

Original Research Article

Allicin-induced modulation of angiogenesis in lung cancer cells (A549)

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Abstract

Purpose: To investigate the effectiveness of allicin as a candidate for lung cancer therapy.

Methods: Allicin solution at different concentrations was tested on A549 lung cancer cell line. Viability and proliferation of A549 cells were determined. The cytotoxic effects of allicin solution on A549 lung cancer cells were quantitatively determined using Alamar blue assay. Cell proliferation and cell cycle were measured by DAPI-flow cytometry analysis in order to investigate the possible cell signalling pathway targeted by allicin. The level of expression of VEGF-A protein was determined with confocal laser scanning microscopy.

Results: Allicin inhibited lung cancer proliferation and down-regulated the protein expression of VEGF, but had no significant cytotoxic effects on A549 cells. Flow cytometric results showed that allicin induced cell cycle arrest of A549 cells at the G1 phase.

Conclusion: These results indicate that allicin exerts anti-proliferative effects on A549 lung cancer cells. Thus allicin, an active component of garlic, might be a promising therapy against lung cancer metastasis.

Keywords: A549 cells, Allicin, qPCR Taqman assay, VEGF-A protein expression, Garlic, Lung cancer

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INTRODUCTION

The use of garlic (*Allium sativum*) in traditional medicine dates to back to 16th century BC [1]. Research in recent decades demonstrate that garlic promotes well-being through reduction of blood level of bad cholesterol (LDL-cholesterol) which is linked to incidents of heart disease [2-5]. Studies have shown that allicin possesses antioxidant properties [5]. It is one of the most biologically active principles isolated from garlic, and its structure and properties were described by Gavallito and Bailey in 1944 [6,7].

Several pharmacological properties have been ascribed to allicin: it possesses antioxidant, anti-inflammatory, antibiotic, antiviral, and anti-proliferative potential, and it enhances the viability of chondrocytes [8]. In addition, it has been reported that allicin inhibits cancer cells [1], and induces apoptosis via caspase-dependent and caspase-independent pathways through the enhancement of accumulation of reactive oxygen species [9]. Indeed, studies have demonstrated that allicin kills cancer cells and induces apoptosis in several cancer cell lines, as well as hepatocarcinoma, and lung cancer [6,7].

Combined use of allicin and cisplatin or carboplatin resulted in enhanced growth inhibitory effect on SW1736 and HTh7 cells [10]. Furthermore, allicin has been reported to inhibit human renal clear cell carcinoma via suppression of the HIF pathway [11]. Moreover, allicin inhibited the proliferation of human mammary (MCF-7), endometrial (Ishikawa), and colon (HT-29) cancer cells [6]. Interestingly, allicin can stimulate the immune system to produce some bioactive anticancer factors which inhibit tumour cells, and can also stimulate the release of many cytokines which enhance the immune system [5,6,12].

Vascular endothelial growth factor (VEGF), first isolated from the pituitary gland [13] is a glycoprotein which is important in angiogenesis and vascular permeability, and it promotes endothelial proliferation and tumour growth. It is known that VEGF increases the permeability of blood vessels during tumour growth, thereby creating new blood vessels [14]. A study has reported that *Melissa officinalis* extract (MOE) significantly decreased the expression of VEGF-A in A549 and MCF-7 cells [15]. The current research was designed to study the effect of allicin extract on lung cancer cells with a view to assessing its potential as an alternative therapeutic approach for inhibiting overexpression of VEGF-A.

EXPERIMENTAL

Lung cancer cultivation

Human alveolar basal epithelial adenocarcinoma cells (A549 cell line, ATCC#CCL-185) were obtained from RMIT University, Australia. The cells were maintained at 37 °C in complete DMEM medium (Life Technologies, Australia) containing 10 % foetal bovine serum (FBS) and 1% penicillin/streptomycin (10,000 U/mL, Life Technologies, Australia) in a 5 % CO₂ incubator.

Assessment of cell viability

The A549 cells were seeded at 2 x 10⁴ cells/100 µL in 96-well plates overnight and treated with 5, 12, 25, 50 and 100 µg/mL of allicin (Sigma-Aldrich, Australia). Evaluation of cells was performed 24 h after treatment at 37 °C and in an atmosphere of 5 % CO₂. Cell viability was determined with AlamarBlue® assay [16].

