

## ORIGINAL ARTICLE

# Anticancer effects of $\alpha$ -Bisabolol in human non-small cell lung carcinoma cells are mediated via apoptosis induction, cell cycle arrest, inhibition of cell migration and invasion and upregulation of P13K/AKT signalling pathway

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## Summary

**Purpose:** Non-small lung cancer (NSCLC) is one of the leading causes of cancer-related deaths world over. Exempting operable cases the treatments for NSCLC mainly include chemotherapy and radiotherapy. However, the survival rate for NSCLC is still far from satisfactory. Moreover, chemotherapy has lot of associated side effects. Therefore, there is an urgent need to look for novel and more viable treatment options. Against this background, the present study was designed to evaluate the anticancer activity of  $\alpha$ -Bisabolol against NSCLC.

**Methods:** Cell viability was assessed by MTT assay. Apoptosis was determined by DAPI and annexin V/propidium iodide (PI) staining. Mitochondrial membrane potential (MMP) and cell cycle analysis were determined by flow cytometry. Cell migration was investigated by wound healing assay and protein expression was evaluated by western blotting.

**Results:**  $\alpha$ -Bisabolol exerted significant anticancer activity on A549 NSCLC cells with IC<sub>50</sub> of 15  $\mu$ M. The anticancer effects of  $\alpha$ -Bisabolol were found to be due to G2/M cell cycle arrest and mitochondrial apoptosis.  $\alpha$ -Bisabolol also inhibited cell migration of A549 cells dose-dependently. Moreover, the results showed that  $\alpha$ -Bisabolol could inhibit the PI3K/AKT signalling pathway in a dose-dependent manner. The results of the present study indicate that  $\alpha$ -Bisabolol exerted selective anticancer effects on A549 cells via induction of cell cycle arrest, mitochondrial apoptosis and inhibition of PI3K/Akt signalling pathways.

**Conclusions:** This molecule showed promising anticancer features and could be developed as a potent lead candidate for the management and treatment of NSCLC.

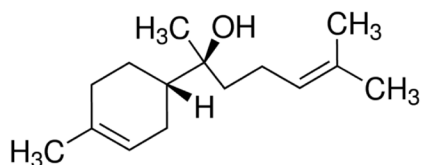
**Key words:** apoptosis,  $\alpha$ -bisabolol, cell cycle arrest, cell migration, non-small lung cancer, PI3K/AKT

## Introduction

Plants synthesize a diversity of chemical scaffolds which include, but are not limited to a wide array of flavours and fragrances [1]. The molecules responsible for the flavour and the fragrance of the compounds are usually synthesized from plant essential oils. It has been reported that there around 3,000 plants that produce essential oils and only around 300 are considered commercially important [2]. Quite recently, the plant essential oils and their

constituents are not only used as food preservatives and additives, but also find their application in the pharmaceutical industry [3]. In plants they play several essential roles such as repelling of predators/herbivores and attraction of pollinators [4]. The pharmaceutical exploitation of essential oils is mainly based on the presence of several constituents which show a number of bioactivities. These constituents are considered of importance in

the development of products that promote human health. It has been reported that several essential oils have been used to prevent malaria by preventing the growth of mosquito [5].  $\alpha$ -Bisabolol (Figure 1) is one of the important constituents of several plant species such as *Matricaria chamomilla*, *Eremanthus erythropappus*, and many other plant species.  $\alpha$ -Bisabolol containing oils has been shown to exhibit anti-inflammatory, analgesic, antibiotic and anticancer activities. In addition,  $\alpha$ -Bisabolol has been shown to exhibit low cytotoxicity and its use as ingredient in several of the commercial products has therefore been regarded by FDA as safe [6]. Although anticancer activity of bisabolol has been reported to some extent [7], it has not been evaluated against lung cancer. Therefore, the present study was designed to evaluate the anticancer activity of bisabolol against NSCLC cell line A549. NSCLC is one of the major causes of cancer-related deaths and in USA alone it is the leading cause of mortality due to cancer. Although the treatment of NSCLC involves chemotherapy, it only slightly improves the patient survival rate, but that too at the cost of considerable side effects [8,9]. This study was designed to investigate the anticancer effects of  $\alpha$ -Bisabolol against the A549 NSCLC cell line and decipher the underlying mechanism.



