

Anticancer effects of β -elemene with hyperthermia in lung cancer cells

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Abstract. β -elemene is a novel, plant-derived anticancer drug, which has been used to target multiple solid tumor types. Hyperthermia is an adjuvant therapeutic modality to treat cancer. However, the underlying mechanisms associated with the efficacy of these two treatments are largely unknown. The aim of the present study was to evaluate the effects of β -elemene combined with hyperthermia in lung cancer cell lines. An MTT assay was used to determine cell viability. The cell cycle and apoptosis were analyzed using flow cytometry. The morphology of cells during apoptosis was determined using a transmission electron microscope. The expression levels of P21, survivin, caspase-9, B-cell lymphoma 2 (Bcl-2) and Bcl-2-like protein 4 (Bax) mRNA were detected using quantitative polymerase chain reaction. β -elemene with hyperthermia treatment significantly inhibited the viability and increased the apoptosis rate of A549 cells compared with β -elemene treatment alone ($P<0.01$), and significantly decreased the proportion of cells in S phase compared with the control ($P<0.01$). Morphological observation using transmission electron microscopy indicated cross-sectional features of apoptosis: Chromatin condensation, reduced integrity of the plasma membrane, increased cellular granularity, nuclear collapse and the formation of apoptotic bodies. β -elemene with hyperthermia treatment significantly promoted P21 and Bax mRNA expression ($P<0.01$) and significantly decreased caspase-9, Bcl-2 and survivin mRNA expression ($P<0.01$) in

A549 cells. In conclusion, β -elemene with hyperthermia has a significant inhibitory effect on A549 cells. This occurs through reducing S phase and inducing apoptosis, via an increase in P21 and Bax expression and a decrease in caspase-9, Bcl-2 and survivin expression.

Introduction

Lung cancer is the leading cause of cancer-related mortality throughout the world. Non-small cell lung cancer (NSCLC) in particular has low survival rates and poor outcomes (1). The optimal chemotherapeutic treatments for NSCLC are often limited by dose-related toxicity (2). Developing new treatments is necessary in order to improve the prognosis for NSCLC patients. Traditional Chinese Medicine is believed by some to be beneficial in anticancer treatment and in reducing the side effects of conventional treatment (3-9).

β -elemene (1-methyl-1-vinyl-2,4-di-isopropenyl-cyclohexane) is a novel anticancer drug extracted from the ginger plant, *Curcuma zedoaria*. It has been used to target multiple types of solid tumor, including lung, esophageal and breast cancers, hepatocarcinoma, glioblastoma and melanoma. β -elemene has been reported to inhibit the growth and DNA synthesis of multiple types of tumor cell, resulting in the apoptosis or suppressed growth of tumors, without severe side effects (10-17). Hyperthermia is an adjuvant therapeutic modality in cancer treatment, by which the tumor region is maintained at high temperatures in order to inhibit the growth of cancer cells. It can cause regression of multiple cancer types, including lung, breast, colon and pancreatic cancer (18-21). In addition, hyperthermia causes cancer cells to become more sensitive to the effects of radiation and certain anticancer drugs (22-25). The mechanisms of hyperthermia in treating cancers may be related to inhibiting DNA repair, promoting intracellular accumulation of chemical agents, altering cellular Ca^{2+} homeostasis, inducing cell cycle arrest and apoptosis, increasing membrane permeability and rearranging the cytoskeleton (26-28). Hyperthermia also has direct cytotoxic effects, which provide a number of other clinical advantages, including

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activation of the immune system against tumors, improvement of oxygenation and enhancing drug delivery (29-32). However, the underlying mechanisms associated with its efficacy are largely unknown.

The current study aimed to investigate the efficacy of β -elemene combined with hyperthermia treatment in suppressing lung cancer cell growth. The role of β -elemene and hyperthermia in inhibiting NSCLC cell growth was investigated and cell cycle arrest, apoptosis rates and the expression of key genes were evaluated. The results of the current study could provide a theoretical basis for their use in a novel anticancer therapeutic modality.

Materials and methods

Chemicals and reagents. β -elemene was obtained from Dalian Holley Kingkong Pharmaceutical Co., Ltd. (cat. no. 081152; Dalian, China). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum were obtained from HyClone (GE Healthcare Life Sciences, Logan, UT, USA). Cycletest™ Plus DNA Reagent kit and Annexin V-Fluorescein Isothiocyanate and Propidium Iodide (PI) Apoptosis Detection kits were purchased from BD Biosciences (Franklin Lakes, NJ, USA). All other chemical reagents were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

Cell lines and culture. The NSCLC cell line A549 was purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM supplemented with 10% fetal calf serum, in a humidified incubator (Thermo Fischer Scientific, Inc., Waltham, MA, USA) containing 5% CO₂ at 37°C.