Proliferation assay

The proliferation of the treated lung cancer cell lines (A549) was measured by quantifying their

deoxyribonucleic acid (DNA) content using a Picogreen® assay according to manufacturer's instructions. The cells were trypsinized after treatment at various allicin concentrations for 24 h. The cells were then rinsed two times with cold DPBS, followed by centrifugation for 5 min at 1200 rpm. The cell pellets were subsequently collected for lysis using a NP40 cell lysis buffer (Life Technologies, Australia) for 30 min on ice, and at 10-min intervals, the cell lysates were subjected to a freeze-thaw cycle prior to centrifugation at 13000 rpm for 10 min at 4 °C. A 100-µL portion of clear lysate was aliquoted to a clean 96-well plate and incubated for 5 min with 100 µl of PicoGreen® reagent at room temperature in the dark.

Fluorescence was measured using the multi-mode microplate reader at an excitation wavelength of 480 nm and an emission wavelength of 520 nm. Cell numbers in the sample were determined by reference to a DNA standard curve. The DNA standard curve was obtained using cell lysates containing a known number of control cells.

Cell cycle staining-DAPI

After 24 h of treatment, treated and untreated cells were harvested using Trypel Express (Life Technologies), washed twice in PBS (to get rid of serum proteins) and centrifuged at 1200 x g for 5 min. The pellets (3 x 10⁶ cells) were resuspended in 1 mL PBS (Ca- and Mg-free). Ice-cold 95 % ethanol (3 mL) was added dropwise to the suspension, while vortexing. Then, the cells were fixed in 70 % ethanol solution for 30 min, and centrifuged at 2000 - 2200 x g for 10 min, washed again in 15 mL PBS; and re-centrifuged at 2000 - 2200 x g for 10 min. Thereafter, the cells were counted and re-suspended in 0.5 - 2.0 mL DAPI stain solution, followed by 130-min incubation on ice prior to flow cytometric analysis.

Immunoassay

Immunofluorescence was used to determine VEGF protein expression. Following fixation in paraformaldehyde, the treated and untreated cells were rinsed in phosphate buffered saline buffer (rinsing solution) containing Tween-20 (0.05 %), prior to Triton X-100-mediated permeabilization. They were then blocked for 1 h with bovine serum albumin (1 %), rinsed thrice in the rinsing solution, and incubated with anti-VEGF-A primary antibody (diluted 1: 100) for 1 h, followed by rinsing and incubation for 1 h with secondary antibody, then rinsing and staining with 4', 6-diamidino-2-phenylindole

dihydrochloride. After rinsing, the cells were subjected to laser scanning confocal microscopy.

TaqMan-qPCR assay

In the determination of VEGF-A expression, RNA was extracted from treated and untreated cells using miniRNA kit (Qiagen, Australia) and subjected to reverse transcription to cDNA with QuantiTect (Qiagen, Australia). Then, qPCR was carried out under the following conditions: initial cycling at 95 °C for 10 min; 40 cycles at 95 °C for 15 sec, at 60 °C for 60 sec, and at 72 °C for 30 sec, with GAPDH as internal control. The relative expression of VEGF-A was quantified using the $2^{-\Delta\Delta CT}$ method [17].

Statistical analysis

Data are presented as mean \pm standard deviation (SD). One way analysis of variance (ANOVA) and Duncan's multiple range tests were used for statistical analyses of differences between the various groups, while two-group comparisons were done with Student's *t*-test. All statistical analyses were carried out with GraphPad Prism ver. 6.01. Values of $p < 0.05$ were assumed significant.

RESULTS

To determine the effect of allicin on cell viability, the lung cancer cells (A549) were treated with 5, 12, 25, 50 and 100 $\mu\text{g/ml}$ of allicin for 24 h. Alamar blue assay demonstrated that allicin solution reduced lung cancer cell viability time- and dose-dependently. However, the effect of allicin on the lung cancer cells was not significant. These results are shown in Figure 1.

