Original Article
Inhibitory effect of β-elemene on human breast cancer cells

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Abstract: It has been approved for the clinical application of β-elemene to treat various cancers mainly brain tumors in China. In the present study, we found that β-elemene significantly inhibited the in vitro growth of human breast cancer cells by inducing apoptosis. In addition, β-elemene also induced the conversion of LC3-I into LC3-II as well as the formation of autolysosomes, indicating the activation of autophagy. Interestingly, inhibition of autophagy significantly potentiated the growth-inhibitory effect of β-elemene on breast cancer cells. In summary, β-elemene induced cytoprotective autophagy in human breast cancer cells in addition to apoptosis. Inhibition of autophagy significantly enhanced the cytotoxicity of β-elemene to human breast cancer cells. Therefore, combination of β-elemene with autophagy inhibitors could be a promising strategy for the treatment of breast cancer.

Keywords: β-elemene, breast cancer, apoptosis, autophagy

Introduction

Elemenes including α-, β-, γ-, and δ-elemene, are a group of natural chemical compounds extracted from a variety of medical herbs and plants such as Curcuma WenYuJin [1]. Elemenes contribute to the floral aromas of some plants, and are used as pheromones by some insects. Recently, β-elemene has attracted great interest because of its potent anti-proliferation effects toward some types of cancer cells [2-5]. Based on some small scale clinical trials with low quality in China, β-elemene has been approved by the State Food and Drug Administration of China for anti-cancer treatment. Up-to-now, β-ELE has been clinically used to treat leukemia and carcinomas in brain, breast, liver and other tissues.

It seems that β-elemene exerts its anti-cancer effect through multiple mechanisms. For example, β-elemene can induce apoptosis or cell cycle arrest [5-7], and reverse epithelial-mesenchymal transition (EMT) [8] and multidrug resistance (MDR) [9-11] in various cancer cells. Recently, it was frequently reported that autophagy could be induced by chemotherapeutic and targeted therapy drugs as a cytoprotection mechanism to count-act anti-cancer drugs [12-14]. In the present study, we found that β-elemene induced cytoprotective autophagy in breast cancer cells in addition to apoptosis. Inhibition of autophagy significantly enhanced the cytotoxicity of β-elemene, indicating that combination of β-elemene with autophagy inhibitors could be a promising strategy for cancer treatment.

Materials and methods

Reagents and antibodies

The chemicals such as β-elemene and Chloroquine were provided by J&K chemical Ltd. (Shanghai, China). The primary antibodies against microtubule-associated protein 1 light chain 3 (LC3) and GAPDH were bought from Santa Cruz.

Cell cultures

The breast cancer cell lines (Bcap37, MBA-MD-231) were bought from cell bank (Chinese Academy of Sciences). Monolayer culture of
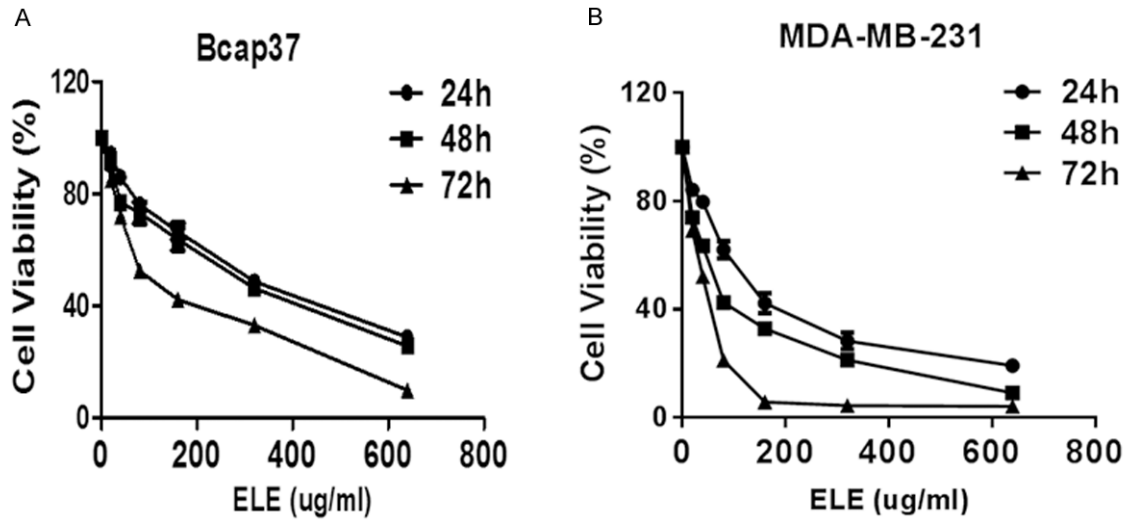
Figure 1. β-elemene inhibits the viability of breast cancer cells. Bcap37 (A) and MDA-MB-231 (B) cells were treated with varied concentration of β-elemene for different times as indicated. The cell viability was determined by MTS assay. The statistical significance was analyzed by One-Way ANOVA test. ELE: β-elemene.

cancer cells was maintained in DMEM supplemented with 10% fetal bovine serum 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin and incubated at 5% CO_2, 37°C, and 95% humidity.

