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β-Elemene induces apoptosis as well as protective autophagy in human non-small-cell lung cancer A549 cells

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Abstract

Objectives β-Elemene, a novel traditional Chinese medicine, has been shown to be effective against a wide range of tumours. In this study, the antitumour effect of β-elemene on human non-small-cell lung cancer (NSCLC) A549 cells and the mechanism involved have been investigated.

Methods Cell viability and apoptosis were measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and flow cytometry, respectively. Protein expression was assayed by Western blotting. Autophagy was evaluated under fluorescence microscopy and transmission electron microscopy.

Key findings β-Elemene inhibited the viability of A549 cells in a dose-dependent manner. This suppression of cell viability was due to the induction of apoptosis. Further study showed that β-elemene inhibited the activity of the PI3K/Akt/mTOR/p70S6K1 signalling pathway, and at the same time it triggered a robust autophagy. The autophagy was characterized by the accumulation of punctate LC3 dots in the cytoplasm, morphological changes, and the increased levels of LC3-II as well as Atg5-Atg12 conjugated proteins. Inhibition of autophagy with chlorochine significantly enhanced the antitumour effect of β-elemene.

Conclusions Our data indicated that β-elemene inhibited the activity of the PI3K/Akt/mTOR/p70S6K1 signalling pathway in human NSCLC A549 cells, which resulted in apoptosis as well as protective autophagy. A combination of β-elemene with autophagy inhibitor might be an effective therapeutic option for advanced NSCLC.

Introduction

Lung cancer is the leading cause of cancer death worldwide. Approximately 85% of lung cancer cases are non-small-cell lung cancer (NSCLC), with most NSCLC patients being diagnosed at late stage, thus missing the opportunity for surgery.[1] Combinational chemotherapy is the major treatment for advanced NSCLC, but the overall survival time is less than 1 year.[2] Thus, new agents with high antitumour effect on NSCLC are urgently needed.

Recently, some traditional Chinese medicines have exhibited promising anticancer effects on leukaemia and some solid tumours. Among these drugs, elemene (1-methyl-1-vinyl-2,4-diisopropenyl-cyclohexane), an extract from the ginger plant *Rhizoma zeodaria*, is a good candidate for a cancer treatment.[3] β-Elemene, the major component of elemene, has been shown to be effective against a variety of tumours *in vitro* and *in vivo*.[4–6] Clinical trials with β-elemene have shown that it can effectively treat certain types of tumours and presents fewer side effects than other cytotoxic agents.[7,8] The high efficacy towards tumour cells and the low toxicity to normal tissues make β-elemene a potential tumour-specific cancer therapy.

Although the detailed mechanisms by which β-elemene kills tumour cells are not clear, recent studies have shown that β-elemene could inhibit cell proliferation by inducing apoptosis and cell-cycle arrest.[3,9,10] The apoptosis was associated with the down-regulation of anti-apoptotic proteins such as Bcl-2, Bcl-X(L) and XIAP.[3,4,11,12] Yet how upstream signalling pathways regulate β-elemene-induced apoptosis is not clearly understood. PI3K/Akt/mTOR is a major signalling pathway that regulates cell survival, growth and apoptosis.[13] It can be

induced by a wide range of stimuli and often acts as an anti-apoptotic signal. Inhibition of PI3K/Akt/mTOR activity has been reported to inhibit cell proliferation and enhance the apoptosis-inducing ability of certain anticancer drugs.[14,15] This indicates that the PI3K/Akt/mTOR signal might be a good target for cancer therapy.

