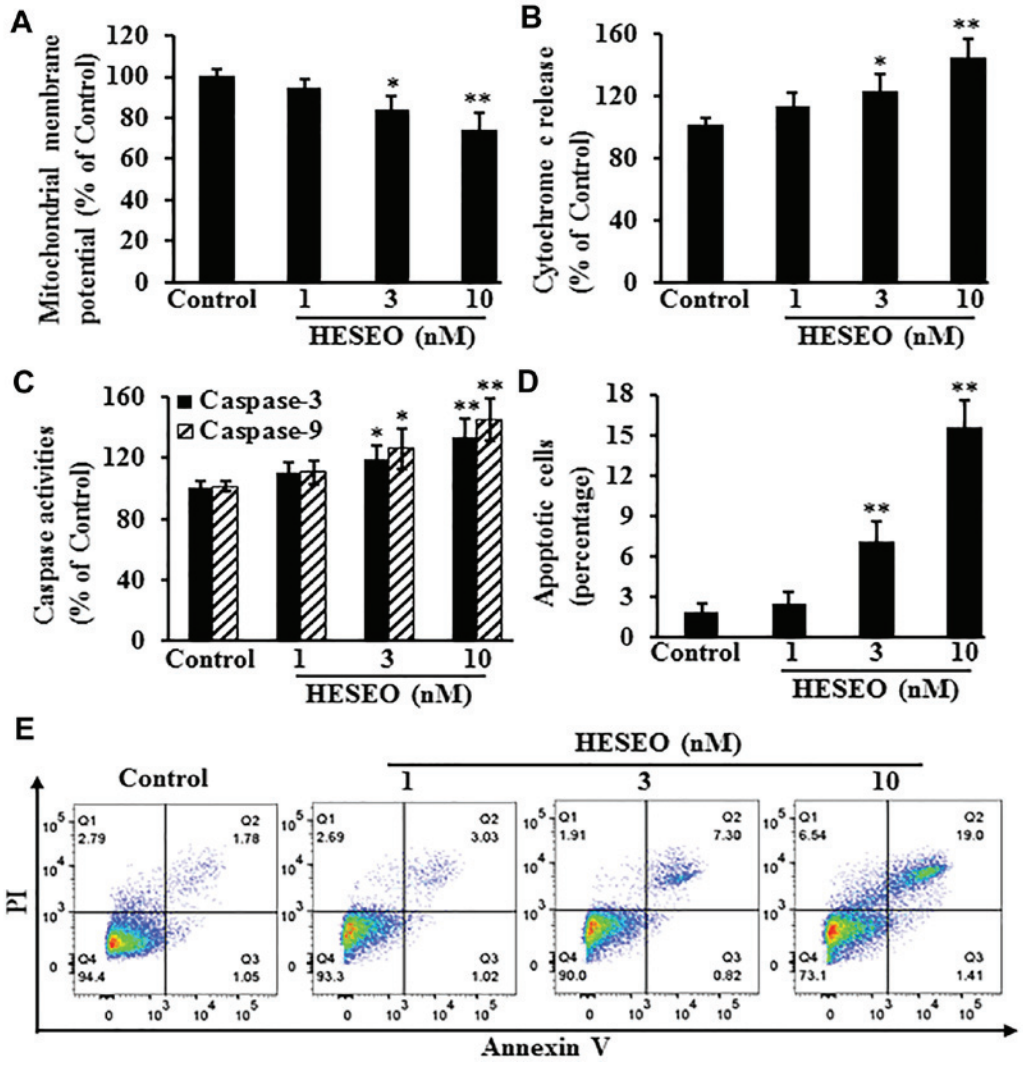

Figure 2. HESEO induces MG-63 cell apoptosis. After treatment with HESEO for 48 h, the levels of (A) MMP depolarization, (B) cytochrome *c* release, (C) caspase activity and (D) apoptosis were determined. (E) Representative dot plots indicating the level of apoptotic cells Data are presented as the mean ± SD (n=3). *P<0.05, **P<0.01 vs. control group. HESEO, 15-hydroxy-6α,12-epoxy-7β,10αH,11βH-spiroax-4-ene-12-one; MMP, mitochondrial membrane potential.

