Diallyl trisulfide induces apoptosis and inhibits proliferation of A549 cells in vitro and in vivo

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Lung cancer is the leading cause of cancer-related mortality all over the world. In recent years, pulmonary adenocarcinoma has surpassed squamous cell carcinoma in frequency and is the predominant form of lung cancer in many countries. Epidemiological investigations have shown an inverse relationship between garlic (Allium sativum) consumption and death rate from many cancers. Diallyl trisulfide (DATS) is one of the garlic-derived compounds (also known as: organosulfur compounds, OSC). DATS can induce apoptosis and inhibit the growth of many cancer cell lines. Our study demonstrated that the apoptotic incidents induced by DATS were a mitochondria-dependent caspase cascade through a significant decrease of the anti-apoptotic Bcl-2 that resulted in up-regulation of the ratio of Bax/Bcl-2 and the activity of caspase-3, -8, and -9. Eventually, DATS induced the apoptosis and inhibited the proliferation in a concentration- and time-dependent manner. Furthermore, by establishing an animal model of female BALB/c nude mice with A549 xenografts, we found that oral gavage of DATS significantly retarded growth of A549 xenografts in nude mice without causing weight loss or any other side effects compared with the control group. All the evidence both in vitro and in vivo suggested that DATS could be an ideal anti-cancer drug.

Keywords: diallyl trisulfide; lung adenocarcinoma; A549 cell; apoptosis

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Introduction

Lung cancer has been the leading cause of cancer-related mortality not only in China, where a peak is still expected in its incidence, but also around the world for decades [1,2]. Unfortunately, the majority of lung cancer patients are not diagnosed until the disease has progressed to an advanced stage [2]. Squamous-cell carcinoma (SCC), adenocarcinoma, small cell carcinoma and large cell carcinoma are the four most common histological subtypes of lung cancer. The incidence rates for three major types of female lung cancer (SCC, adenocarcinoma, and small cell carcinoma) have increased, with an especially rapid increase in adenocarcinoma which is now the predominant type in women while SCC prevails in men [3]. This is probably because of the changes in smoking habits and tobacco products (e.g. lower tar contents and use of a filter or inhalation) [4,5], but also slightly different characteristic of tumorigenesis for each pathological type should be considered as well. As women have an increased susceptibility to adenocarcinoma, more studies are needed to throw light upon the molecular mechanism and eventually find the ideal therapeutic method.

Garlic (Allium sativum) has been reported to have been used in treating some diseases. A number of epidemiological investigations showed an inverse relationship between garlic consumption and death rate for malignant tumors of the digestive tract [6–8], especially the mortality from gastric cancer in China [9]. A wealth of laboratory evidence for possible lung cancer-preventive biological mechanisms of a series of organosulfur compounds (OSC), including water-soluble sulfur compounds such as S-allylcysteine (SAC) and S-allymercaptocysteine (SAMC) and oil-soluble sulfur compounds, namely diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS), and ajoene, has been published [10–13]. But, whether the biochemistry and toxicology of individual allyl sulfides are different depending on the number of sulfur atoms in the molecule have not been sufficiently concluded. However, DATS, containing sulfane sulfur in its structure, has...
stronger biological activity than SAC, SMAC and DAS, DADS [11,14–16]. Increasing evidence suggested that DATS showed a potential ability to inhibit proliferation and induce apoptosis of hepatocellular carcinoma and colon and prostate cancer [16–18]. It has been shown that DATS induced apoptosis by inducing proapoptotic proteins and decreasing expression of anti-apoptotic proteins in human lung cancer cell lines H358 and H460, without knockdown of Bax and Bak proteins, but not in the normal human bronchial epithelial cell line [13]. However, the exact pathway by which DATS induces apoptosis and inhibits the growth of lung cancer cells lines while normal cells can survive is still not clear. Conflicting results have occasionally come out even with same cell line [19,20]. Moreover, systematical studies and research data of DATS both in vitro and in vivo are relatively rare.

So, further studies about the chemopreventive effects of DATS on lung cancer are needed. In this study, it was hypothesized that DATS treatment may reduce the progress of lung cancer by enhancing the expression and activity of caspase-3, -8, and -9 expression and inhibiting bcl-2 levels. A number of in vivo and in vitro studies were designed and carried out to test this hypothesis. DATS contributes to the treatment of lung cancer and may be as an ideal cancer chemopreventive agent for lung cancer.