**Figure 1.** Chemical structure of  $\alpha$ -Bisabolol.

## Methods

### Chemicals, reagents and cell cultures

$\alpha$ -Bisabolol and other chemicals were of reagent-grade and purchased from Sigma Chemical Co. (St. Louis, Missouri, USA) unless otherwise mentioned. NSCLC cell line A549 was procured from Cancer Research Institute of Beijing, China, and was cultured continuously in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics (streptomycin 100  $\mu$ g/ml and penicillin G 100 U/ml) and maintained at 37°C.

### MTT assay for the determination of antiproliferative activity of $\alpha$ -Bisabolol

The antiproliferative effect of  $\alpha$ -Bisabolol against NSCLC A549 and normal FR2 lines was investigated by MTT assay. All cells were cultured at the density of  $1 \times 10^6$  cells/well in 96 well plates for 12 hrs. The cells were then exposed to 0-100  $\mu$ M  $\alpha$ -Bisabolol dose for 24 hrs. Thereafter, 20  $\mu$ l of MTT solution was added to each well. Prior to the addition of 500  $\mu$ l of DMSO, the cells

were separated from the medium. For solubilizing the MTT formazan crystals 500  $\mu$ l dimethylsulfoxide were added. Thereafter, absorbance was assessed by ELISA plate reader at 570 nm. Since  $\alpha$ -Bisabolol exhibited lowest  $IC_{50}$  against A549 cells, further experiments were performed only at the of 0, 10, 20 and 40  $\mu$ M concentrations.

### DAPI and annexin V/PI double staining for apoptosis

A549 cells were cultured at a density of  $1 \times 10^6$  cells per well in 6-well plates and then were exposed to 0, 7.5, 15 and 30  $\mu$ M  $\alpha$ -Bisabolol for 24 hrs. This was immediately followed by DAPI staining. Then, the cell samples were examined and photographs were taken via a fluorescence microscope. To estimate the apoptotic cell populations, A549 cells were plated at a density of  $1 \times 10^6$  cells/well in 6-well plates and treated with varied concentrations (0, 7.5, 15 and 30  $\mu$ M) of  $\alpha$ -Bisabolol for 24 hrs. Thereafter, the cells were collected and washed with phosphate buffered saline (PBS). The cells were then incubated with Annexin V/FITC and PI for 15 min and the apoptotic cell populations were estimated by flow cytometry (BDBiosciences, San Jose, CA, USA).

### Cell cycle analysis of A549 Cells

To investigate the distribution of the A549 cells in different phases of the cell cycle, approximately  $1 \times 10^5$  cells in each well in 6-well plates were kept at 37°C overnight to allow the cells to adhere. This was followed by treatment with various concentrations of  $\alpha$ -Bisabolol (0, 7.5, 15 and 30  $\mu$ M) and then the plates were incubated at 37°C for 24 hrs. Thereafter, the cells were trypsinized followed by resuspension in ice-cold PBS. Finally, the cells were treated with ethanol (70%) at -20°C and allowed to fix overnight. Following fixation with ethanol, the cells were treated with ice-cold PBS twice and centrifuged (1000 rpm) for 10 min at 4°C. Afterwards, the cells were resuspended in 1 ml PI/Triton-X 100 solution for 30 min in the dark. Finally, the distribution of the cells at each phase was examined from 10,000 cells in a FacScan flow cytometer (BD Biosciences). The estimation of the percentage of cells in each phase of the cell cycle was carried out by WinMDI software.