were divided randomly into three groups (5 mice/group) and treatment was initiated 14 days after cell injection. Mice were treated with HESEO (1 or 2 mg/kg) by intragastric administration 5 days per week or were treated with 0.5% carboxymethylated cellulose (vehicle; 50 mg/kg) as a negative control. For the combination study, $6x10^5$ K7M2 cells in 100 μl PBS were injected into Balb/c mice through the tail vein. Mice were divided randomly into four groups (5 mice/group) and treatment was initiated 14 days after cell injection. Mice were treated with HESEO (1 mg/kg) by intragastric administration 5 days per week, with MTX (87.5 mg/kg) by intraperitoneal injection weekly, or with the combination treatment. Mice treated with vehicle served as the negative control.

K7M2 cells ($5x10^5$) were subcutaneously injected into Balb/c mice. When tumor volume reached 50-100 mm^3, tumor-bearing mice were equally divided into three different groups by tumor volume (5 mice/group). Mice were treated with HESEO (1 or 2 mg/kg) by intragastric administration 5 days per week or were treated with vehicle as a negative control. Once tumor volume reached 1,500 mm^3 or the maximum diameter exhibited by a single subcutaneous tumor reached 20 mm, all tumor-bearing mice were sacrificed to collect tumors for analysis.

Statistical analysis. Data are presented as the mean ± SD and were analyzed using SPSS version 12.0 (SPSS, Inc.). Data were analyzed by one-way analysis of variance followed by Dunnett's post-hoc test. For the subcutaneous tumor model, one-way analysis of variance followed by Dunnett's post-hoc test was used to compare differences among the groups. For the tail vein injection tumor model, Kaplan-Meier survival analysis was used to compare differences among the groups and P-values were calculated using the log-rank test. P<0.05 was considered to indicate a statistically significant difference. Samples were measured in triplicate and experiments were repeated three times.

Results

HESEO inhibits the proliferation of osteosarcoma cells. Cell proliferation was measured by counting the cell number after