**Materials and Methods**

**Cell culture**

The human lung cancer cell line (A549, Shanghai Institute of Biochemistry and Cell Biology, Shanghai, China) was cultured in Roswell Park Memorial Institute (RPMI) 1640 (Sigma, St Louis, USA) containing 10% FBS, 1% penicillin–streptomycin at 37°C in a 5% CO₂ humidified cell culture incubator.

**MTT assay**

Cell viability was estimated by methyl thiazolyl tetrazolium (MTT) assays to determine the cytotoxicity effect of DATS on A549 cells. The cells were plated into 96 well (5 x 10⁵ cells/ml) and treated at four concentrations (0, 25, 50, and 100 μM) of DATS (Alis Chemicals Co. LTD, Shanghai, China) for 1 h. Then the medium of each well was replaced and incubated for 48 h in new RPMI 1640 medium. All cells were determined by MTT assay as previous described [11]. Results were shown as mean ± standard deviation (SD) of 3 independent experiments (n = 5).

**RNA isolation and real-time PCR for caspase-3, -8, -9, and Bax**

The cells were harvested and total RNA was extracted using the TRIzol Kit (Invitrogen, Carlsbad, USA). The expressions of caspase-3, -8, -9, and Bax were quantified by real-time polymerase chain reaction (PCR) using a Bio-Rad iQ5 real-time PCR system with EvaGreen Supermix (Bio-Rad, Hercules, USA) according to the instruction manual. The primer for GAPDH, Bax, and caspase-3, -8, and -9 are as follows: GAPDH, forward, 5'-AGTCTGGTGTGAACGATTTG-3', reverse, 5'-TGTA GACCTGTAGTTAGGCTCA-3'; Bax, forward, 5'-ATGG GCTGGACACTGGACTTC-3', reverse, 5'-GAGCGAGGC GGTTAGAC-3'; caspase-3, forward, 5'-TGGTACACTCC ACAGCACCTGTTA-3', reverse, 5'-CATGGCACAAA GCGACTGGATGAA-3'; caspase-8, forward, 5'- TTTCA GCAGTTCAGGGTGTGA-3', reverse, 5'-CTGTT AATCCACGACTTTGGAG-3'; caspase-9, forward, 5'-GTTGCTCTTCTGCGAACTTTGGAGA-3', reverse, 5'-GTT GGCTTCGACAATTTGCT-3'. Results are mean ± standard error mean (SEM) of 3 repeat experiments (n = 5) and GAPDH was used as a reference transcript.

**Caspase-3, -8, and -9 activity**

Caspase-3, -8, and -9 activities in the cells were assayed using colorimetric assay kits (Beyotime, Shanghai, China) as previously described [21]. A549 cells were cultured alone or with 100 μM DATS for 72 h and the activities of Caspase-3, -8, and -9 were determined. The experiment was performed in triplicate.

**Western blot analysis**

A549 cells were treated with or without DATS for 48 h, and the protein was extracted from all groups of cells and applied to a 12% sodium dodecyl sulfate (SDS)–polyacrylamide gel. Then the proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane. After blocking in 5% not-fat milk for 1 h, the membrane was incubated overnight with primary antibodies, including mouse anti-Bcl-2 antibody (1 : 500, Abcam, Cambridge, USA) and rabbit anti-Bax antibody (1 : 1000, Abcam). Following incubation with horseradish peroxidase-conjugated antibody (Cell Signaling, Beverly, USA) and washing three times with Tris-HCl-buffered salt solution with Tween (TBST), the immunoactive proteins were detected by chemiluminescence (Amersham Biosciences, Piscataway, USA).

**Animal experiments**

Forty female BALB/c nude mice (6 week old) were obtained from the Institute of Zoology, Chinese Academy of Sciences (Beijing, China). The mice were housed under specific pathogen-free conditions, and food and water were given *ad libitum* throughout the experimental periods. Animal protocols were according to the permission and experimental rules of the animal ethics committee of Shandong University.