### Boyden Chamber assay for cell migration

Cell migration assay was carried out by Boyden chamber assay with some modifications. Cells at the density of  $5 \times 10^4$  cells/well were suspended in 2% FBS medium and placed in the upper chamber of 8  $\mu$ m pore size transwells. Afterwards, RPMI-1640 medium supplemented with 10% FBS was added to the lower chamber. This was followed by incubation of 24 hrs. On the upper surface of the membrane, unigrated cells were removed, while on the lower surface of the membrane the migrated cells were fixed in methanol (100%) and Giemsa-stained. The cell migration was estimated by counting the number of the migrated cells under microscope.

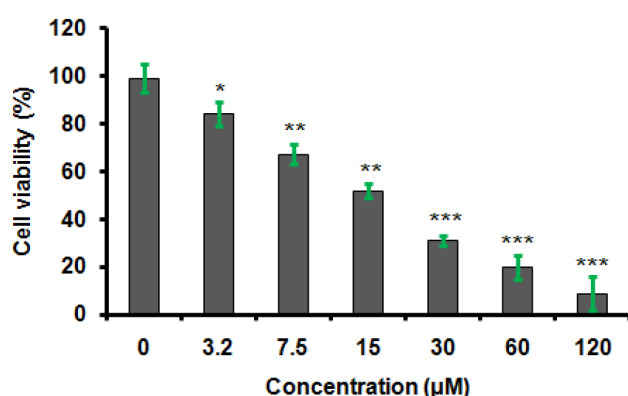
### Determination of protein expression by western blotting

Protein expression was determined by western blot analysis. Briefly, proteins present in the cell extracts were resolved by SDS-PAGE. This was followed

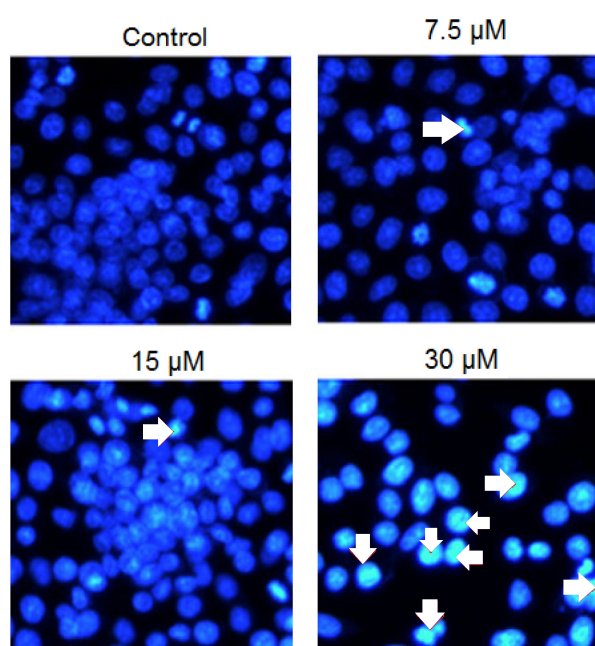
by transference on nitrocellulose membrane. Thereafter the membrane was treated with non-fat milk (5%) in PBS and incubated with suitable primary antibody for 2 hrs at room temperature followed by secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hr at room temperature. The western blots were then observed in an ECL western blot analysis system (Amersham Biosciences).

#### Statistics

The experiments were performed in triplicate and presented as mean  $\pm$  standard deviation (SD). Student's t-test with the help of GraphPad prism 7 software was used for analyses. Results were considered significant at \* $p < 0.01$ , \*\* $p < 0.001$  and \*\*\* $p < 0.0001$ .



**Figure 2.** Effect of indicated concentrations of  $\alpha$ -Bisabolol on cell viability. All experiments were carried out in triplicate and shown as mean  $\pm$  SD. Results were considered significant at \* $p < 0.001$ , \*\* $p < 0.001$  and \*\*\* $p < 0.0001$ .



**Figure 3.** Induction of apoptosis by  $\alpha$ -Bisabolol at indicated concentrations as depicted by DAPI staining. All experiments were carried out in triplicate. The results show that  $\alpha$ -Bisabolol induces apoptosis in A549 cells in a concentration-dependent manner. Arrows show apoptotic cells.

