

RESEARCH ARTICLE

The Synergistic Anticancer Effect of Artesunate Combined with Allicin in Osteosarcoma Cell Line *in Vitro* and *in Vivo*

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Abstract

Background: Artesunate, extracted from *Artemisia annua*, has been proven to have anti-cancer potential. Allicin, diallyl thiosulfinate, the main biologically active compound derived from garlic, is also of interest in cancer treatment research. This object of this report was to document synergistic effects of artesunate combined with allicin on osteosarcoma cell lines *in vitro* and *in vivo*. **Methods:** After treatment with artesunate and allicin at various concentrations, the viability of osteosarcoma cells was analyzed by MTT method, with assessment of invasion and motility, colony formation and apoptosis. Western Blotting was performed to determine the expression of caspase-3/9, and activity was also detected after drug treatment. Moreover, in a nude mouse model established with orthotopic xenograft tumors, tumor weight and volume were monitored after drug administration via the intraperitoneal (i.p.) route. **Results:** The viability of osteosarcoma cells in the combination group was significantly decreased in a concentration and time dependent manner; moreover, invasion, motility and colony formation ability were significantly suppressed and the apoptotic rate was significantly increased through caspase-3/9 expression and activity enhancement in the combination group. Furthermore, suppression of tumor growth was evident *in vivo*. **Conclusion:** Our results indicated that artesunate and allicin in combination exert synergistic effects on osteosarcoma cell proliferation and apoptosis.

Keywords: Artesunate - allicin - osteosarcoma cell line - apoptosis

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Introduction

Osteosarcoma is one of the most common malignant bone tumors in children and adolescents (Meyers et al., 1998; Bacci et al., 2000; Hauben et al., 2003; Chano et al., 2004; Marina et al., 2004; Benassi et al., 2007). The present therapeutic strategies in clinical applications have low efficiency under the current osteosarcoma therapy (Huang et al., 2012; Choeyprasert et al., 2013). Therefore, new drugs for osteosarcoma therapy are urgently needed to explore.

Artesunate, is a derivative of artemisinin isolated from *Artemisia annua* L, has been used to treat malaria, especially against cerebral malaria (Padilla-Camberos et al., 2010; Bachmeier et al., 2011; Berdelle et al., 2011; Jin et al., 2011; Mota et al., 2011; Jiang et al., 2012; Zhou et al., 2012), new report shown that it has a variety of biological activities, such as hepatoprotective, antioxidative, anti-inflammatory, antidiabetic, antiallergic, and antibacterial effects (Li et al., 2009; Bat-Chen et al., 2010; Du et al., 2010; Sertel et al., 2010; Zhang et al., 2010; Hamacher-Brady et al., 2011; Cheng et al., 2013; Li et al., 2013). Allicin, diallyl thiosulfinate, is the main biologically active compound derived from garlic (Oommen et al., 2004; Arditti et al., 2005; Park et al., 2005; Zhang et al., 2006;

Miron et al., 2012). Allicin became an object of interest due to its potential to confer a vast spectrum of health beneficial effects including: anti- microbial, anti-fungal and anti-parasitic, anti-hypertensive, cardioprotective, anti-inflammatory and anti-cancer activities (Zhang et al., 2010; Cha et al., 2012; Louis et al., 2012; Wang et al., 2012).

The present study aimed to evaluate the antiproliferative artesunate and allicin in human osteosarcoma cell lines and to analyze its synergistic antitumor effect.

Materials and Methods

Cell lines, cell culture and reagents

Human osteosarcoma lines (MG-63, U2OS, 143-B, SaOS-2 and HOS) were obtained from American Type Culture Collection (ATCC, USA). Cells were cultured immediately after the thawing of the cells at a concentration of 5×10^6 cells/mL in RPMI 1640 culture medium (Hyclone, USA) supplemented with 10% FBS (Hyclone, USA), 100 U/mL penicillin and 100 U/mL streptomycin. Cells were incubated in a humidified atmosphere containing 5% carbon dioxide at 37 °C. Artesunate, provided by Guilin South Pharmaceutical Company Limited (purity > 99.0%, Guilin, Guangxi,

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China). Artesunate was dissolved in 1 ml of 5% sodium bicarbonate to obtain 1 mM stock solution, and then diluted in medium to the appropriate working concentration. Allicin (Xuzhou Ryen Pharma. Co., Ltd., China, 15 mg/ml) were used directly.

Cell viability assay

The viability of osteosarcoma lines after artesunate, allicin and their combination treatment were analyzed by MTT method. Briefly, cells were seeded into 96-well plates at a density of 4.0×10^3 cells per well, incubated for 24 h before the drug treatment. After 24 h, 48 h, 72 h treatment with varying doses of drug or its combination. After treatments, discard medium, wash with PBS and incubated with MTT (5 mg/mL in phosphate-buffered saline), incubated for another 4 h at 37 °C. Discard the medium, DMSO was added to dissolve MTT formazan crystals and absorbance was measured using a multiwell plate reader (BioTek, Winooski, VT, USA) at 570 nm. Cell viability was calculated as percentage of viable cells in total population.