A549 cells were used to establish the mouse tumor model. Briefly, a single-cell suspension of A549 cells was prepared. Then, the mice were subcutaneously injected...
with A549 cells (5 x 10^6/mouse) suspended in 100 μl PBS. The mice were randomly divided into two groups. One group orally received 6 μM [22] DATS in 100 μl PBS every other day (DATS group, n = 20). The other group was given 100 μl PBS (control group, n = 20). The general state of the animals was observed. On the 30th day, the mice were killed and the incidence and volume of tumors were assayed.

**Statistical analysis**

The data were shown as mean ± SD. Statistical significance was established by One-way analysis of variance and t-test. Statistical analyses were carried out using SPSS 13.0 software. P < 0.05 was considered significant.

**Results**

**DATS treatment inhibits the survival of A549 cells in vitro**

The effect of DATS on cancer cells’ survival was assayed by MTT, and it was found that A549 cells were significantly inhibited after incubation with DATS (P < 0.05). Furthermore, this effect on tumor cells of DATS was dose-dependent (Fig. 1).

**DATS treatment induces the expression of Bcl-2 in vitro**

Bcl-2 plays a central role in apoptosis, proliferation, and invasion of tumor cells. In this study, the protein expression of Bcl-2 was examined to explore the inherent mechanism of DATS on A549 cells. The result showed that DATS treatment led to a marked down-regulation of Bcl-2 protein expression in A549 cells (Fig. 2, P < 0.05).

As shown in Fig. 2, three concentrations and times were used in this experiment. We found that 100 μM DATS achieved a larger effect compared with other two groups (P < 0.05), showing that the effect of DATS on A549 cells was dose-dependent. Compared with the control group and the 24 h group, Bcl-2 expression was more significantly decreased at 48 h than that in the 24 h group (P < 0.05). However, the expression of Bcl-2 was not further decreased at 72 h (P > 0.05). So, 100 μM DATS for 48 h was used for the further experiment.

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**Figure 1** Cell viability of A549 cells determined by MTT assay after DATS incubation. The A549 cells were treated at four concentrations (0, 25, 50, and 100 μM) of DATS for 1 h. Results were shown as mean ± SD of three independent experiments (n = 5). *P < 0.05 vs. the control group, †P < 0.05 vs. 25 μM DATS group, ‡P < 0.05 vs. 50 μM DATS group.

**Figure 2** Bcl-2 assayed by western blot in vitro. (A) Bcl-2 protein expression after DATS treatment at different concentrations (0, 25, 50, and 100 μM) for 48 h. (B) Quantitative analysis of (A). *P < 0.05 vs. the control group, †P < 0.05 vs. 25 μM DATS group, ‡P < 0.05 vs. 50 μM DATS group. (C) Bcl-2 protein expression after 100 μM DATS treatment for different times (0, 24, 48, and 72 h). (D) Quantitative analysis of (C). *P < 0.05 vs. the control group, †P < 0.05 vs. 24 h group.
DATS treatment increases the expression and activity of caspase-3, -8, and -9 in vitro

After incubation with 100 μM DATS for 48 h, mRNA expressions of caspase-3, -8, and -9 were more markedly increased in DATS-treated A549 cells than in the control group [Fig. 3(A–C), P < 0.05]. Also, we assayed the activity of caspase-3, -8, and -9 in the cells. After incubation with 100 μM DATS for 48 h, the activity of caspase-3, -8, and -9 was significantly enhanced in comparison with the control group [Fig. 3(D–F), P < 0.05].

DATS treatment increases the expression of Bax in vitro

mRNA and protein expression of Bax were measured after incubation with or without 100 μM DATS for 48 h. We found that Bax mRNA and protein levels were more significantly increased in DATS-treated A549 cells than those in the control group (Fig. 4, P < 0.05).

DATS treatment inhibits the growth of lung cancer cells in vivo

The anti-tumor effect of DATS using the BALB/c nude mice model was assessed. As shown in Fig. 5, a single injection of DATS induced a marked reduction in the incidence and volume of tumors in the mice compared with those in the control group (Fig. 5, P < 0.05). Although neither remarkable inhibitory of angiogenesis nor obvious metastasis could be roughly observed by nude eyes, related experiments in vivo and clinical trial are undergoing in our laboratory and hospital.