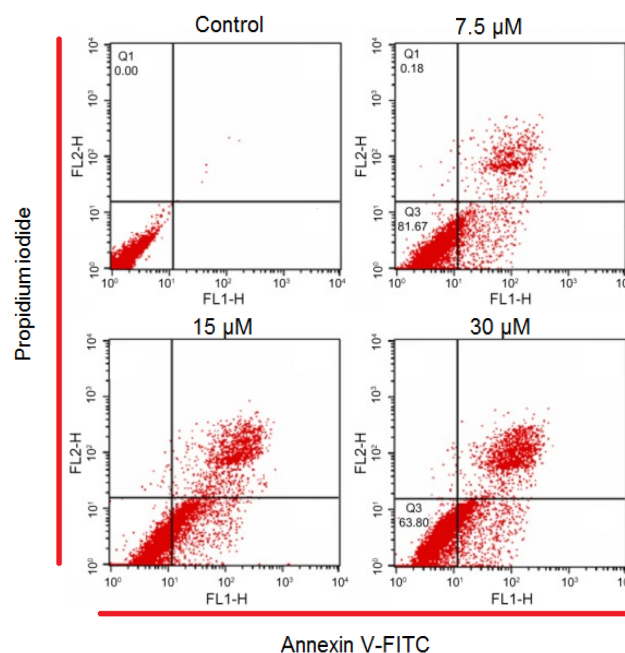
## Results

### *$\alpha$ -Bisabolol exerts antiproliferative effects on A549 cells*

To determine the antiproliferative activity of  $\alpha$ -Bisabolol on A549 NSCLC cell line, cancer cells were exposed to  $\alpha$ -Bisabolol at different concentrations and  $IC_{50}$  was determined.  $\alpha$ -Bisabolol displayed significant anticancer effects against A549 cells with an observed  $IC_{50}$  15  $\mu$ M. The effect of  $\alpha$ -Bisabolol on the cell viability showed a dose dependent trend (Figure 2).

### *$\alpha$ -Bisabolol causes apoptosis in A549 cells*

We further investigated if the anticancer effects of  $\alpha$ -Bisabolol were due to induction of apoptosis. Therefore,  $\alpha$ -Bisabolol-treated A549 cells were subjected to DAPI staining. It was observed that  $\alpha$ -Bisabolol caused apoptosis in A549 cells in a concentration-dependent manner as it was obvious from the increased number of cells with white colour nuclei (Figure 3). Estimation of apoptotic cell population was determined by flow cytometry as indicated in Figure 4. The percentage of apoptotic cells increased from 2.15% in the control to 48.5% at 30  $\mu$ M concentration of  $\alpha$ -Bisabolol. To examine if apoptosis followed the mitochondrial pathway we determined the protein expression of Bax and Bcl-2. The results showed that the protein



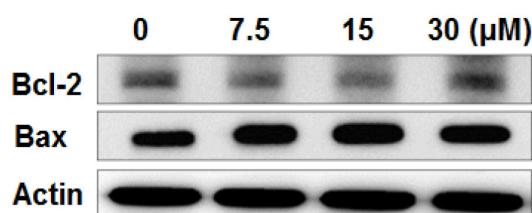
**Figure 4.** Estimation of apoptotic cell populations at indicated concentrations of  $\alpha$ -Bisabolol by annexin V/PI staining followed flow cytometry. Experiments were carried out in triplicate. The Figure shows that the apoptotic cell populations increase with increase in the concentrations of  $\alpha$ -Bisabolol.