Cell viability assay (MTS)

Cell growth was determined by CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI, USA) according to the protocol provided. Cells were seeded into 96-well plates (cultured overnight for adherent cells) and treated with chemicals with different concentrations. After 24-h, 48-h, 72-h incubation, 20 μl MTS (5 mg/ml) was added into each well respectively. The absorbance was then measured using a model ELX800 Micro Plate Reader (Bio-Tek Instruments, Inc.) at 570 nm.

Electron microscopy

Treated cells were washed and fixed for 30 min in 2.5% glutaraldehyde. The samples were treated with 1.5% osmium tetroxide, dehydrated with acetone and embedded in Durcupan resin. Thin sections were poststained with lead citrate and examined in the TECNAI 10 electron microscope (Philips, Holland) at 60 kV.

Western blot analysis

Western blotting of LC3 was carried out as previously reported [13]. The protein was applied

to a proper concentration of SDS-polyacrylamide gel, transferred to a PVDF membrane, and then detected by the proper primary and secondary antibodies before visualization with a chemiluminescence kit. Visualization was done with Image Quant LAS-4000 (Fujifilm, Tokyo, Japan) using image Multi-Gauge Software (Fujifilm, Tokyo, Japan).

Cell apoptosis analysis

The human breast cancer cell lines were seeded into 6-well plates ($3 × 10^5$/well) and treated with either the diluent control (DMSO) or ELE at various concentrations. At the end of incubation, the cells on each well were trypsinized, washed with PBS and stained with Annexin V-FITC Apoptosis Detection Kit (BD Pharmingen). The cell apoptosis was determined by the flow cytometry (Becton Dickinson, Mountain View, CA, USA).

Statistical analyses

Unless otherwise stated, data were expressed as the mean ± SD, and analyzed by Student's t test.

Results

β-elemene inhibited viability of breast cancer cells

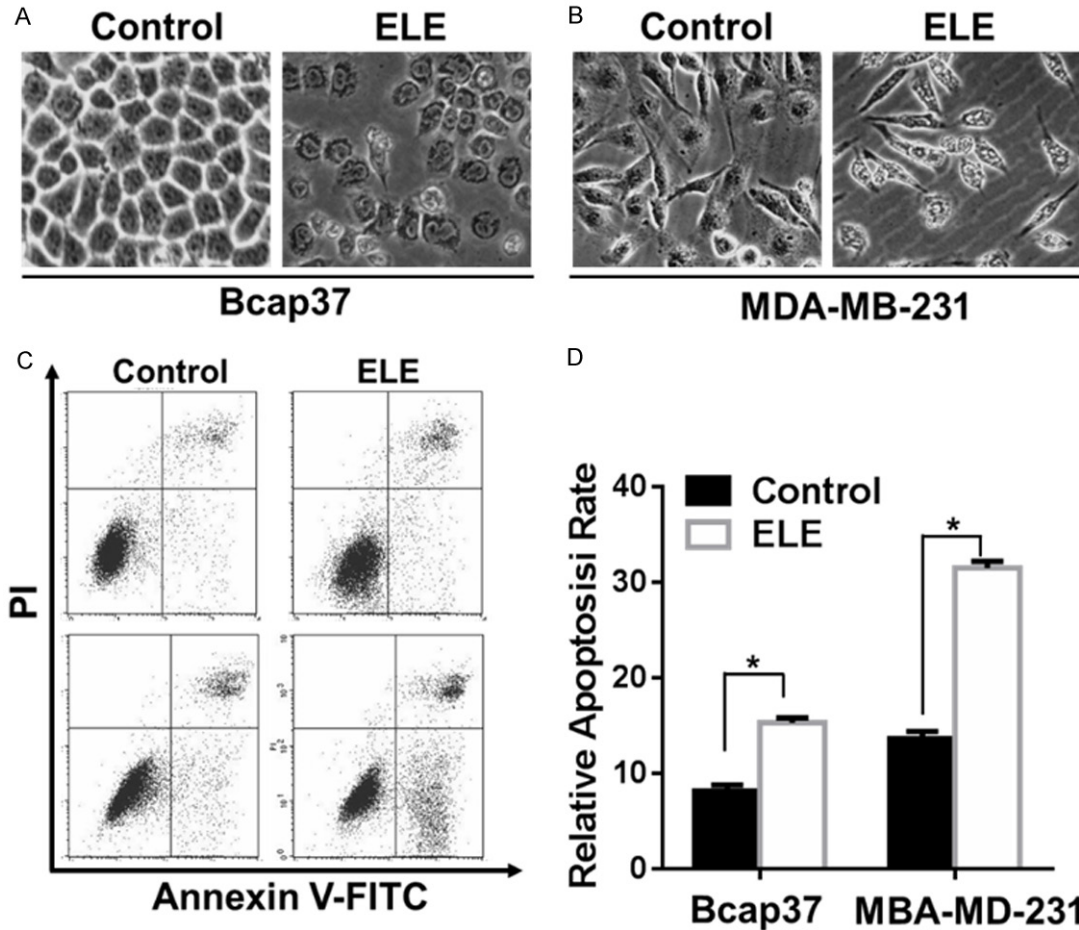We started to evaluate the effect of β-elemene on breast cancer cells by evaluating the viability