Meanwhile, mTOR is also a key regulator of autophagy.[16] Autophagy is an intracellular degradation system that delivers cytoplasmic constituents to the lysosome.[17] It plays an important role in cellular homeostasis through the elimination of damaged old organelles as well as the turnover of long-lived proteins.[18] When cells encounter environmental stresses such as nutrient starvation or anticancer-drug treatment, autophagy can be activated and protects cells by preventing them from undergoing apoptosis. The mechanisms by which autophagy inhibits apoptosis is not clear; one possibility is the sequestration of damaged cellular organs in autophagosomes. Recent studies have shown that some chemotherapeutics known to activate apoptosis also induce autophagy; inhibition of autophagy by pharmacological inhibitors can enhance the antitumour effects of certain cytotoxic agents.[19–21] In this case autophagy serves as a protective response against stress conditions and promotes cell survival. However, dysregulated or excessive autophagy may lead to autophagic cell death, the type II programmed cell death to distinguish it from apoptosis. Thus, autophagy is a process that can lead to cell death or paradoxically allows cells to escape cell death. The point at which autophagy becomes autophagic cell death remains unclear.

In this study, we have investigated whether β-elemene inhibited proliferation and induced apoptosis in human NSCLC A549 cells. The impacts of β-elemene on PI3K/Akt/mTOR signalling pathway was further investigated. More importantly, we illustrated the important role of autophagy in the anticancer effect of β-elemene.

Materials and Methods

Reagents and antibodies

β-Elemene was obtained from Yuanda Pharmaceuticals (Dalian, China). Propidium iodide, RNase and chlorochine were purchased from Sigma-Aldrich (St Louis, MO, USA). Hoechst33342 was from Invitrogen (Carlsbad, CA, USA). Anti-Bcl-2, anti-Bax, anti-Survivin, anti-actin and anti-Akt antibodies were purchased from Santa Cruz Biotechnology (CA, USA). Anti-LC3, anti-Beclin 1 and anti-Atg5 (reactivates with both Atg5 and Atg5-Atg12 conjugated proteins) antibodies were from Novus Biological (Littleton, CO, USA). Anti-PARP, anti-phospho-Akt (Ser-473), anti-phospho-mTOR (Ser-2448), anti-mTOR, anti-phospho-p70S6K1 (Thr-389), and anti-p70S6K1 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

Cell cultures

The human NSCLC cells A549 were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) containing 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 mg/ml) at 37°C under an atmosphere of 95% air and 5% CO_2. The cells were subcultured every 2–3 days and were all from the logarithmic phase of growth.

Cell viability assay

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded at 5×10^4 cells/well in 96-well plates, incubated overnight and then exposed to the indicated concentrations of β-elemene for the indicated times. Thereafter, 20 μl MTT solution (5 mg/ml) was added to each well, and the cells were incubated for another 4 h at 37°C. The cells were then lysed in 200 μl dimethyl sulfoxide, and the optical density was measured at 570 nm with a microplate reader (Model 550; Bio-Rad Laboratories, Hercules, CA, USA).

Analysis of apoptosis

Cells were seeded at 3×10^5 cells/well in six-well plates, incubated overnight and then exposed to the indicated concentrations of β-elemene for the indicated times. Cells were collected and incubated with 1 μg/ml Annexin V for 15 min in the dark, and then incubated with 1 μg/ml propidium iodide for 10 min. Finally, the samples were evaluated by flow cytometry and the data were analysed using WinMDI software. Annexin V positive cells were considered to be the apoptotic cells.

Fluorescence microscopy

Cells were seeded and treated with 10 or 50 μg/ml β-elemene for 24 h in Lab-Tek chamber slides (Nunc S/A, Polylabo, Strasbourg, France). Cells were then fixed in 3.3% paraformaldehyde for 20 min, permeabilized with 0.2% Triton X-100 for 3 min and blocked with 5% bovine serum albumin (BSA). The slides were incubated with anti-LC3 antibody for 1 h and then reacted with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG for 45 min. The nucleus was stained with Hoechst33342 for 5 min (Invitrogen), and the images were obtained with a fluorescence microscope (Olympus, Japan).