Invasion and motility assays

For invasion assay, filters were coated with Matrigel™ (Sigma) diluted to 1 mg/mL in serum-free RPMI1640 medium. The inserts were incubated at 37 °C for 1 h to allow gel polymerisation. MG-63 and U2OS cells were harvested and suspended with RPMI containing 10% FBS. Cells were incubated at 37 °C for 24 h. Afterwards, the inner side of the filter was wiped with a wet swab to remove the cells while the outer side of the insert was rinsed with PBS and stained with 0.25% crystal violet (Sigma) for 10 min, rinsed again and then allowed to dry. The filters were then viewed under microscope and the percent of area occupied by migrated or invading cells. The procedure for carrying out motility assays was identical to the protocol used for invasion assays with the exception that the inserts were not coated with matrigel.

Colony formation analysis

MG-63 and U2OS cells were seeded into 6-cm dishes. After 6 h incubation, they were treated with concentrations of artesunate, allicin alone or in combination. Colonies were fixed with methanol and stained with 1.25% Giemsa and 0.125% crystal violet for counting. Cell survival was expressed in relation to the untreated control.

Western blotting

Cells were plated at a density of 3×10^5 /mL in 6-well plates the day before treatment, and then exposed to an artesunate, allicin or a combination of the two drugs. Following centrifugation and sonication, cell extracts were clarified at 12000 rpm for 10 min. Protein concentrations were measured using a BCA assay Kit. Protein samples (40 µg), diluted with SDS sample buffer, were separated by 10% polyacrylamide gel electrophoresis, and followed by electro-blotting on a polyvinylidene difluoride (PVDF) membrane. After blocking with non-fat milk, the membrane was probed overnight at 4 °C with primary antibodies following: anti-Caspase 3, anti- Caspase 9 (Cell Signaling), immunoreactivity was detected using

anti-mouse IgG conjugated peroxidase, then visualized by with the ECL kit (Pierce, Rockford, IL, USA) following to the manufacturer's instructions.

Caspase activity measurement

The assay is based on the cleavage of the chromogenic substrates, DEVD-pNA and LEHD-pNA, by caspase-3 and caspase-9, respectively. Colorimetric assay kit (BioBox, Nanjing, China) was used to detect the activity of caspase-3 and caspase-9 following the manufacturer's instruction. P MG-63 and U2OS cells were seeded into 96-well white opaque plates. The next day, cells were treated with artesunate and allicin. At the end of the incubation, cells were lysed in lysis buffer on ice and centrifuged for 5 min at 10000 rpm. Caspase substrate solution containing the specific peptide substrate was then added to the supernatant; incubate for 2 h at 37 °C before measurement by microplate reader.

In vivo assay

Animal experiment was approved by the Institutional Animal Care and Use Committee, animal care was in strict compliance with the institutional guidelines. BALB/c nude mice were purchased from the Shanghai Laboratory Animal Center of the Chinese Academy Sciences. MG-63 cells were harvested, washed with PBS, and re-suspended in PBS, then mice were injected subcutaneously (s.c.) into each posterior hind flank region with approximately 6.0×10^6 cells, one site per mouse. Xenografts were allowed to grow, and treatment was started when the injected cell mass reached a mean volume of 150 mm³. After tumor formation, the mice were randomized into four groups (N = 5 per group), and administered artesunate (50 mg/kg per day, i.p.), allicin (5 mg/kg per day, i.p.), combination (50 mg/kg artesunate plus 5 mg/kg per day allicin, i.p.), saline only in control group. Tumor growth was monitored every 5 days. The width (Wid) and the length (Len) of the tumors were measured using a slide caliper and the volume (V) of each tumor was calculated as follows: $V = \text{Wid}^2 \times \text{Len} / 2$.

Statistical analysis

All data are expressed in text as mean ± standard deviation (SD). Statistical analysis of the difference between treated and untreated groups was performed with Student's t-test. Values of $P < 0.05$ were considered as significant differences.