Figure 2. ELE induces the apoptosis of breast cancer cells. A. The morphological changes of Bcap37 and MDA-MB-231 cells treated with β-elemene (320 μg/ml) for 24 hours were observed under light microscopy. B and C. The apoptosis of Bcap37 and MDA-MB-231 cells treated with β-elemene (320 μg/ml) for 24 hours were analyzed by PI and Annexin V staining. The statistical significance was analyzed by Student's t test. The asterisks indicate statistical significance ($P < 0.05$). ELE: β-elemene.

of breast cancer cells treated with MTS assay before and after β-elemene treatment. As shown in **Figure 1A** and **1B**, β-elemene significantly inhibit the viability of Bcap37 and MBA-MD-231 cells in both time-and dose-dependent manners (**Figure 1**, $P < 0.05$, One-Way ANOVA test).

β-elemene induced apoptosis of breast cancer cells

Some cancer cells became floating after the exposure to β-elemene (**Figure 2A** and **2B**), indicating that β-elemene may induce the apoptosis of breast cancer cells. Indeed, PI and Annexin V double staining results confirm the induction of apoptosis by β-elemene (**Figure 2C** and **2D**). However, there are still many live cells

left, indicating that β-elemene may induce other changes in addition to the apoptosis of breast cancer cells.

β-elemene induced autophagy in breast cancer cells

Therefore, we asked whether autophagy was induced after β-elemene treatment. First, we evaluated the conversion of LC3-I into LC3-II before and after β-elemene treatment by western blotting analysis. As shown in **Figure 3**, β-elemene can induce the switch of LC-I to LC-II in a dose-dependent manner in both Bcap37 and MBA-MD-231 cells, indicating that autophagy might be activated by β-elemene (**Figure 3A** and **3B**). Next, we confirmed the activation of autophagy by electronic microscopy exami-
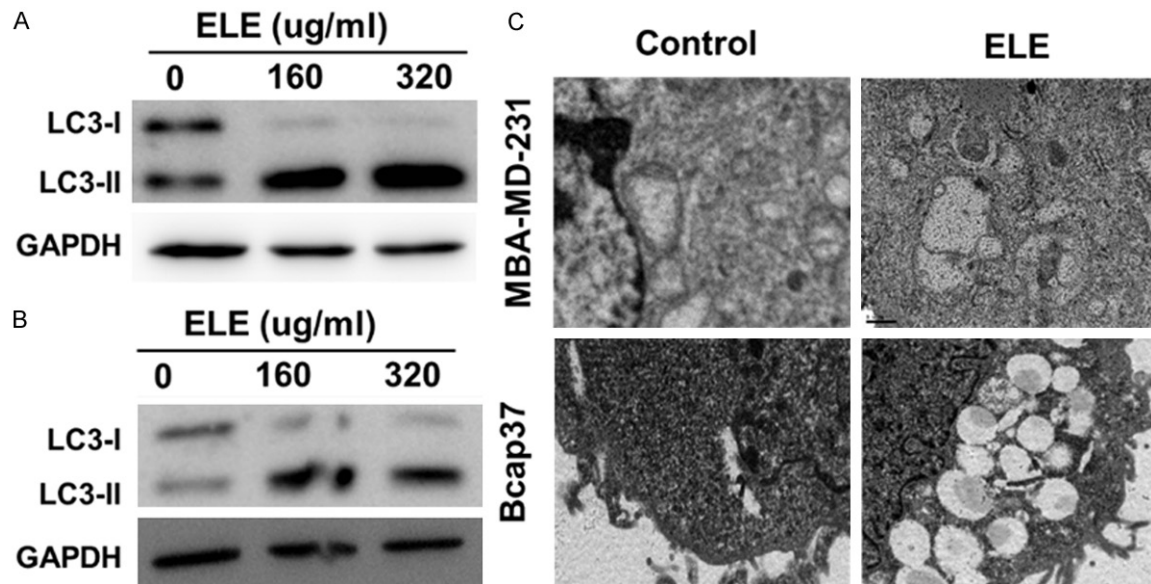
Figure 3. ELE activates autophagy in breast cancer cells. LC3 in Bcap37 (A) and MDA-MB-231 (B) cells treated with indicated concentration for 24 hours were detected by western blotting. (C) The changes in ultra-microstructure of Bcap37and MDA-MB-231 cells treated with β-elemene (320 μg/ml) for 24 hours were observed under electron microscopy. ELE: β-elemene.