MTT assay. Cells were seeded in 96-well plates at 8,000 cells/well and cultured overnight at 37°C. Various concentrations of β -elemene (0, 15, 30, 60, 125, 250 and 500 μ g/ml) were added and the plates were incubated at different temperatures (37 and 42°C) for 1.5 h in a humidified atmosphere containing 5% CO₂. After 24 h, 5 mg/ml MTT was added to each well for 4 h. Then, the level of formazan was assessed by measuring the absorbance using a Tecan microplate reader (Tecan Group Ltd., Männedorf, Switzerland) at 490 nm.

Cell cycle and apoptosis assay. The experimental groups were as follows: 15 μ g/ml β -elemene; 60 μ g/ml β -elemene; hyperthermia; hyperthermia and 15 μ g/ml β -elemene; and hyperthermia and 60 μ g/ml β -elemene. Hyperthermia groups were incubated at 42°C for 1.5 h. Cells were treated with or without β -elemene and/or hyperthermia according to their experimental group at 37°C for 24 h. The control group received neither β -elemene nor hyperthermia. Then the cells were harvested and incubated in 10 μ g/ml RNase for 30 min at 37°C and stained with 50 μ g/ml PI for 1 h at 4°C in the dark. Cell cycle analysis was performed on a fluorescence-activated cell sorting Calibur flow cytometer (BD Biosciences). Cells were resuspended in DMEM, then stained with PI and Annexin V. The flow cytometer was used to analyze the samples for apoptosis.

Morphology of apoptotic cells. Cells were collected and fixed with 2.5% glutaraldehyde in 0.02 M phosphate-buffered saline (pH 7.4) at 4°C for 4 h and post-fixed in 1% osmic acid for 1 h, dehydrated in an ascending acetone series and subsequently embedded in epoxy resin (Sigma-Aldrich; Merck KGaA) at 62°C for 48 h. Ultrathin 70-nm sections were stained with uranyl acetate and lead citrate. The ultrastructural organization was observed using a JEM-1200EX transmission electron microscope (JEOL, Ltd., Tokyo, Japan).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Cells were cultured in 6-cm culture capsules and treated according to the aforementioned group conditions for 24 h. The ABI 7500 Real Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used for RT-qPCR assays. Total RNA was extracted from A549 cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). P21, survivin, caspase-9, B-cell lymphoma 2 (Bcl-2) and Bcl-2-like protein 4 (Bax) mRNA levels were measured using an SYBR PrimeScript RT-PCR Kit (Takara Bio, Inc., Otsu, Japan).

Statistical analysis. All experiments were performed in triplicate. Data are presented as the mean \pm standard error of the mean. Differences among treatment groups were analyzed using the Student's t-test and SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

β -elemene with hyperthermia inhibits proliferation of A549 cells. β -elemene inhibited the growth of A549 cells in a dose-dependent manner, and the half maximal inhibitory concentration (IC₅₀) was 35.43 μ g/ml. β -elemene (500 μ g/ml) with hyperthermia significantly increased the inhibition of A549 cells compared with β -elemene (500 μ g/ml) treatment alone ($P < 0.01$; Fig. 1).

β -elemene with hyperthermia decreases S phase of A549 cells. Flow cytometric analysis revealed the effect of β -elemene with or without hyperthermia on cell cycle distribution. Treatment with hyperthermia significantly decreased the percentage of cells in S phase at all concentrations of β -elemene ($P < 0.01$; Fig. 2). The percentage of cells at S phase after β -elemene (60 μ g/ml) treatment and hyperthermia decreased from 35.60 to 30.75% compared with the control group. Hyperthermia treatment without β -elemene resulted in a significant reduction in S phase cells ($P < 0.01$), but treatment with hyperthermia and β -elemene does not appear to have had a significant effect compared with β -elemene alone (Fig. 2).

β -elemene with hyperthermia induces apoptosis in A549 cells. β -elemene treatment was found to increase the rate of apoptosis in A549 cells ($P < 0.01$; Fig. 3). Hyperthermia treatment also significantly increased the rate of apoptosis in A549 cells compared with the control (0 μ g/ml β -elemene) or with β -elemene treatment alone (15 or 60 μ g/ml) (all $P < 0.01$; Fig. 3).

Furthermore, morphological observation of β -elemene with hyperthermia-induced apoptosis in A549 cells using

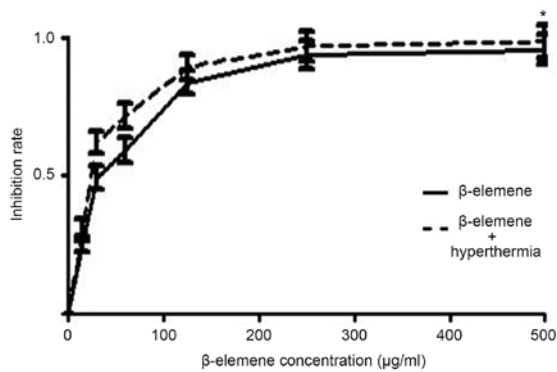


Figure 1. Inhibition rates of β -elemene with or without hyperthermia in A549 cells. Percentage of cell viability was determined using an MTT assay. * $P < 0.01$ vs. corresponding β -elemene treatment without hyperthermia.