Results

Artesunate and allicin synergistically enhanced the inhibitory effect on osteosarcoma cancer cells *in vitro*

The viability of MG-63 and U2OS cells treated with artesunate and allicin was analyzed by MTT analysis. The growth of MG-63 and U2OS cells was significantly inhibited by artesunate (Figure 1A) and allicin (Figure 1B) in a dose dependent manner. To evaluate the synergistic effect of artesunate and allicin in combination, the viability of MG-63 cells were analyzed. The viability was significantly decreased in combination compared with single drug used (Figure 1C). Osteosarcoma cancer cell line 143-B, SaOS-2 and HOS were also used to analyze

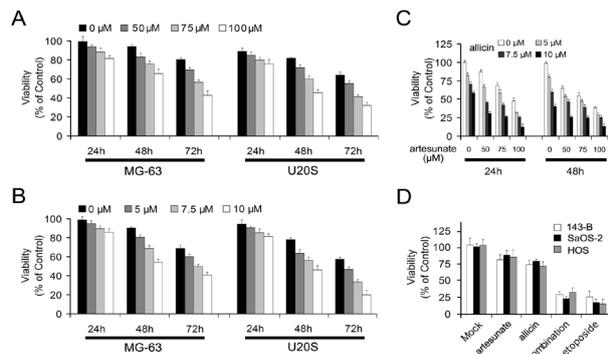


Figure 1. Cell Viability of Artesunate and Allicin Combination on Osteosarcoma Cells. (A) Viability of MG-63 and U2OS cells treated by artesunate at the concentration of 50, 75 and 100 μM for 24 h, 48h and 72h. (B) Viability of MG-63 and U2OS cells treated by allicin at the concentration of 5, 7.5 and 10 μM for 24 h, 48h and 72h. (C) Viability of MG-63 cells treated by artesunate (50, 75 and 100 μM) and allicin (5, 7.5 and 10 μM) combination at the concentration of for 24 h and 48h. (D) Artesunate and allicin combination on other osteosarcoma cells (143-B, SaOS-2 and HOS) for 48 h treatment ($P < 0.05$)

the synergistic effect, and this synergistic effect still exists after artesunate and allicin in combination (Figure 1D).

Artesunate and allicin synergistically induce osteosarcoma cancer cells apoptosis

The key feature of malignant cells is their motility and invasion to distant sites, invade surrounding tissue, and its colony formation ability. The motility assays and invasion assays were performed after drug combination. As is shown in Figure 2, combination treatment of artesunate and allicin suppressed MG-63 and U2OS cells' motility (Figure 2A) and invasion (Figure 2B) when compared to drugs used separately ($P < 0.05$), moreover, the combination decreased the colony numbers of MG-63 and U2OS cells (Figure 2C). Lastly, to confirm the apoptosis induction after combination, MG-63 and U2OS cells were treated with the drugs and examined by FCM analysis. At the concentrations tested, artesunate and allicin combination still elicited significant apoptosis compared to drug used separately (Figure 2D) ($P < 0.05$).

Caspase-3/9 expression and activity significantly up-regulated in combination treatment

Caspase cascades activation is a classic signals

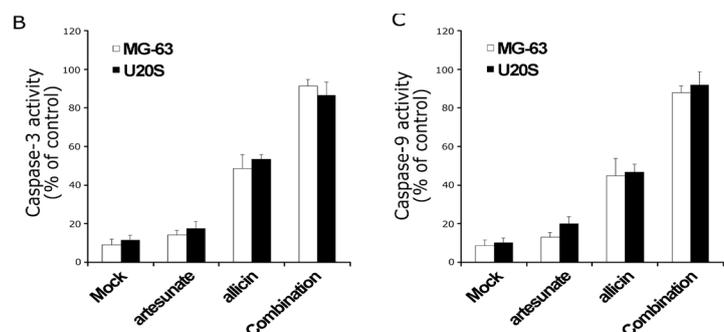
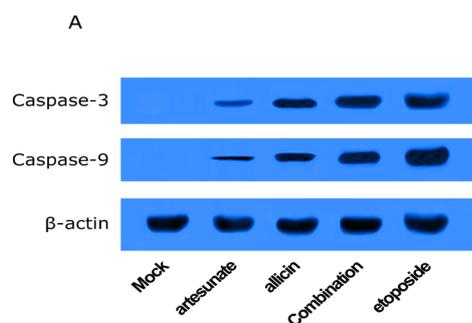


Figure 3. Caspase-3 and Caspase-9 Expression and Its Activity after Combination of Artesunate and Allicin on MG-63 and U2OS Cells. (A) Caspase-3 and Caspase-9 protein expression level detected by Western blotting. (B) Caspase-3 activity detection after drug alone or combination (75 μM artesunate plus 7.5 nM allicin) treatment. (C) Caspase-9 activity detection after treated with single drug or combination (75 μM artesunate plus 7.5 nM allicin)