nation. As shown in **Figure 3C**, typical autophagosomes were presented in β-elemene -treated cells rather than untreated cells, confirming the induction of autophagy by β-elemene.

Inhibition of autophagy promotes growth-inhibitory effect of β-elemene

To explore the relevance of autophagy in β-elemene-induced growth inhibition, we determined the effect of β-elemene on breast cancer cells before and after the suppression of autophagy. While autophagy inhibitor chloroquine (CQ) alone had no significant effect on breast cancer cells, the inhibitory effect of β-elemene on cell viability was significantly enhanced in both breast cancer cell lines s after CQ treatment (Bcap 37 in **Figure 4A** and MBA-MD-231 in **Figure 4D**). Consistently, apoptosis induced by β-elemene were increased after CQ treatment (Bcap 37 **Figure 4B**, **4C**, and MBA-MD-231 in **Figure 4D**, **4E**), indicating that autophagy in response to ELE treatment played a cyto-protective role to help cancer cells survive under unfavorable conditions.

Discussion

Although β-elemene has been approved by the State Food and Drug Administration of China and widely used for the clinical management of

patients with various cancers mainly brain tumors, the mechanism of the anti-cancer effect of β-elemene remains largely unknown. In addition, it would be interesting to know whether β-elemene can improve the clinical efficacy of commonly used treatment modalities such as chemotherapy and targeted therapy. In this study, we found that β-elemene inhibited the viability of breast cancers in vitro in a dose- and time-dependent manner (**Figure 1**). Such an inhibition can be attributed to the induction of both apoptosis (**Figure 2**) and cell cycle arrest (data not shown). These data indicates that β-elemene could be used potentially for the treatment of patients with breast cancer, which is one of the most commonly diagnosed cancer and the first leading cause of cancer-related deaths in women [15].