Transmission electron microscopy

Cells were treated with 50 μg/ml β-elemene for 24 h and collected by trypsinization, then fixed with 2.5% phosphate-

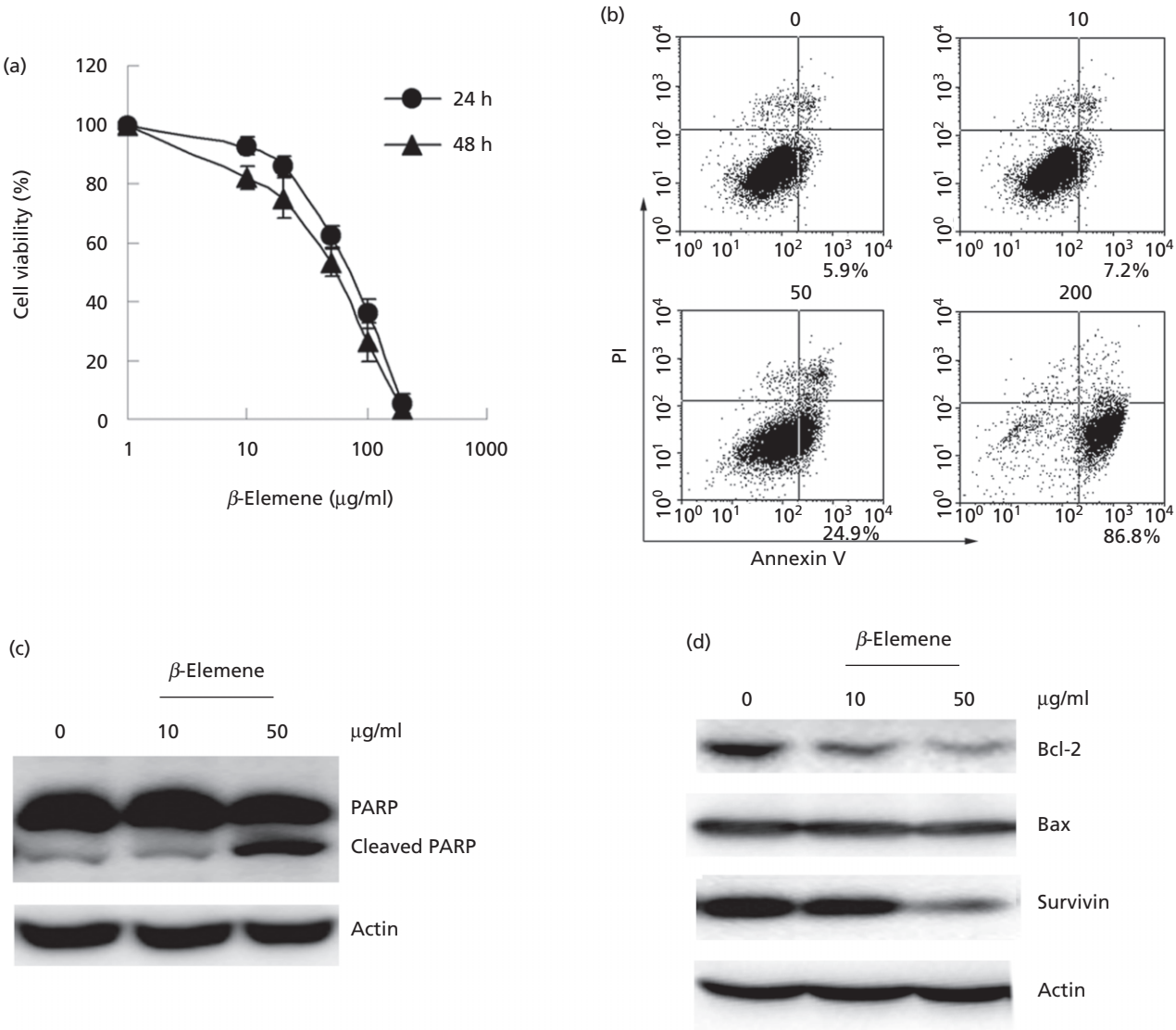

Figure 1 Effects of β-elemene on cell viability and apoptosis of A549 cells. (a) A549 cells were treated with β-elemene at the indicated concentrations for 24 or 48 h, and the cell viability was analysed by MTT assay. Dots: mean of three independent experiments; bars: SD. (b) A549 cells were treated with 10, 50 or 200 μg/ml β-elemene for 24 h, and apoptosis was determined by flow cytometry followed by propidium iodide (PI)-Annexin V staining. (c) Cells were treated with either 10 or 50 μg/ml β-elemene for 24 h, and the cleavage of poly ADP ribose polymerase (PARP) was detected by Western blotting. (d) Cells were treated with 10 or 50 μg/ml β-elemene for 24 h, and the expression of Bcl-2, Bax and Survivin were detected by Western blotting. The results were representatives of three independent experiments. Actin was used as loading control.