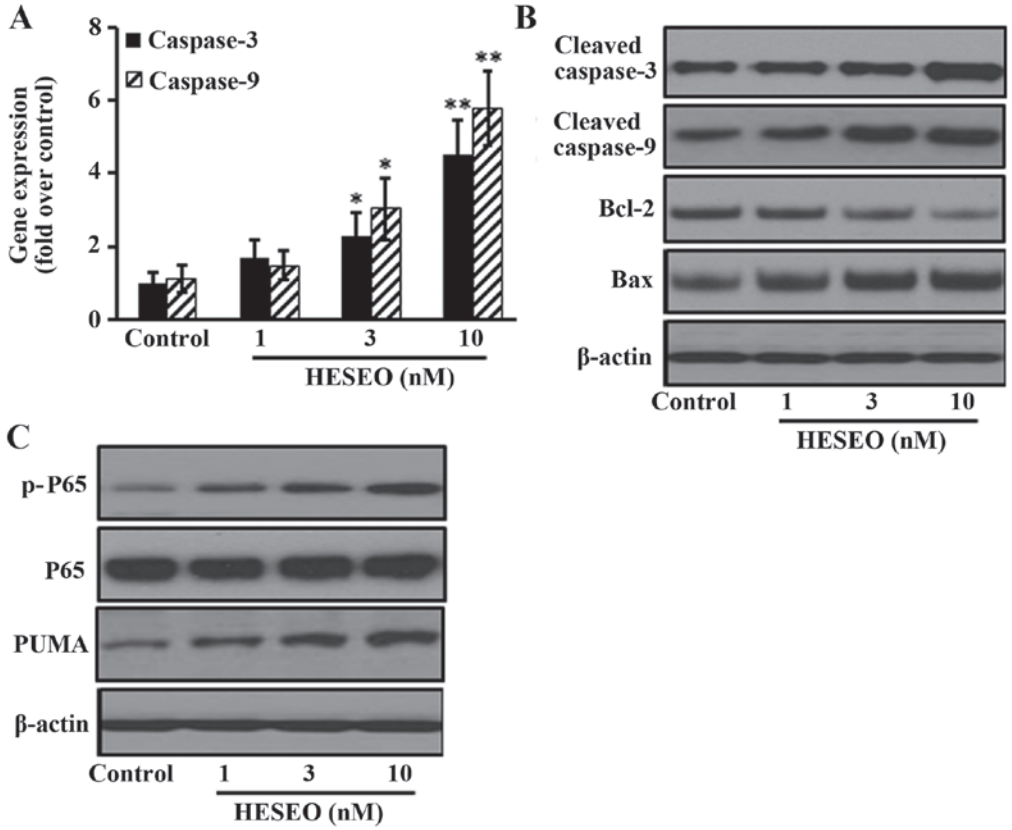

Figure 3. HESEO modulates apoptosis-related gene and protein expression in MG-63 cells. After treatment with HESEO for 48 h, HESEO (A) increased caspase gene expression; (B) increased pro-apoptotic protein expression and decreased anti-apoptotic protein expression; and (C) increased p-P65 and PUMA protein levels. Data are presented as the mean ± SD (n=3). *P<0.05, **P<0.01 vs. respective control group. HESEO, 15-hydroxy-6α,12-epoxy-7β,10αH,11βH-spiroax-4-ene-12-one; p-, phosphorylated; PUMA, p53 upregulated modulator of apoptosis.

72 h. HESEO demonstrated anti-proliferative activities on MG-63, SaOS2, HOS and U2OS cells when compared with vehicle control (DMSO; Fig. 1).

HESEO induces apoptosis of MG-63 cells. After treatment, levels of apoptosis-related markers were detected. Compared with the DMSO control, HESEO reduced the MMP (Fig. 2A), and increased the release of cytochrome *c* (Fig. 2B), caspase-3/9 activities (Fig. 2C) and the percentage of apoptotic cells (Fig. 2D and E).

HESEO induces apoptosis through increasing the expression of NF-κB p-P65 and PUMA. After HESEO treatment, the expression of pro-apoptotic genes was significantly increased when compared with control treatment (Fig. 3A). The expression of pro-apoptotic proteins (cleaved caspase-3, cleaved caspase-9, Bax) was also increased, whereas the expression levels of anti-apoptotic proteins (Bcl-2) were decreased with HESEO treatment compared with in the control group (Fig. 3B). HESEO treatment also increased the expression levels of NF-κB p-P65 and PUMA compared with in the control group (Fig. 3C).

HESEO increases the survival time and decreases the tumor burden of K7M2 tumor-bearing mice. The anti-tumor effects of HESEO were further explored in syngeneic tumor models. K7M2 tumor-bearing mice were treated with 1 or 2 mg/kg HESEO. HESEO treatment at 2 mg/kg significantly increased the survival time of tumor-bearing mice with lung metastasis (Fig. 4A) and decreased the tumor burden of tumor-bearing mice in a subcutaneous tumor model (Fig. 4B and C) when compared with control treatment. Furthermore, HESEO treatment increased NF-κB p-P65 levels and PUMA expression in tumor tissues of mice subcutaneously injected with cells (Fig. 4D). In addition, combination treatment with HESEO and the chemotherapeutic agent MTX significantly increased the survival time of tumor-bearing mice with lung metastasis over either treatment alone (Fig. 4E).