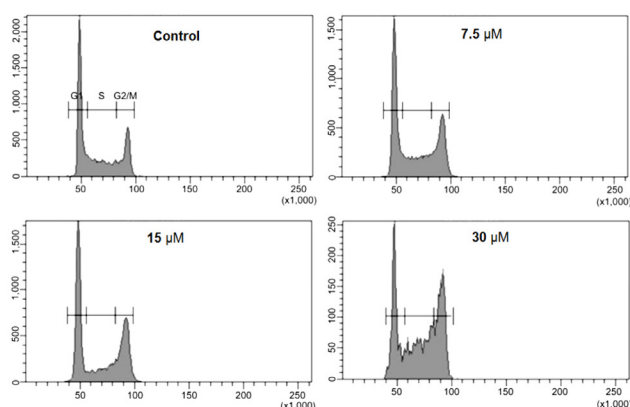
expression of Bax was upregulated in a concentration-dependent manner and that of Bcl-2 decreased (Figure 5).

#### *$\alpha$ -Bisabolol induces G2/M cell cycle arrest of A549 cells*

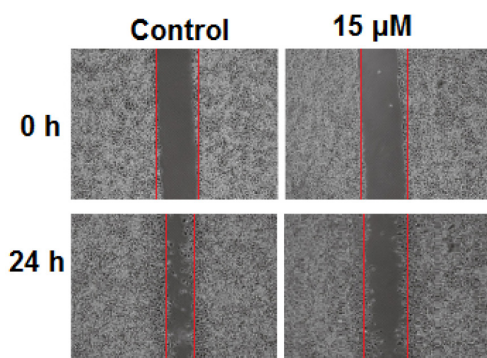
To assess the effect of  $\alpha$ -Bisabolol on the cell cycle phase distribution of A549 cells, the cells were exposed to 0, 7.5, 15 and 30  $\mu$ M of  $\alpha$ -Bisabolol



**Figure 5.** Effect of  $\alpha$ -Bisabolol on the protein expression of Bax and Bcl-2 as observed by western blot analysis. The experiments were carried out in triplicate. The Figure depicts that  $\alpha$ -Bisabolol decreased the expression of Bcl-2 and increased the expression of Bax concentration-dependently.



**Figure 6.**  $\alpha$ -Bisabolol triggers G2/M cell cycle arrest at indicated doses. Experiments were carried out in triplicate and show that  $\alpha$ -Bisabolol triggers G2/M cycle arrest of A549 cells concentration-dependently.



**Figure 7.** Effect of  $\alpha$ -Bisabolol on the migration of A549 cells at indicated concentrations. The experiments were carried out in triplicate and show that  $\alpha$ -Bisabolol inhibits the migration of A549 cells.

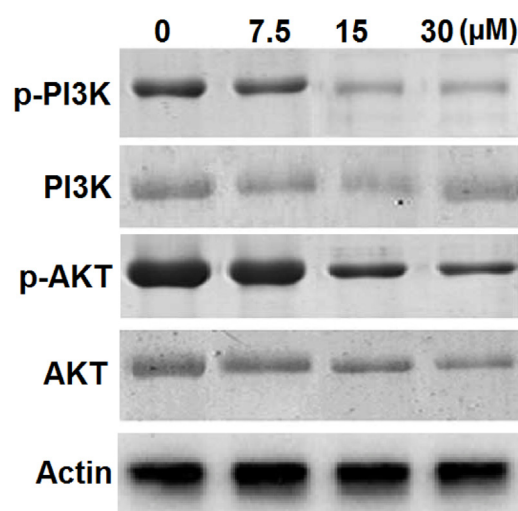
for 24 hrs. It was revealed that the number of cells at G2 phase increased in a dose-dependent manner causing cell cycle arrest (Figure 6). At 30  $\mu$ M there was a marked increase in sub-G1 phase cells.

#### *$\alpha$ -Bisabolol suppresses cell migration of A549 cells*

We also investigated the effect of  $\alpha$ -Bisabolol on cell migration of A549 cells (Figure 7). The results of cell migration assay after  $\alpha$ -Bisabolol treatment at 30  $\mu$ M concentration for 24 hrs, indicated that  $\alpha$ -Bisabolol reduced the motility and migration of the of A549 cells in a dose-dependent manner (Figure 7).