buffered gluteraldehyde, postfixed in 1% phosphate-buffered osmium tetroxide. Cells were then embedded, sectioned, double stained with uranyl acetate and lead citrate, and analysed using a JEM-1200EX transmission electron microscope (JEOL, Tokyo, Japan).

Western blotting

Cells were solubilized in 1% Triton lysis buffer on ice. Cell lysate proteins (50 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membranes (Immoblin-P; Millipore, Billerica, MA, USA). The membranes were blocked with 5% skim milk in TBST buffer (10 mM Tris (pH 7.4), 150 mM NaCl, 0.1% Tween 20), incubated with the indicated primary antibodies and reacted with horseradish peroxidase-conjugated secondary antibodies. The proteins were visualized with enhanced chemiluminescence reagent (SuperSignal Western Pico Chemiluminescent Substrate; Pierce, Rockford, IL, USA).

Statistical analysis

All the presented data were confirmed in at least three independent experiments and are expressed as the mean ± SD. Statistical comparisons were made by Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

β-Elemene inhibited the viability and induced apoptosis in A549 cells

The effect of β-elemene on the viability of A549 cells was first detected. As shown in Figure 1a, β-elemene inhibited cell viability in a dose-dependent manner. The IC50 values of β-elemene at 24 and 48 h were 53.5 and 36.5 μg/ml, respectively. Flow cytometry assays showed an increased apoptotic population among the cells treated with β-elemene (Figure 1b). To understand the molecular mechanism involved in β-elemene-induced apoptosis, the effects of β-elemene on apoptosis-associated proteins were investigated. Under the treatment of 50 μg/ml β-elemene for 24 h, poly ADP ribose polymerase (PARP) was cleaved to its active form, which confirmed the apoptosis induced by β-elemene (Figure 1c). β-Elemene had little effect on the expression of Bax, but decreased significantly the levels of Bcl-2 and Survivin (Figure 1d). These data indicated that β-elemene inhibited the viability of A549 cells through inducing apoptosis, which was associated with the down-regulation of anti-apoptotic proteins.

β-Elemene inhibited the PI3K/Akt/mTOR/p70S6K1 signalling pathway

PI3K/Akt is one of the most important signalling pathways in regulating survival and apoptosis. After treatment with 40 μg/ml β-elemene for 24 h, the level of phosphor-Akt was obviously down-regulated, leading to the down-regulation of downstream phosphor-mTOR as well as phosphor-p70S6K1 (Figure 2). A low dose (10 μg/ml) of β-elemene had little effect on PI3K/Akt/mTOR/p70S6K1 activity. These data indicated that β-elemene-induced apoptosis might have been due to its inhibition of the PI3K/Akt/mTOR/p70S6K1 signalling pathway.

β-Elemene induced autophagy in A549 cells

It has been well documented that mTOR inhibition could result in autophagy, thus we investigated if there was any autophagy in the cells treated with β-elemene. A549 cells were treated with 10 or 50 μg/ml β-elemene for 24 h, and the localization of LC3 was evaluated under fluorescent microscopy. As shown in Figure 3a, in the cells treated with 10 μg/ml β-elemene, over 20% of cells were observed with LC3-positive puncta. In the cells treated with 50 μg/ml β-elemene,