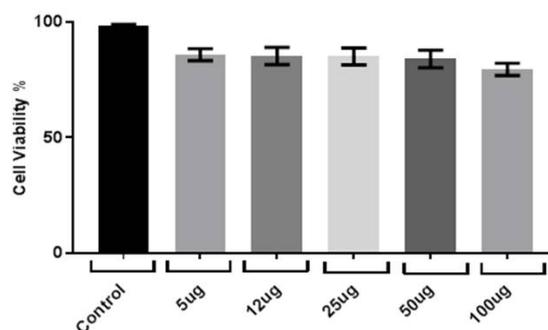


Figure 1: Effect of allicin on the viability of A549 cancer cell line. Cell toxicity was determined using AlamarBlue® assay. Viability decreased with increased allicin concentrations, but was not statistically significant. Data were collected from multiple independent experiments and represented as mean \pm standard deviation

To evaluate the effect of allicin treatment on lung cancer cell proliferation, A549 cells were treated with different concentrations of allicin, and cell numbers were monitored for 24 h. As shown in Figure 2, allicin significantly reduced the proliferation and growth of A549 cells. When compared with untreated controls, allicin reduced the proportion of cells in the S phase cells and increased the proportion of cells in G2 phase due to cell arrest at the G1 phase (Figure 3).

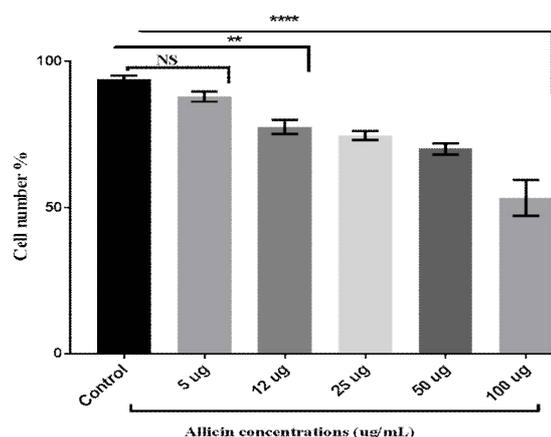


Figure 2: Effect of allicin on metabolic activity of lung cancer cells (A549), as measured using an PicoGreen® assay. Data are presented as mean \pm SD

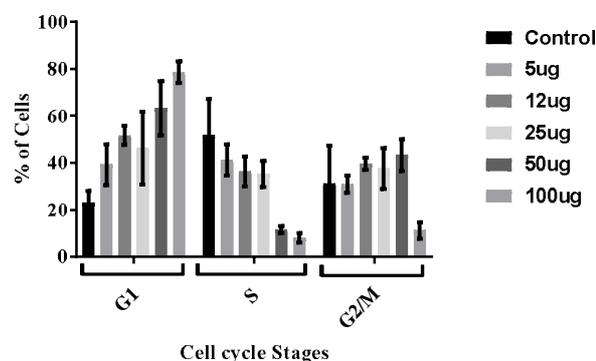


Figure 3: Effect of allicin on A549 cell cycle phases. Cells were arrested at G1 phase. Data from multiple independent experiments are presented as mean \pm SD

To study the impact of allicin on lung cancer cells which overexpress VEGF-A protein, the cells were exposed to different concentrations of allicin for 24 h. Thereafter, they were examined microscopically and photographed to track the VEGF protein marker using immunoassay. The results confocal microscopy (Figure 4) revealed that after allicin treatment, VEGF-A (greenish colour) levels were reduced in A549 cells, when compared to control cells. This indicates that VEGF-A expression was significantly decreased, implying that allicin reversed the overexpression of VEGF-A overexpression induced effects.

Thus, allicin exhibited a protective and anticancer property.

DISCUSSION

Many studies on cancer cell lines have revealed that allicin has anticancer properties. It seems to be able to kill cancer cells and prevent their proliferation and growth. In the present study, exposure of lung cancer cells (A549) to allicin resulted in time- and dose-dependent decreases in viability as depicted in AlamarBlue® assay. However, allicin did not produce any toxic effect on the lung cancer cells. These results are in agreement with previous findings [5, 9]. In contrast, some studies reported anti-tumour effect of allicin and its toxic effect on mammalian cell lines [18,19].

In the present study, cell proliferation decreased with increasing concentrations of allicin, possibly due to increased stress on the cells at higher levels of allicin concentrations. The allicin-induced reduction in proliferation also exerted a proportionate effect on metabolism in the A549 cells. This is in agreement with previous studies that demonstrated allicin-induced declines in the percentages of actively dividing MCF-7 and ZR-75 cells [20]. In another study, it was reported that the percentage of cells in G0/G1 phase decreased with increase in the S phase population of chondrocytes when lung cancer cells were exposed to allicin [8]. As shown in the present study, cells arrested at G1 phase were significantly high in all treated cells, relative to untreated cells. These correspond to high proportion of apoptotic cells. Some studies reported that allicin induced cell arrest of gastric cancer cells in M phase; this is could be correlated to the up-regulated expressions of p21WAF1 and p16INK4 genes [21].