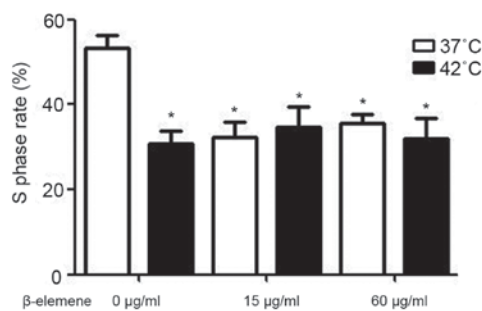


Figure 2. Cell cycle distribution induced by β -elemene with or without hyperthermia. The percentage of cells in S phase in different treatment groups was evaluated by flow cytometry. * $P < 0.01$ vs. control group (37°C, 0 µg/ml β -elemene).

transmission electron microscopy showed cross-sectional features of apoptosis. Hyperthermia and β -elemene treatment showed increased levels of chromatin condensation, nuclear collapse and apoptotic body formation compared with the control group or β -elemene treatment alone (Fig. 4).

β -elemene promotes P21 and Bax expression and suppresses caspase-9, Bcl-2 and survivin expression in A549 cells. RT-qPCR was used to identify the expression of P21, survivin, caspase-9, Bax and Bcl-2 genes. The results indicated a significant increase in P21 and Bax mRNA expression in the β -elemene (60 µg/ml) and hyperthermia treatment group compared with the β -elemene and control groups ($P < 0.01$; Fig. 5). Furthermore, a significant decrease in caspase-9, Bcl-2 and survivin mRNA expression was observed in the β -elemene (60 µg/ml) with hyperthermia group compared with the β -elemene and control groups ($P < 0.01$). These data suggested that β -elemene triggered apoptosis *in vitro* through an apoptosis pathway related to the aforementioned genes.

Discussion

The antitumor effect of β -elemene has been demonstrated in multiple types of tumor, particularly in lung cancer treatment, but the underlying mechanism remains unclear (10-17). Hyperthermia can be a highly effective cancer treatment, particularly when combined with chemotherapy, radiotherapy

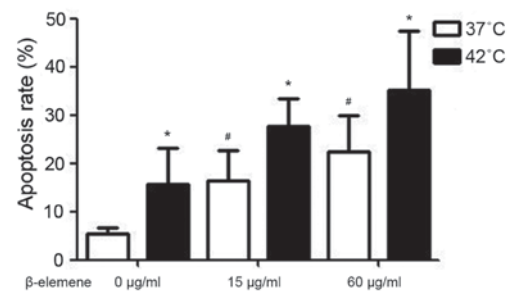


Figure 3. Apoptosis induced by β -elemene with or without hyperthermia. The percentage of apoptotic cells in different treatment groups was evaluated by flow cytometry. * $P < 0.01$ vs. control group (37°C, 0 µg/ml β -elemene), * $P < 0.01$ vs. corresponding β -elemene treatment without hyperthermia.

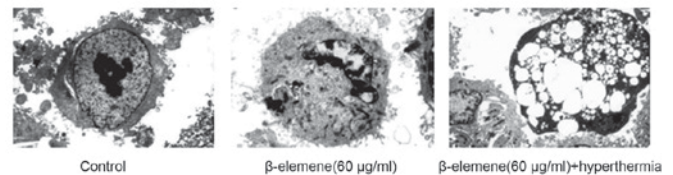


Figure 4. Transmission electron microscopy of cell groups. Higher levels of cell shrinkage, chromatin condensation, nuclear collapse and apoptotic bodies are visible in the 60 µg/ml β -elemene group and 60 µg/ml β -elemene + hyperthermia group compared with the control group.

or immunotherapy (33,34). Hyperthermia can inhibit DNA repair, promote intracellular accumulation of chemical agents, increase membrane permeability, alter cellular Ca^{2+} homeostasis, induce cell cycle arrest and apoptosis, and rearrange the cytoskeleton (25,27,28,30). Hyperthermia treatment has previously been found to affect various cellular targets, including DNA, proteins, membranes and the cytoskeleton, of carcinoma cells (35).