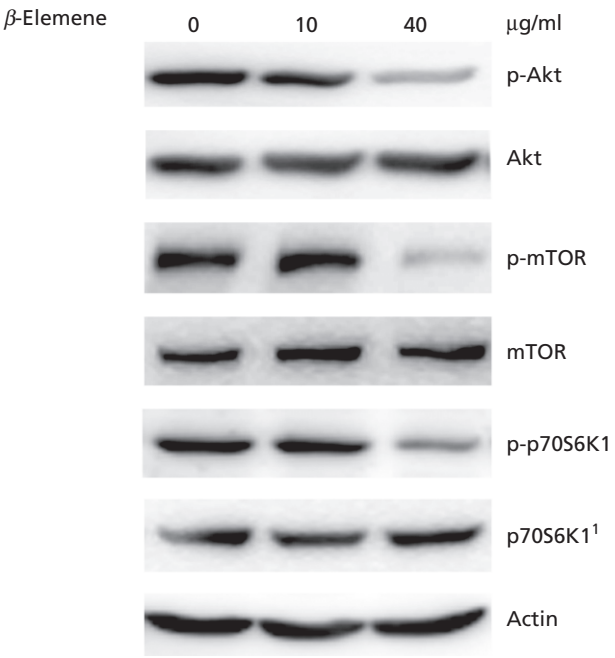

Figure 2 Effects of β-elemene on the PI3K/Akt/mTOR/p70S6K1 signalling pathway. A549 cells were exposed to 10 or 40 μg/ml β-elemene for 24 h, and the expression of phosphor-Akt (p-Akt), phosphor-mTOR (p-mTOR) and phosphor-p70S6K1 (p-p70S6K1) was detected by Western blotting. The results were representatives of three independent experiments. Actin was used as loading control.

more than 80% of cells showed LC3-positive puncta. The formation of autophagosomes was further confirmed by transmission electron microscopy. Upon treatment of 50 μg/ml β-elemene many double membrane enclosed vesicles containing engulfed organelles (autophagic vesicles) were observed in the cytoplasm (Figure 3b). Meanwhile, β-elemene treatment significantly increased the levels of LC3-II and Atg5-Atg12 conjugated protein as demonstrated by Western blotting. The expression of other Atgs such as Beclin 1 and Atg5 was scarcely affected (Figure 3c).These data indicated that β-elemene treatment not only resulted in apoptosis, but also induced autophagy.

Contribution of autophagy to the antitumour effects of β-elemene in A549 cells

Since autophagy can result in both survival and cell death, we investigated how β-elemene-induced autophagy contributed to its antitumour effects. A549 cells were treated with either 50 μg/ml β-elemene or 20 μM chlorochine (an inhibitor of autophagy which blocks the fusion of autophagosomes with lysosomes, stopping autophagy at the late phase), or co-treated with β-elemene and chlorochine for 24 h. Cell viability assays showed that co-treatment with β-elemene and