Discussion

As a genetically unstable and highly malignant mesenchymal bone tumor, osteosarcoma is characterized by structural chromosomal alterations (18-20). Although advances in multimodality treatment, consisting of radiation and chemotherapy, have led to a relatively good prognosis, the 5-year survival rate for patients with osteosarcoma has reduced in the past two decades, due to the presence of pulmonary metastasis in 40-50% of patients (21,22). Therefore, to improve survival levels, it is imperative to search for effective treatment strategies. In the present study, HESEO, a natural compound extracted from the endophytic fungus *Penicillium* sp. FJ-1 isolated from *Avicennia marina*, was found not only to inhibit the proliferation of MG-63 cells, but also to inhibit the proliferation of other human osteosarcoma cells when compared with the control group. Furthermore, HESEO induced apoptosis of

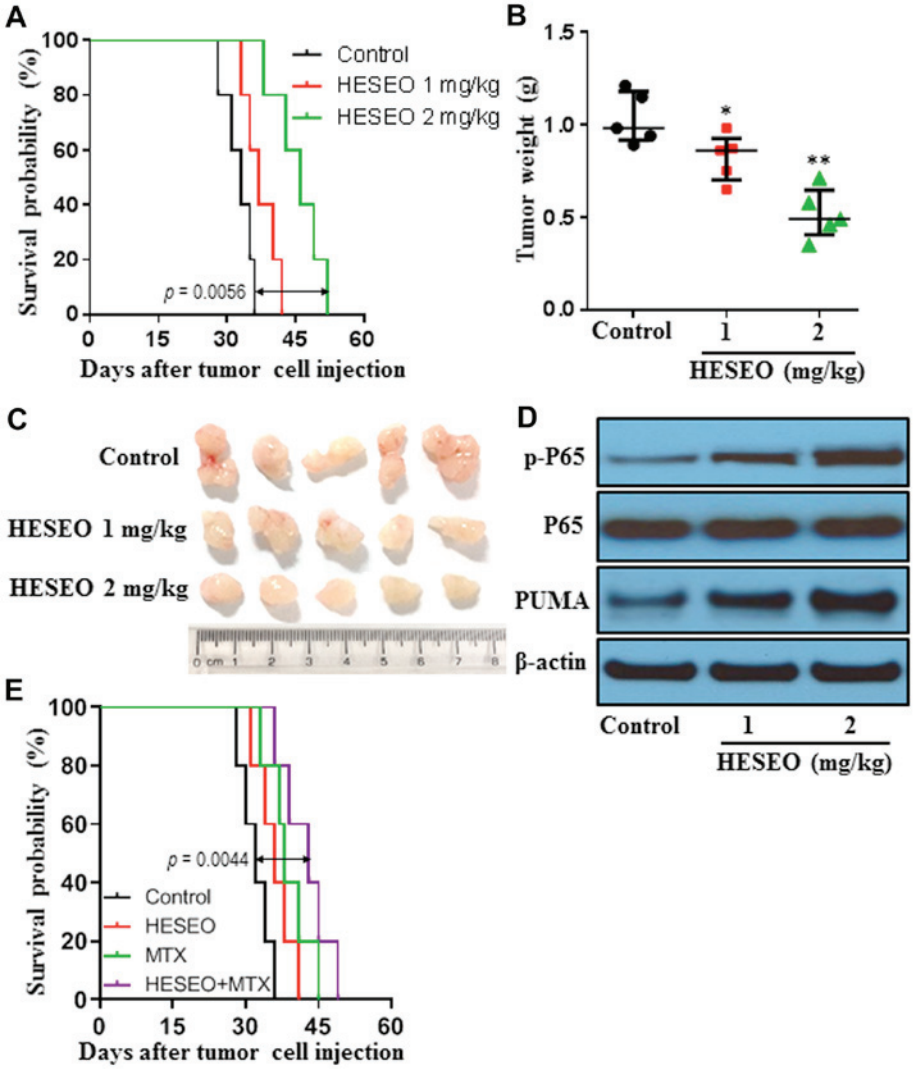

Figure 4. HESEO treatment increases the survival time and decreases the tumor burden of K7M2 tumor-bearing mice. (A) Kaplan-Meier survival analysis was performed to compare differences among groups and the P-value was calculated with the log-rank test. (B) HESEO treatment significantly decreased the tumor burden of tumor-bearing mice in a subcutaneous tumor model; (C) representative images of tumors. (D) HESEO treatment increased NF-κB p-P65 levels and PUMA expression in tumor tissues of mice subcutaneously injected with cells (E). Combined treatment with HESEO and MTX significantly increased the survival time of tumor-bearing mice with lung metastasis over either treatment alone. Data are presented as the median ± interquartile range (n=5). *P<0.05, **P<0.01 vs. control group. HESEO, 15-hydroxy-6α,12-epoxy-7β,10αH,11βH-spiroax-4-ene-12-one; MTX, methotrexate; p-, phosphorylated; PUMA, p53 upregulated modulator of apoptosis.