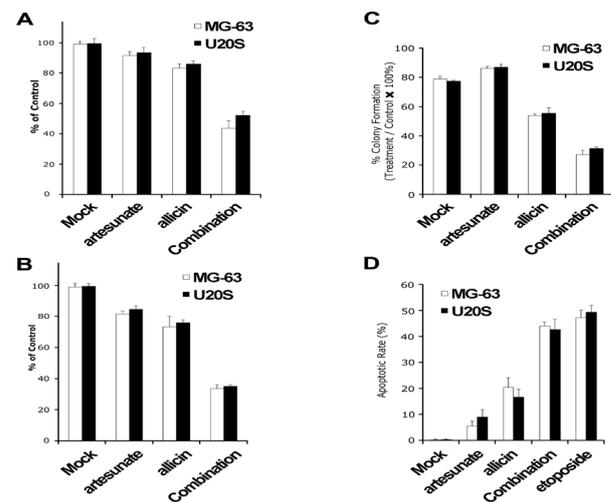


Figure 2. Motility, Invasion, Colony Formation and Apoptosis on Osteosarcoma Cells Treated by Artesunate and Allicin Combination. (A) Motility of MG-63 and U2OS after drug alone or combination (75 μM artesunate plus 7.5 nM allicin) exposure for 48 h. (B) Invasion of MG-63 and U2OS after drug alone or combination (75 μM artesunate plus 7.5 nM allicin) treated for 48 h. (C) Colony formation of MG-63 and U2OS after drug alone or combination (75 μM artesunate plus 7.5 nM allicin) used for 48 h. (D) Apoptotic rate of MG-63 and U2OS after drug alone or combination (75 μM artesunate plus 7.5 nM allicin) treatment for 48 h

during apoptotic generation, in Figure 3, caspase-3 and caspase-9 expression was significantly increased after the artesunate and allicin combination treatment in MG-63 cells (Figure 3A), and the activity of caspase-3 (Figure 3B) and caspase-9 (Figure 3C) in MG-63 and U2OS increased significantly with drug combination, which means that the activation of caspases was triggered in MG-63 and U2OS cells.

Combination suppresses *in vivo* tumor growth

The antitumor effect of artesunate and allicin in combination was investigated in a osteosarcoma cancer MG-63 cells bearing mice. After implantation for one week, we chose 40 mice with tumor xenografts and randomly divided them into four groups. The present study has shown that osteosarcoma cell growth was significantly inhibited in the combination group in tumor volume (Figure 4A) and weight (Figure 4B) compared with the group of the drug used separately ($P < 0.05$).

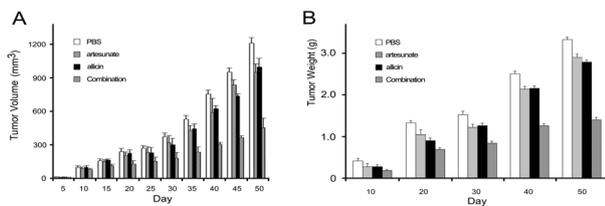


Figure 4. Therapy for Osteosarcoma Model (MG-63 osteosarcoma cells bearing mice). (A) Tumor volume of mouse model under different treatment. (B) Tumor weight of mouse model on different therapies

Discussion

Osteosarcoma is the most important primary malignant tumor of bone especially in children (Hauben et al., 2003). Until recently, a 5-year survival rate of 20% for treatment through surgical intervention alone was considered acceptable (Meyers et al., 1998; Bacci et al., 2000; Benassi et al., 2007). In the past decade, the survival of patients with osteosarcoma has increased, due to rapid advances in neoadjuvant chemotherapy (Chano et al., 2004; Marina et al., 2004). Although combination of surgery with chemotherapy has noticeably improved the survival rate of osteosarcoma patients, the application of anticancer drugs is still associated with significant adverse reactions, for instance acquisition of drug-resistant phenotypes, necessitating the development of new chemotherapeutic agents (Huang et al., 2012; Choeprasert et al., 2013). Recently, tumor therapy by traditional Chinese herb is becoming more and more attractive (Li et al., 2007; 2008; 2013; Youns et al., 2009; Louis et al., 2012; Miron et al., 2012; Cheng et al., 2013).

The present study demonstrates that artesunate and allicin combination exerts potent to suppress the osteosarcoma cells effects both *in vitro* and *in vivo*. The caspase signaling cascades are central to the process of apoptosis, and the extrinsic and intrinsic cascades are triggered by caspase-3 and caspase-9. The combination inhibited the proliferation and induced apoptosis of osteosarcoma cells in motility, invasion and colony formation by caspase-3 and caspase-9 activation. At last, we found that an i.p administration of artesunate and allicin combination suppressed tumor growth *in vivo*.

In conclusion, in present study, we demonstrate that artesunate combined with allicin can synergistically suppress the growth of human osteosarcoma cells *in vitro* and *in vivo*. It appears that this combination induces apoptosis in human osteosarcoma cells by increasing the activation of the Caspase-3/9. Our data suggest the potential clinical use of artesunate and allicin combination in the treatment of patients with osteosarcoma.

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