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Allicin suppresses human glioblastoma cell growth by inducing cell cycle arrest and apoptosis, and by promoting autophagy

Oratai Weeranantanapan*, Kankawi Satsantitham, Pishyaporn Sritangos and Nuannoi Chudapongse

School of Preclinical Sciences, Institute of Science, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand

*Corresponding author: oratai@g.sut.ac.th

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Abstract: Glioblastoma is the most aggressive cancer that occurs in the brain and spinal cord. In the present study, we investigated the effect of allicin, an organosulfur compound obtained from garlic (Allium sativum), on glioblastoma cell growth. When human glioblastoma DBTRG-05MG cells were incubated with different concentrations of allicin for 24 h, cell growth was suppressed in a dose-dependent manner. The results from image-based cytometry assays suggested that allicin caused S and G2/M phase cell cycle arrest and induced apoptosis. Autophagy detection studies showed that allicin also promoted this mechanism. Because cell migration is a key process during tumor formation, the effect of allicin on glioblastoma cell migration was also examined. After allicin treatment, the migration ability of cells decreased when compared with the control after 24 h. Taken together, the present results suggested that allicin caused S and G2/M phase cell cycle arrest and induced apoptosis. Autophagy detection studies showed that allicin also promoted this mechanism. Because cell migration is a key process during tumor formation, the effect of allicin on glioblastoma cell migration was also examined. After allicin treatment, the migration ability of cells decreased when compared with the control after 24 h. Taken together, the present results suggested that allicin inhibited human glioblastoma cell growth by inducing S and G2/M phase cell cycle arrest, apoptosis and autophagy. Our findings suggest that allicin suppressed glioblastoma cell growth through multiple target pathways. Therefore, allicin potentially serves as an alternative therapeutic candidate or could be synergistically used in combination with the standard drug for the treatment of glioblastoma multiforme.

Keywords: allicin; glioblastoma; apoptosis; autophagy; cell cycle arrest

INTRODUCTION

Allicin is a major bioactive compound of freshly crushed garlic (Allium sativum). A number of studies have shown that allicin has antimicrobial [1], antiinflammatory [2], as well as anticancer activities [3-5] in a wide range of cancer cell types.

Glioblastoma multiforme (GBM) is one of the most aggressive common brain tumors in adults. Brain malignancies often exhibit poor prognosis as malignant glioblastoma cells are able to migrate and invade the surrounding brain area easily.

It was reported that allicin-induced glioblastoma cell death resulted from apoptosis [6]. However, it has been known that the mode of cell death is not only apoptosis. Autophagy is an intracellular degradation mechanism using hydrolytic enzymes in the lysosomes to remove dysfunctional or unnecessary cytoplasmic materials under certain stress conditions. After autophagy induction, the cell initially delivers cytoplasmic components to lysosomes via a double membrane-bound vesicle that surrounds intracellular organelles [7] known as the autophagosome (also called autophagic vacuole or autolysosome). Autophagy-related proteins, including LC3 and Beclin 1, play a role in forming autophagosomes [8]. Fusion of the autophagosome and lysosome leads to the formation of the autolysosome. Degradation of cytoplasmic...
materials occurs by the action of hydrolytic enzymes within the autolysosome. It has been shown that autophagy is also one of the forms of cell death. A great number of studies have reported that natural compounds are capable of inducing autophagy [9]. In recent years, increasing evidence has supported the involvement of allicin in inducing autophagy in several types of cancer cells, including hepG2 human liver carcinoma cells [10,11] and thyroid cancer cells [12]. However, to the best of our knowledge, allicin-mediated autophagic cell death of glioblastoma cells has not been demonstrated yet.

The present study aimed to examine the effect of allicin on cell growth and to investigate the cell death mechanism underlying the allicin-induced anticancer effect on human glioblastoma cells. Herein we demonstrated that allicin suppressed DBTRG-05MG cell growth. We then examined the mechanism underlying allicin-mediated cell death. We found that allicin did not only induce apoptosis but also caused cell cycle arrest and autophagy. Moreover, allicin inhibited the migration of human glioblastoma cells.