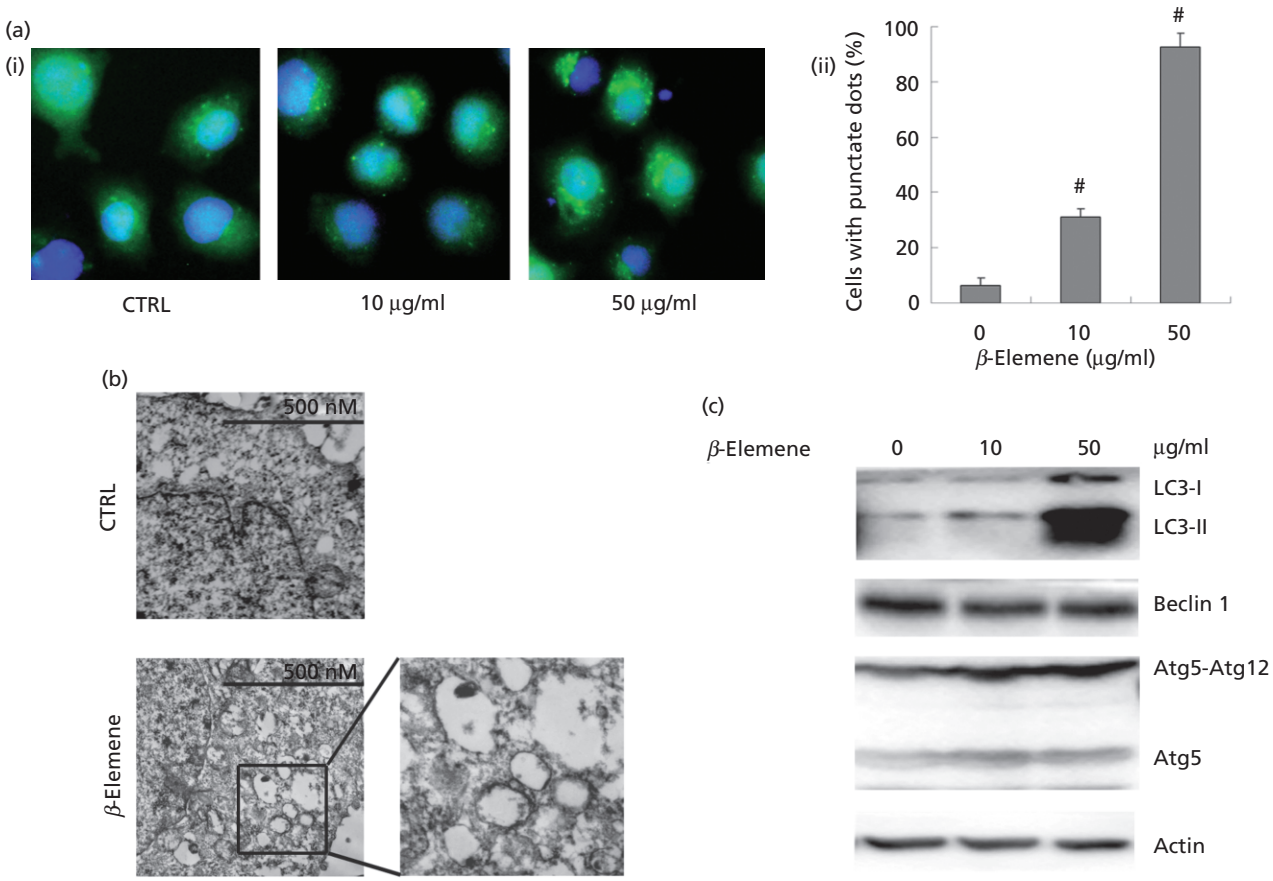

Figure 3 β-Elemene induced autophagy in A549 cells. (a) A549 cells were treated with either 10 or 50 μg/ml β-elemene for 24 h, stained and observed under a fluorescence microscope as described in Materials and Methods (i). CTRL, control. The percentage of cells with punctuate dots was represented with a histogram (ii). Columns: mean of three independent experiments; bars: SD. #$P < 0.01$ vs untreated cells. (b) Cells were treated with 50 μg/ml β-elemene for 24 h, then harvested and subjected to transmission electron microscopy as described in Materials and Methods. (c) After the cells were exposed to 10 or 50 μg/ml β-elemene for 24 h, cell lysates were subjected to Western blotting with specific antibodies. The results were representatives of three independent experiments. Actin was used as loading control.

chlorochine significantly decreased cell viability, compared with the cells treated with β-elemene alone (47.0 ± 3.0% vs 66.1 ± 4.6%, $P < 0.05$) (Figure 4a). Flow cytometry assays showed that co-treatment with β-elemene and chlorochine also increased the apoptotic population (Figure 4b). These data indicated that inhibition of autophagy enhanced the antitumour effects of β-elemene in A549 cells, which suggested that in the context of β-elemene treatment, autophagy was protective and prevented cells from undergoing apoptosis.

Discussion

β-Elemene as a novel traditional Chinese anticancer herb has shown broad antitumour effects *in vitro* and *in vivo*.[3–6,9–12] It has been approved by the State Food and Drug Administration of China for the treatment of malignant effusion and some solid tumours. Li *et al*.[4] reported that β-elemene induced cell cycle arrest and apoptotic cell death in human NSCLC H460 cells. In our study, we found that β-elemene inhibited the viability of another type of NSCLC cell, the A549 cell, by inducing apoptosis without altering the cell cycle distribution. This may have been due to the different regulating mechanism of cell cycle in different types of cell.