The present study investigated whether combining β -elemene treatment with hyperthermia could improve its anti-tumor activity. It was found that β -elemene with hyperthermia could inhibited the growth of A549 cells significantly more than β -elemene treatment alone. Furthermore, it was shown in the present study that β -elemene with hyperthermia altered the cell cycle distribution, significantly decreasing the percentage of cells in S phase compared with the control. Cell cycle regulation serves a key function in cell proliferation, and P21 is considered to be a key cell cycle checkpoint protein (36,37). In the present study, it was demonstrated that β -elemene with hyperthermia significantly increased the expression of P21, which resulted in a decreased S phase in the A549 cells. This suggests that P21 mediates cell proliferation via decreasing S phase in lung cancer cells.

Caspase-3 is a key executor in apoptosis and therefore serves a crucial function in programmed cell death. Apoptosis is related to the activation of Bax, which then binds to Bak to induce the release of cytochrome C. The release of cytochrome C is related to the cleavage of caspase-9. Interaction between caspase-9 and caspase-3, -6 and -7 activates the latter caspases in cells. Finally, activated caspase-3 causes DNA breakage and induces cell apoptosis (38-40). In this study, β -elemene with hyperthermia decreased caspase-9 expression and increased Bax expression. It is proposed that β -elemene

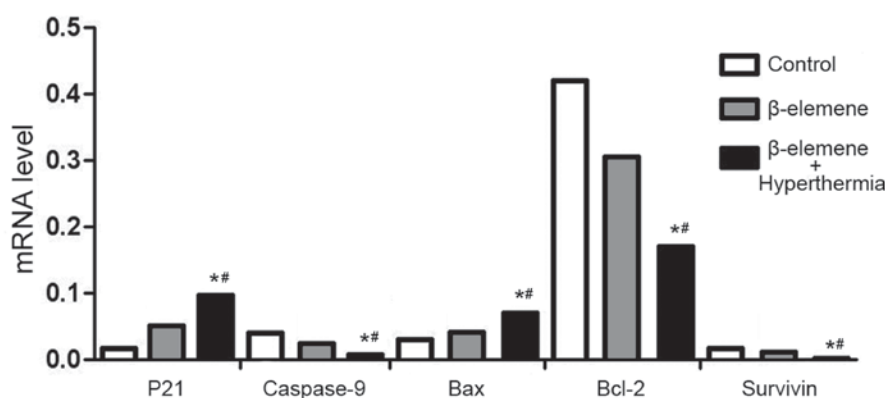


Figure 5. The mRNA levels of P21, Bcl-2, Bax, caspase-9 and survivin following β -elemene (60 μ g/ml) treatment with hyperthermia. * $P < 0.01$ vs. control, # $P < 0.01$ vs. β -elemene. Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-like protein 4.

with hyperthermia decreased caspase-9 activation through the induction of Bax expression. This suggests that β -elemene and hyperthermia-induced apoptosis involves a caspase-dependent pathway.

Bcl-2 can maintain cell survival by limiting the pro-apoptotic effects of Bax and suppressing the release of cytochrome C from mitochondria (41,42). Therefore, the Bcl-2/Bax ratio can induce the apoptosis of cells. It has been indicated that the Bcl-2/Bax ratio may be more critical in determining apoptosis than either protein alone (43,44). Furthermore, certain Bcl-2 family proteins have previously been found to be modulated in apoptosis progression. The balance between pro- and anti-apoptotic members of the Bcl-2 family proteins decides the fate of the cell, and an increased proportion of pro-apoptotic proteins results in apoptotic cell death (45,46). In the present study, β -elemene with hyperthermia induced apoptosis in A549 cells, and also increased Bax expression and decreased Bcl-2 expression. These findings suggest that Bcl-2 family proteins play a critical role in β -elemene and hyperthermia-induced cell death in lung cancer cells.

Survivin is a member of the inhibitor of apoptosis family. It is expressed in ~60 different human tumor types, with the highest levels of expression observed in breast and lung cancer cell lines (47-49). The survivin protein inhibits caspase activation, thereby leading to negative regulation of apoptosis. This has been demonstrated by the disruption of survivin induction pathways leading to an increase in apoptosis and a decrease in tumor growth (50,51). Survivin inhibits both Bax and Fas-induced apoptotic pathways and blocks apoptosis by directly inhibiting caspases (52). Consistent with this, in the present study, β -elemene with hyperthermia was found to both suppress the expression of survivin and induce apoptosis.

In conclusion, β -elemene with hyperthermia was shown to have a significant inhibitory effect in A549 cells. This occurs through decreasing S phase and inducing apoptosis, with a simultaneous increase in P21 and Bax mRNA expression and a decrease in caspase-9, Bcl-2 and survivin mRNA expression. The present study was a preliminary exploration into the anticancer effects of β -elemene with hyperthermia in lung cancer cells. Further research into the effects β -elemene and hyperthermia treatment would be valuable in improving therapeutic strategies for lung cancer.

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