MATERIALS AND METHODS

Reagents and antibodies
Allicin, goat polyclonal secondary antibody to rabbit IgG (Alexa Fluor 488), and fluoroshield mounting medium with DAPI were purchased from Abcam (Cambridge, MA, USA). MTT or 3(4,5-dimethyl-thiazol-2,5-diphenyl tetrazolium bromide and anti-LC3 antibody were purchased from Sigma (St. Louis, MO, USA). Tali® cell cycle and Tali® cell-apoptosis kits were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA)

Cell culture
The human glioblastoma cell line DBTRG-05MG was obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were grown in RPMI containing 10% fetal bovine serum (FBS) supplemented with 1% penicillin-streptomycin. Cell culture medium and supplements were purchased from Corning Inc. (NY, USA). The cultured cells were incubated at 37°C in a humidified incubator with 5% CO₂.

Cell viability assay
Cell viability was examined using an MTT assay, as the mitochondria succinate dehydrogenase enzymes in living cells are capable of reducing the yellow water-soluble substrate MTT to the purple formazan products; these products are proportional to the viable cell number and inversely proportional to cell toxicity. The DBTRG-05MG cells were seeded in 96-well plates at a density of 10⁴ cells/well for 24 h and were then washed with phosphate-buffered saline (PBS) once. Cells were treated with allicin at 0.5, 1, 2, 5, 10, 20 and 30 μg/mL concentrations in serum-free RPMI media for 24 h. After incubation, MTT reagent was added to each well and incubated for 4 h. DMSO was added to solubilize the formazan crystals. The microplate reader was used to measure the optical density [8] at a wavelength of 540 nm. The experiments were repeated in triplicate.

Cell cycle analysis
DBTRG-05MG cells were seeded on 6-well plates at a density of 3x10⁵ cells/well for 24 h. The cells were treated with 5, 10 and 15 μg/mL of allicin for 24 h. After treatment, the cells were collected by trypsinization. To investigate the cell cycle distribution of different groups, a Tali® cell cycle kit was used according to the manufacturer’s instruction. Briefly, after centrifugation, the cells were washed and fixed in ice-cold 70% ethanol at -20°C overnight. The cells were centrifuged and washed with DPBS. The Tali® cell cycle solution was used to stain cells in the dark for 20 min at room
temperature. The stained cells were examined using the Tali® image-based cytometer (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The experiments were repeated in triplicate.

Apoptosis assay
DBTRG-05MG cells were seeded on 6-well plates at a density of 3x10^5 cells/well for 24 h. The cells were treated with 5, 10 and 15 μg/mL of allicin for 24 h. After treatment, the cells were collected by trypsinization. The cells were then stained with Annexin V and propidium iodide (PI) according to the manufacturer's instructions. Briefly, the cell pellet was resuspended in 1X Annexin binding buffer (ABB), followed by the addition of Annexin V Alexa Fluor 488. The stained cells were incubated in the dark for 20 min at room temperature. After centrifugation, the cell pellet was again resuspended in 1X ABB and incubated with PI in the dark for 3 min, at room temperature. Cells from 20 random fields were observed to examine apoptotic cell death using a Tali® image-based cytometer (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The experiments were repeated in triplicate.

Autophagy detection
In this study, we investigated autophagic cell death using two different methods. First, we observed intracellular autophagy by transmission electron microscopy (TEM). In brief, DBTRG-05MG cells were treated with 5, 10 and 15 μg/mL of allicin for 24 h and then harvested by trypsinization. After centrifugation, the cell pellet was directly fixed in 1% glutaraldehyde for at least 2 h at 4°C. The cells were postfixed in 1% osmium tetroxide for 30 min. Ultrathin sections were collected and stained with uranyl acetate and lead citrate. The samples were viewed on an FEI Tecnai G2 20 electron microscope (FEI company, OR, USA). To confirm the TEM results, autophagy was detected by the observation of LC3 expression using the immunofluorescent assay. DBTRG-05MG cells were treated with 5, 10 and 15 μg/mL of allicin in 24-well plates for 24 h. The cells were fixed with 4% paraformaldehyde for 20 min, washed 3 times with 0.1% Triton X-100 in PBS and incubated with LC3 antibody (1:200) overnight. The next day, the primary antibody was removed and washed again with 0.1% Triton X-100 in PBS. The secondary antibody conjugated with Alexa Fluor 488 (1:1000) was used to detect LC3 with a Nikon Ti-E confocal microscope (Nikon, Tokyo, Japan) and 40× and 60× oil objective lenses. Thirty images from each condition were quantified as the percentage of LC3 positive cells using ImageJ software.