In all treated cells, the protein expression of VEGF-A was low in A549 cells, when compared with control cells. This finding is consistent with a previous report which showed that allicin caused down-regulation of VEGF-A protein marker in lymph angiogenesis [22]. The results of the present study confirm that allicin indeed downregulates VEGF-A protein expression, suggesting that it acts as an anti-tumour drug. Real-time PCR results demonstrated that VEGF-A expression and overexpression were significantly decreased by allicin, which point to its anticancer property. These results are in agreement with previous findings [11,22]. The results obtained this study also show that VEGF-A expression was significantly increased in control cells, even though allicin led to the reduction of VEGF-A in treated cells thereby inhibiting cell proliferation. Therefore, there was a strong correlation between the reduction in VEGF-A expression, and the results from laser

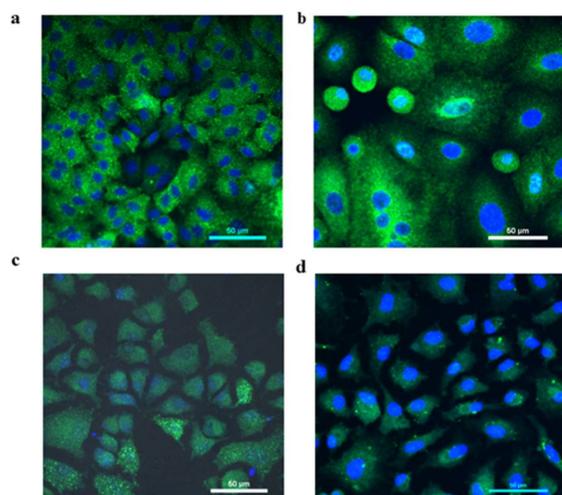


Figure 4: Immunofluorescence analysis of VEGF-A protein expression in untreated (a) and treated lung cancer cells. A549 cells were treated with 5 µg/mL (b), 50 µg/mL (c), 100 µg/mL (d) of allicin. Nucleated cells are in blue colour (DAPI). The protein level of VEGF-A was decreased, relative control cells. Scale bar represents 50 µm

Real-time PCR was done to confirm the effect of allicin on lung cell lines that over-express VEGF-A. Cells were treated for 24 h with 100 µg/ml, which was the concentration that yielded the highest inhibition of VEGF-A expression (as shown in confocal microscopy images). The VEGF-A gene was then measured using Taqman assay to track the vascular endothelial growth factor expression. The VEGF-A expression was significantly decreased (Figure 5), indicating that allicin can change the VEGF-A overexpression, thereby revealing the anticancer potential of allicin.

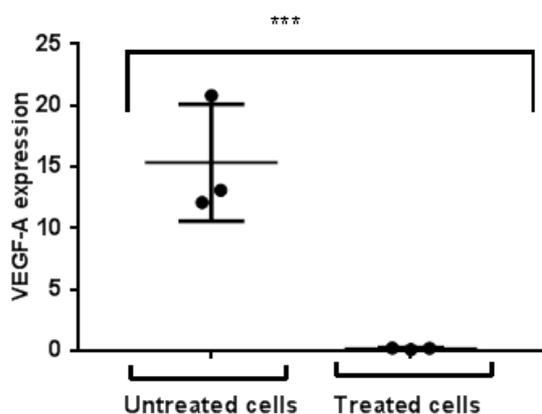


Figure 5: Reduction of VEGF-A expression in A549 cell line by allicin treatment. Cells were treated with 100 µg/mL allicin

scanning confocal microscopy and real time PCR.

CONCLUSION

The present study provides evidence that allicin possesses anti-proliferative effects when administered to lung cancer cell lines (A459), through down-regulation of vascular endothelial growth factor. Thus, allicin may be an effective treatment for lung cancer. Nevertheless, more investigations into other cell signalling pathways are required through *in vivo* testing of allicin to confirm it's as an effective treatment for lung cancer.

DECLARATIONS

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Competing interests

The authors declare that no competing interests exist with regard to this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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