#### *$\alpha$ -Bisabolol inhibits PI3K/Akt signalling pathway*

PI3K/Akt signalling cascade is considered as one of the crucial pathways that could be targeted for the treatment of cancer. In the present study we examined the effect of  $\alpha$ -Bisabolol on the expression of some of the important proteins of this pathway. Our results showed that, in comparison to the untreated cells,  $\alpha$ -Bisabolol-treated cells exhibited a dose-dependent downregulation of p-PI3K and p-AKT proteins (Figure 8). However, PI3K and Akt expression levels remained more or less unaffected. Therefore, we believe that that  $\alpha$ -Bisabolol induced anticancer effect partly via PI3K/Akt signalling cascade.



**Figure 8.** Effect of  $\alpha$ -Bisabolol on PI3K/AKT pathway of A549 cells at indicated concentrations. The experiments were performed in triplicate and show that  $\alpha$ -Bisabolol inhibits this pathway.

## Discussion

Plants extracts and plant-derived molecules are considered to possess important pharmacological potential. Plants synthesize a wide array of secondary metabolites for their own defence and

these metabolites have also been shown to exhibit health-promoting effects on human [10]. Among these metabolites, essential oils have been shown to exhibit considerable antiproliferative effects due to the presence of a number of biochemical constituents [11]. For instance, the essential oil of *Salvia* and *Artemisia* species exhibits strong anticancer activity [12,13]. These essential oils are rich sources of  $\alpha$ -Bisabolol.

In the current study we evaluated the anticancer properties of purified  $\alpha$ -Bisabolol against NSCLC line A549. The results showed that  $\alpha$ -Bisabolol exhibits significant anticancer activity against these cells with an  $IC_{50}$  of 15  $\mu$ M. Our results are also supported by previous studies wherein  $\alpha$ -Bisabolol has been shown to inhibit the growth of several types of cancer cells such as pancreatic cancer cells.

Previously, it has been reported that many of the essential oils induce apoptosis in cancer cells. For example the essential oil of *Curcuma wenyujin* induces apoptosis in cancer cells [14,15]. Therefore, we also checked whether  $\alpha$ -Bisabolol triggers apoptosis in A549 cells. For this, we carried out DAPI and annexin V/PI double staining and the results showed that  $\alpha$ -Bisabolol triggered apoptosis in A459 cells in a concentration-dependent manner which was also associated with alteration in the Bax/Bcl-2 expression ratio.

Furthermore, essential oils and their constituents have also been reported to induce cycle arrest in cancer cells [16]. For instance, Germacrone, a constituent of several essential oils induces cell cycle arrest [17]. We therefore, also evaluated the effect of  $\alpha$ -Bisabolol on the cell cycle phase distribution

of A549 cells and the results clearly showed that  $\alpha$ -Bisabolol could induce G2/M cell cycle arrest in these cells.

Molecules that can inhibit the migration of cancer cells are considered potent anticancer agents [18], therefore we evaluated whether  $\alpha$ -Bisabolol could exert its effects on the migration of A549 cells. It was observed that in wound healing assay  $\alpha$ -Bisabolol could significantly inhibit the migration of A549 cells. These results suggest that  $\alpha$ -Bisabolol could prove useful in preventing the metastasis of cancer cells *in vivo* as well. PI3K/AKT is one of the important signalling pathways that has been found to be responsible for tumorigenesis and progression of several cancers. Evaluation of the protein expression by western blotting revealed that  $\alpha$ -Bisabolol could inhibit the expression of p-AKT and p-PI3K. However, the expression levels of AKT and PI3 remained almost unaltered. Taken together, these results show that  $\alpha$ -Bisabolol could prove a potent anticancer agent.

## Conclusion

From our results we conclude that  $\alpha$ -Bisabolol induces apoptosis and cell cycle arrest in A549 cells.  $\alpha$ -Bisabolol also inhibits cell migration. These results suggest that  $\alpha$ -Bisabolol could prove an important molecule for the treatment of NSCLC and deserves further research endeavours.

## Conflict of interests

The authors declare no conflict of interests.

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