Migration assay
The DBTRG-05MG cells were seeded on 6-well plates at a density of 3x10^5 cells/well in 3 mL of medium supplemented with 10% FBS for 24 h. A sterile 200-μl pipet tip was used to scratch a straight line through a cell monolayer. The wells were washed with PBS three times to remove detached cells. To selectively address the antimigratory effect of allicin, only the concentration that insignificantly affected cell growth was selected. The cells were incubated with serum-free media containing 0.5 μg/mL of allicin and were allowed to migrate for 24 h. Cell migration was monitored at the indicated time-points by phase contrast inverted microscopy (Olympus, Tokyo, Japan) using a 4× objective lens. The experiments were repeated in triplicate. The numbers of migratory cells in the scratch area from 10 images of each condition were counted using ImageJ software.

Statistical analysis
Values are expressed as the mean±standard error of the mean (SEM). Multiple group comparisons were made using one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test using SPSS version 18 software. A p value of <0.05 indicated statistical significance.
RESULTS

**Allicin inhibits human glioblastoma cell growth in a dose-dependent manner**

As allicin has been reported to exhibit antiproliferative effects on several cell types, including glioblastoma cells, the MTT assay was performed to investigate the effect of allicin on cell viability of the glioblastoma cell line DBTRG-05MG. The cell growth inhibitory effect of allicin on DBTRG-05MG cells is demonstrated in Fig. 1A. Allicin, at concentrations from 1 μg/mL, significantly suppressed glioblastoma cell growth. The IC\textsubscript{50} value of allicin was 6.23 μg/mL. We showed that allicin treatment induced morphological changes in a dose-dependent manner. These morphological changes included cell rounding, cytoplasmic shrinkage and cell death (Fig. 1B). These results indicated that allicin has a cell growth inhibitory effect on DBTRG-05MG cells.

**Allicin induces S and G2/M cell cycle arrest in human glioblastoma cells**

Since the cell cycle arrest and apoptosis are two major causes leading to the inhibition of cell growth, we first investigated the effect of allicin on cell cycle distribution using the Tali® cell cycle kit. As shown in Fig. 2, allicin increased the percentage of cells in G\textsubscript{2}/M and S phases and decreased the proportion of cells in G\textsubscript{0}/G\textsubscript{1}. Allicin at a concentration of 15 μg/mL significantly increased (p<0.01) the population of DBTRG-05MG cells in G\textsubscript{2}/M and S phases when compared with untreated control. These results indicated that allicin induced S and G2/M cell cycle arrest in DBTRG-05MG cells.

**Allicin promotes apoptosis of human glioblastoma cells**

To investigate the cell death mechanism, an apoptosis assay was performed using a Tali® image-based cytometer. As shown in Fig. 3A, the number of apoptotic cells (green) increased when the cells were treated with allicin. Allicin at a concentration of 15 μg/mL significantly increased (p<0.01) the percentage of apoptotic cells labeled with annexin V when compared with the untreated control group (Fig 3B).

**Allicin induces autophagy in human glioblastoma cells**

Interestingly, the results from the apoptosis assay were inconsistent with the results of the MTT assay. The percentage of apoptotic cells was low even in cells treated with 15 μg/mL of allicin, whereas the results of the MTT assay showed that the IC\textsubscript{50} value was 6.23 μg/mL. Therefore, we hypothesized that there would be other cell death mechanisms involved in which allicin affects DBTRG-05MG cell death. Autophagy detection was performed. As shown in Fig. 4A, autophagic vacuoles were observed in TEM images of DBTRG-05MG cells treated with allicin (arrow). Moreover, the LC3 immunostaining images in Fig. 4B revealed puncta formation of LC3, which represents autophagosomes (arrow), in the cytoplasm of cells. Interestingly, they were obviously present in DBTRG-05MG cells treated with 10 and 15 μg/mL of allicin. As shown in Fig. 4C, the percentage of LC3 positive cells significantly increased when the cells were treated with 10 and 15 μg/mL of allicin (p<0.001). This is the first evidence showing that allicin induced autophagy in human glioblastoma cells.