The mechanism by which β-elemene kills cells is still not clear. It has been reported that the apoptosis induced by β-elemene was associated with the down-regulation of anti-apoptotic proteins such as Bcl-2, Bcl-X(L) and XIAP.[3,11,12] In our study, not only was the expression of Bcl-2 down-regulated by β-elemene, the expression of Survivin, a member of the inhibitor of apoptosis family of proteins (IAPs) was also significantly down-regulated. This indicated that the down-regulation of these anti-apoptotic proteins might have contributed to the anticancer effect of β-elemene on A549 cells.

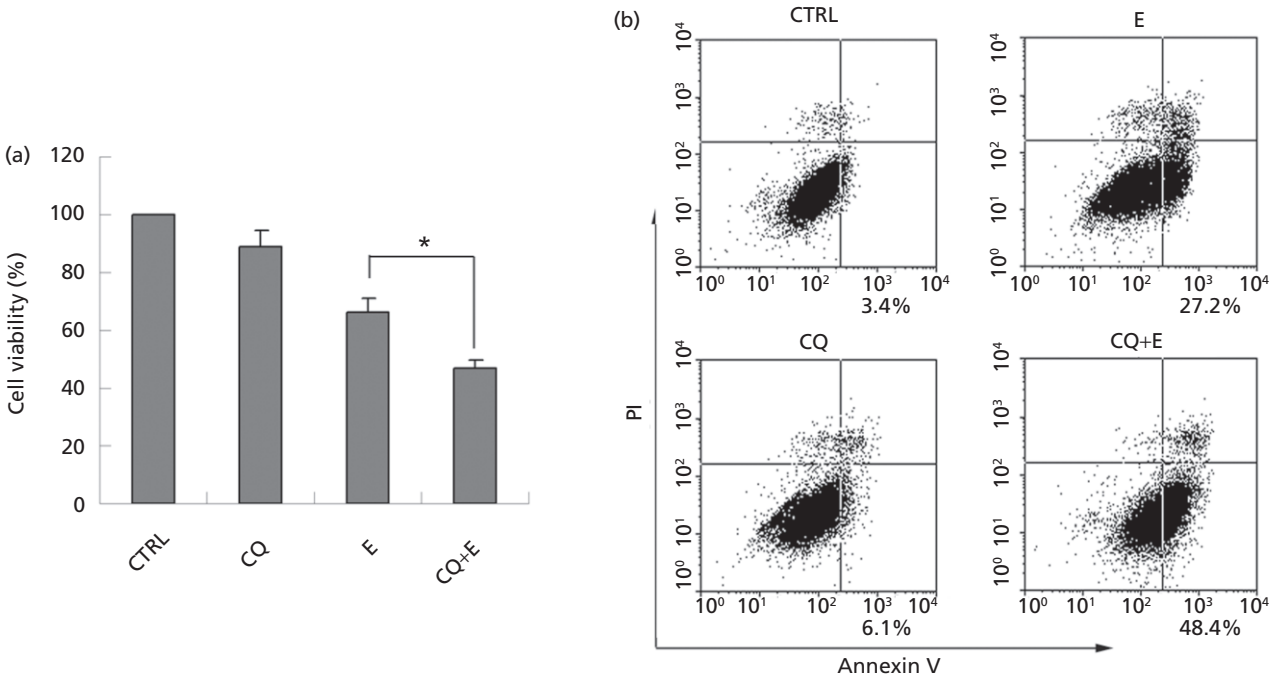

Figure 4 Inhibition of autophagy enhanced the antitumour effect of β-elemene. (a) A549 cells were exposed to either 50 μg/ml β-elemene (E) or 20 μM chlorochine (CQ), or a combined treatment of β-elemene and chlorochine for 24 h, and the cell viability was measured by MTT assay. CTRL, control. Columns: mean of three independent experiments; bars: SD. *$P < 0.05$ vs cells treated with β-elemene alone. (b) Cells were treated with the indicated agents for 24 h, and apoptosis was analysed by flow cytometry assay.