Interestingly, we found β-elemene induced autophagy in addition to apoptosis (**Figure 3**). Autophagy is morphologically characterized by the appearance of "double-membrane" vacuoles (autophagosomes) in the cytoplasm. In addition, the mammalian homologue of LC3, a homologue of Apg8p essential for autophagy in yeast, is found to be a specific biochemical marker for autophagy. Newly synthesized LC3 termed LC3-1 is evenly distributed throughout the cytoplasm. Upon induction of autophagy,
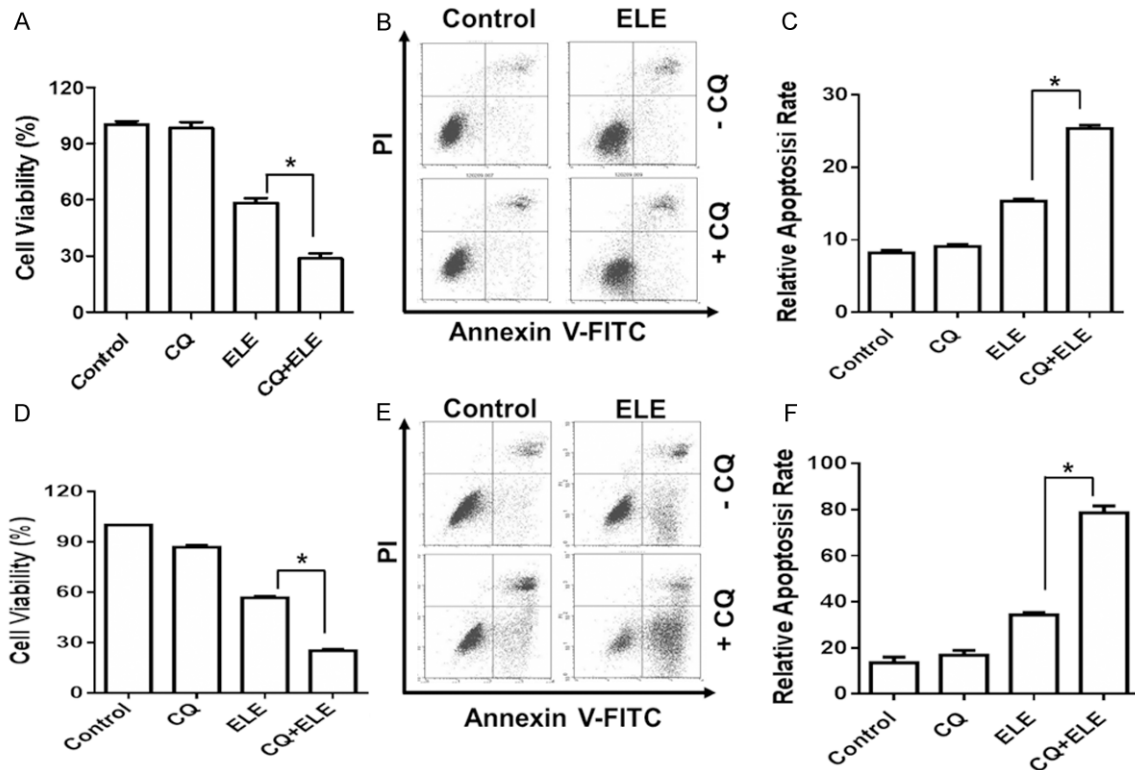
Figure 4. Autophagy inhibition enhances the sensitivity of breast cancer cells to ELE. The viability of Bcap37 (A) and MDA-MB-231 (D) cells treated with or without Chloroquine (10 μm) and β-elemene (320 μg/ml) were analyzed by MTS assay. The apoptosis of Bcap37 (B and C) and MDA-MB-231 (E and F) cells treated with or without Chloroquine (10 μm) and β-elemene (320 μg/ml) were analyzed by PI and Annexin V staining. The statistical significance was analyzed by Student's t test. The asterisks indicate statistical significance ($P < 0.05$). ELE: β-elemene; CQ: Chloroquine.

some LC3-I is converted into LC3-II, which is tightly bound to the autophagosomal membranes forming ring-shaped structures in the cytosol and degraded after the maturation of autophagosomes. We found that β-elemene indeed induced the conversion of LC3-II from LC3-I (**Figure 3B**). The accumulation of LC3-II can result from the blockage of the autophagy flux so that the degradation of LC3-II was hampered [16]. However, the analysis of ultrastructure by electron microscopy revealed that mature autolysosomes formed in breast cancer cells treated with β-elemene, indicating that β-elemene indeed activates rather than inhibits autophagy. Although it was designated as programmed cell death type II, autophagy was recently found to promote cellular survival under unfavorable stress conditions such as deprivation of amino acids, revealing a new role of autophagy in cancer development. In consistence, we found that inhibition of autophagy induced by β-elemene could potentiate the anti-cancer effect of β-elemene. Therefore,

β-elemene-induced autophagy functions as a stress response to protect cells from apoptosis. A number of cancer therapeutics including DNA-damaging chemotherapeutics, endocrine therapies such as tamoxifen and radiation therapy have been found to induce autophagy in vitro and in vivo [17-19]. Recently, it was found that autophagy can be activated and protected tumor cells from targeted therapies, such as the imatinib mesylate in Philadelphia chromosome-positive cells [20], trastuzumab in breast cancer [21], Src family kinase inhibitors in prostate cancer [22], proteasome inhibitors in prostate cancer [23]. All of these studies indicate that the combination of anti-autophagy strategies could potentially improve the clinical efficacy of current anti-cancer regiments including β-elemene.

In summary, β-elemene is a potential drug for breast cancer by including apoptosis. Inhibition of autophagy has the potential to improve the clinical efficacy of β-elemene for cancer treat-

ment. However, how β-elemene simultaneously initiates apoptosis program and activates autophagy to maintain cell survival remains further studies.

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Disclosure of conflict of interest

None.

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