MG-63 cells, which may be due to the increased expression of NF-κB p-P65 and PUMA, and increased the survival time and decreased the tumor burden of osteosarcoma tumor-bearing mice when compared with control treatment. Other pathways, such as PI3K-AKT-mTOR, RAS-RAF-MAF and cell cycle regulation, will be explored in future studies.

Mitochondrial dysfunction has been shown to induce apoptosis and is suggested to be central to the apoptotic pathway (23,24). As a group of proteolytic enzymes at the end of the apoptotic signaling pathway, the caspase family plays an essential role in apoptosis. Initiator caspases initiate the apoptosis signal while the executioner caspases carry out the mass proteolysis that leads to apoptosis (25). Notably, the Bcl-2 family exerts its pro-apoptotic (through Bax and Bak) or anti-apoptotic (through Bcl-2 and Bcl-X_L) activities mainly through the mitochondrial pathway. When cells are stimulated, intracellular Bax translocates to the mitochondrial outer membrane and forms a Bax/Bcl-2 heterodimer with Bcl-2 to induce the release of cytochrome *c* from the mitochondria to the cytoplasm, thereby inducing the downstream apoptosis cascade pathway (26,27). The present study indicated that HESEO treatment caused MG-63 cell damage, characterized by reduced MMP expression, increased cytochrome *c* release, increased caspase activity, marked downregulation of anti-apoptotic protein Bcl-2 expression, and upregulation of the expression of pro-apoptotic proteins Bax and caspase-3/caspase-9.

When inactive, NF-κB remains in the cytoplasm bound to specific inhibitory proteins. Upon activation, NF-κB translocates to the nucleus and binds to κB consensus sequences to modulate numerous target genes (28). As a mediator of cell survival, NF-κB signaling acts downstream of the TNF-α receptor. Notably, the TNF-α signal bifurcates downstream of the TNF-α receptor into at least two axes, one of which activates NF-κB to trigger cell death (29). As a target of NF-κB and a critical mediator of TNF-α-induced

apoptosis, PUMA has been demonstrated to be induced in response to DNA-damaging agents, such as commonly used chemotherapeutic drugs and irradiation (30,31). The pro-apoptotic function of PUMA is mediated by its interactions with anti-apoptotic Bcl-2 family members, such as Bcl-2 and Bcl-X_L, leading to Bax/Bak-dependent mitochondrial dysfunction, cytochrome *c* release and caspase activation. The role of PUMA in DNA damage-induced apoptosis has been confirmed using PUMA-knockout cells and mice (32-34). MG-63 cells are p53-deficient, but anti-tumor drugs (such as doxorubicin) can increase PUMA protein expression after 48 h (35). In the present study, MG-63 cells were treated with HESEO for 48 h before PUMA expression was determined. Similarly to doxorubicin, HESEO could increase PUMA expression. Furthermore, the present study reported that HESEO treatment increased NF-κB p-P65 levels, which may induce apoptosis of MG-63 cells.

In conclusion, the present study demonstrated that HESEO, isolated from the endophytic fungus *Penicillium* sp. FJ-1 of *Avicennia marina*, may inhibit osteosarcoma cell proliferation, and increase survival time and decrease tumor burden in tumor-bearing mice, suggesting the scientific rationale to develop HESEO as a therapeutic agent against osteosarcoma.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CFN and SZ designed and conceived the current study. TZA, ZL, CY, XQH, PCL and JS performed the experiments and analyzed the data. TZA, ZL, CFN and SZ prepared the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were performed in compliance with the Chinese legislation on the use and care of laboratory animals, and were approved by The Ethical Committee on Animal Care and Use of Guizhou Medical University (protocol no. 2017-0163).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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