Unlike the influence on apoptotic protein, the effect of β-elemene on signalling transduction has not been well documented. Yao *et al*.[5] reported that the antitumour effect of β-elemene in glioblastoma cells depended on p38 mitogen-activated protein kinases (MAPKs) activation. Zhu *et al*.[22] reported that β-elemene inhibited proliferation of human glioblastoma cells via activation of MKK3 and MKK6. However, little is known about the effect of β-elemene on signalling pathways other than MAPKs. PI3K/Akt/mTOR is another major signalling pathway in regulation of proliferation and apoptosis. Once activated, Akt phosphorylates downstream target mTOR, leading to the activation of ribosomal p70S6 kinase (p70S6K1) and translation initiation factor 4E binding protein 1 (4EBP1). The activated p70S6K1 and 4EBP1 then lead to the up-regulation of a number of growth factors, promote cell growth and cell cycle progress.[23] Previous studies have shown that inhibition of PI3K/Akt/mTOR activity could result in proliferation inhibition and apoptosis induction. In our study β-elemene inhibited the phosphorylation of Akt, mTOR and p70S6K1, and induced apoptosis. Moreover, recent studies have suggested that Survivin is positively regulated by the PI3K/Akt/p70S6K1 pathway.[24] This may explain why β-elemene down-regulated the expression of Survivin in A549 cells. Taken together, the inhibition of PI3K/Akt/mTOR/p70S6K1 and Survivin by β-elemene finally led to the induction of apoptosis.

Meanwhile, mTOR is also a key regulator of autophagy. Inhibition of mTOR activity by some agents has been reported to activate autophagy.[16] In this study β-elemene treatment led to a robust autophagy, which was evident by the increase of punctate LC3 dots and by the cellular morphology. Other than mTOR, autophagy is also regulated by a group of evolutionarily conserved proteins, the Atg proteins.[17] Accumulated evidence suggests that the induction of autophagy is associated with the up-regulation of certain Atg proteins.[5,20,25] Zhu *et al*.[20] reported that increased transcription of Atg5 could lead to autophagy. Thyagarajan *et al*.[25] reported that triterpene-induced autophagy was accompanied by the up-regulation of Beclin 1. In our study β-elemene did not alter the level of either Beclin 1 or Atg5, but enhanced the expression of Atg5-Atg12 conjugated protein, and increased the ratio of LC3-II to LC3-I. These data indicated that autophagy induced by β-elemene in A549 cells was associated not only with the inhibition of mTOR activity, but also with the up-regulation of certain Atg proteins.

Autophagy is important in cell death decisions.[26] It has been documented that some anticancer agents induced protective autophagy and prevented cell death; inhibition of autophagy could enhance the anticancer effect of certain drugs.[26–28] But sometimes it can also kill cells. Li *et al*.[29] reported that tephrosin induced autophagic cell death in A549 cells. Saiki *et al*.[30] reported that caffeine induced

apoptosis by enhancement of autophagy. In our study, inhibition of autophagy by chlorochine significantly reduced cell viability and enhanced apoptosis, indicating the autophagy induced by β-elemene was a protective response. These data suggested that in A549 cells, the inhibition of PI3K/Akt/mTOR activity by β-elemene resulted in two opposite consequences: on the one hand, it inhibited cell proliferation and induced apoptosis; on the other hand, it activated a protective autophagy and protected cells from undergoing apoptosis. Inhibition of protective autophagy might be a good way to enhance the antitumour effect of β-elemene.

Conclusions

Taken together, our study demonstrated that β-elemene inhibited the proliferation of human NSCLC A549 cells by inducing apoptosis. The anticancer effect of β-elemene was associated with the down-regulation of anti-apoptitic proteins Bcl-2 and Survivin. More importantly, the PI3K/Akt/mTOR/p70S6K1 signalling pathway was inhibited by β-elemene, which also led to the activation of a protective autophagy. Inhibition of autophagy significantly enhanced the apoptosis-inducing ability, which suggests that the combination of β-elemene with an autophagy inhibitor might be useful for the treatment of advanced NSCLC.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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