**Allicin decreases human glioblastoma cell migration**

Due to the fact that cell migration is an important process during tumor formation, the effect of allicin on the migratory capacity of DBTRG-05MG cells was investigated by the cell migration assay. As shown in Fig. 5, allicin treatment at concentrations of 0.5 μg/mL caused significant inhibition of the migration of DBTRG-05MG cells after 24 h. This result indicated that allicin inhibited the migration of human glioblastoma cells.

DISCUSSION

GBM is one of the most incurable forms of neoplasms. There are many studies that have reported novel treatments of GBM; however, it is challenging to develop a better,
more effective or alternative strategy for GBM treatment. Allicin, derived from garlic extract, potentially has a therapeutic value due to its antioxidant [2, 13], antiinflammatory [2] and antiproliferative effects [14-16]. The current study demonstrated that allicin inhibited cell growth, exhibited antimigration activity, and induced cell cycle arrest, apoptosis and autophagy in the DBTRG-05MG glioblastoma model.

Uncontrolled cell proliferation is a crucial hallmark of multiple cancers, including glioblastoma. This study found that allicin strongly inhibited DBTRG-05MG cell growth. In comparison to previous studies, our results suggest that DBTRG-05MG glioblastoma cells are more sensitive to allicin than U87MG human glioblastoma cells [6] and U251 human glioblastoma cells [17]. Moreover, allicin exhibited a more potent effect on DBTRG-05MG glioblastoma cells in comparison to non-cancer cell lines, including the NIH/3T3 mouse fibroblast cell line [18] and the INT-407 normal intestinal cell line [19]. Therefore, allicin may be beneficial for the selective treatment of glioblastoma while sparing healthy tissue.

Although the present study is not the first to report that allicin exerts antiproliferative effects on glioblastoma cell lines, we have provided the first evidence suggesting that allicin exerts its effect by inducing S and G2/M phase cell cycle arrest and autophagy in human glioblastoma cells. According to a previous study [6], allicin inhibited U87MG human glioblastoma cell growth by inducing apoptosis, which is consistent with our findings using DBTRG-05MG cells. However, besides apoptosis, our evidence indicates that the mechanism underlying allicin-induced glioblastoma cell death also involved S and G2/M phase cell cycle arrest. This finding is consistent with a previous study [5] in which flow cytometry results showed that allicin causes cell cycle arrest in S and G2/M phases in human gastric cancer cells. The allicin-induced S-phase cell cycle arrest implies that the cells are not capable of duplicating their DNA, whereas, allicin-induced cell cycle arrest in the G2/M phase means that allicin probably causes DNA damage. S phase and G2/M phase cell cycle arrest are upstream events that subsequently result in cell death. Since cell cycle arrest and cellular apoptosis are two therapeutic outcomes often targeted by anticancer drugs, it is interesting that allicin is capable of mediating its effects on human glioblastoma cell through the induction of both apoptosis and cell cycle arrest mechanisms.

In addition to apoptosis, our results suggest that allicin activity was associated with autophagy induction, as observed by TEM and immunofluorescent techniques. In comparison to the apoptosis results, the percentage of LC3 positive cells is much higher than that of apoptotic cells, especially in cells that were treated with allicin at high concentrations. Therefore, the LC3 staining results explained why the percentage of apoptotic cells is not prominent. It suggests that allicin induced glioblastoma cell death predominantly via autophagy. This finding is consistent with previous studies showing that allicin can induce autophagy in several cell types, such as thyroid cell lines [12] and human liver cancer cells [10]. It has been widely reported that there is a relationship between apoptosis and autophagy which underlies the process of cancer cell death [20,21].

As previously mentioned, cell migration and invasion play a critical role in the poor prognosis of glioblastoma. The current study demonstrated that allicin decreased the ability of human glioblastoma cell migration. This corroborates previous studies, which showed that allicin suppresses migration of multiple cancers, including cervical cancer cells [22], cholangiocarcinoma cells [23] as well as human renal carcinoma cells [24].
CONCLUSIONS
The data from the present study demonstrated a novel mechanism of the anticancer effect of allicin on human glioblastoma cells via the induction of S and G2/M phase cell cycle arrest and autophagy. This study provides insight into understanding the mechanism of how allicin affects glioblastoma cell death. Furthermore, we demonstrated that allicin exhibits a significant inhibitory effect on human glioblastoma cell migration. Therefore we recommend the use of allicin as a potentially beneficial anticancer agent in the treatment of glioblastoma.

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Author contributions: OW and KS conceived, designed and performed the experiments, analyzed the data. OW, PS and NC wrote the manuscript. All authors read and approved the final manuscript.

Conflict of interest disclosure: All authors declare that they have no conflict of interest.

REFERENCES
Figure Legends

**Fig. 1.** Allicin suppresses human glioblastoma cell growth in a dose-dependent manner. The DBTRG-05MG cells were treated with various concentrations of allicin (0-30 μg/mL) for 24 h. Cell viability was measured by the MTT assay. A – Percentage of cell viability compared with untreated control. Each column represents the mean±SEM from three experiments. IC₅₀=6.23 μg/mL, *p<0.05 compared with the untreated control, and ***p<0.001 compared with the untreated control. B – Morphological changes of allicin-treated DBTRG-05MG cells.

**Fig. 2.** Allicin induces S and G₂/M phase cell cycle arrest in human glioblastoma cells. DBTRG-05MG cells were treated with different concentrations (0-15 μg/mL) of allicin in RPMI serum-free medium for 24 h. The cells were collected and fixed with 70% ethanol. After staining the cells with the Tali® cell cycle solution, the cell cycles of different groups were investigated by the Tali® image-based cytometer. A – Representative corresponding histograms of cell cycle analysis of allicin-treated DBTRG-05MG cells. B – Data are represented as the mean±SEM from triplicate experiments. **p<0.01 compared with the untreated control and ***p<0.001 compared with the untreated control.

**Fig. 3.** Allicin promotes apoptosis of human glioblastoma cells. A – Representative images of apoptosis detection of allicin-treated DBTRG-05MG cells. B – Data are represented as the mean±SEM from three experiments. **p<0.01 compared with the untreated control.

**Fig. 4.** Allicin induces autophagy in human glioblastoma cells. A – Transmission electron microscope (TEM) images of the allicin-treated DBTRG-05MG cells. The cells were treated with different concentrations of allicin (0-15 μg/mL) for 24 h. The cells were harvested and fixed. Ultrathin sections were collected and stained with uranyl acetate and lead citrate. Images of the cells were observed by TEM. Compared with the untreated control group, initial autophagic vacuoles (AVi) were observed in allicin-treated cells. Arrows showed double membranes. B – Allicin induces LC3 punctation in human glioblastoma cells. The DBTRG-05MG cells were treated with different concentrations of allicin (0-15 μg/mL). After 24 h of treatment, the cells were fixed and stained with anti-LC3 antibody. Arrows showed LC3-labeled autophagosomes. C – Quantitative analysis of B showed that the percentage of LC3 positive cells was increased in allicin-treated DBTRG-05MG cells compared with the untreated control. ***p<0.001 compared with the untreated control.

**Fig. 5.** Allicin decreases human glioblastoma cell migration. DBTRG-05MG cell migration was investigated by the migration assay. The cells were treated with 0.5 μg/mL in RPMI serum-free medium and a migration assay was performed. The numbers of migratory cells in the scratch area were counted at different time points (0, 6, 12 and 24 h) after the scratch was made. A – Images of DBTRG-05MG cell migration (magnification of all figures: 4×). Allicin reduced the number of migratory cells during 24 h. B – Quantification of the number of migratory cells at different time points. **p<0.01 compared with the 24 h control.
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