Discussion

In spite of more and more advances in diagnosis and treatment, cancer is still a major health burden. Multistep tumorigenesis includes oncogene activation, telomerase loss, and induction of aneuploidy as critical initiating events [21,23,24]. Each pathological type of tumor is slightly different with its own characteristic of tumorigenesis and consequently epidemiology. In recent years, pulmonary adenocarcinoma, surpassing SCC in frequency, has been the predominant form of lung cancer among males in many countries. Also, among females, the incidence of adenocarcinoma has always been higher than that of SCC all over the world [3]. As multiple signaling pathways are usually dysfunctional in cancer, garlic that is rich in bioactive sulfur-containing compounds that can modulate cancer cascades is certain to be regarded as a potential chemopreventive and chemotherapeutic agent. Water- or oil-soluble sulfur compounds have been used in clinical medicine for a very long time [7]. DATS, which is the major functional component inhibiting tumors, accounts for nearly 45% of oil-soluble garlic extracts. In this study we not only confirmed the molecular mechanism in DATS-induced apoptosis and anti-proliferation of human lung adenocarcinoma A549 cells in vitro but also did further research on the ability of DATS to inhibit growth of A549 xenografts in female BALB/c nude mice, explained as follows.

Considering the evidence that DATS was most effective in apoptosis induction and cell viability reduction among OSC in many other cancers [11,14–16], we chose DATS as the only OSC in our research. The mechanism of DATS–induced apoptosis appeared to varies depending on the type of cell line used [25], even with the same cell line.
inconsistent results have appeared [19,20]. The intrinsic pathway of apoptosis is mediated by mitochondria. Proteins of apoptosis including cytochrome c are released from mitochondria to the cytoplasm to initiate a caspase cascade reaction resulting in apoptosis [26,27]. The Bcl-2 family also plays an important role in regulation of this intrinsic pathway [28,29]. DATS-induced apoptosis in human bladder cancer cells is associated with increased caspase-3 activation, up-regulation of Bax and down-regulation of Bcl-2 [30]. The change of the Bax/Bcl-2 ratio caused activation of caspase-3 [31]. In the present study, the results showed that DATS decreased Bcl-2 protein expression in A549 cells in a concentration- and time-dependent manner. We found that DATS induced A549 cells apoptosis by increasing expression and activity of caspase-3,-8, and -9, decreasing Bcl-2 expression and enhancing Bax levels. In summary, apoptotic incidents induced by DATS were mitochondria-dependent caspase cascades through a significant decrease of the anti-apoptotic Bcl-2 that resulted in up-regulation of the ratio of Bax/Bcl-2 and the activity of caspase-3, -8, and -9. Additionally, our results demonstrated that DATS inhibited the proliferation of A549 cells in a concentration- and time-dependent manner which may have involved the endoplasmic reticulum. Further research is needed to elucidate the potential mechanisms of the interaction between the endoplasmic reticulum and mitochondria of DATS-induced apoptosis in A549 cells. In contrast to the conclusion of De Martino et al. [32] that

### Figure 4
**The mRNA and protein expression of Bax in vitro**
- (A) Bax mRNA expression assayed by real-time PCR;
- (B) Bax protein expression assayed by western blot;
- (C) quantitative analysis of (B). Results are mean ± SEM of 3 repeat experiments (n = 5). *P < 0.05 vs. the control group.

### Figure 5
**Incidence and volume of tumor assayed in female BALB/c nude mice**
- (A) Representative image in the control group;
- (B) representative image in the DATS group;
- (C) the incidence of tumor between the two groups;
- (D) the volume of tumor between the two groups. *P < 0.05 vs. the control group.
water-soluble sulfur compounds were more effective than oil-soluble sulfur compounds in inhibiting cell proliferation, more evidence, similar to the present results in A549 cells, have suggested that DATS treatment caused G2-M phase cell cycle arrest in many kinds of cancer cells [10,18]. After treatment with DATS in a concentration and time gradient way, the proliferation of A549 cells were significantly inhibited.

Cellular systems are good for in obtaining mechanistic insights. The observations that we obtained in cells should be confirmed in animal models to associate with the cellular findings. So based on the above results in vitro, we took DATS as the model drug to test whether DATS administration inhibited growth of A549 xenografts in nude mice. Consistent with similar experimental results about other human cancer cells [17], oral gavage of DATS significantly retarded growth of A549 xenografts in nude mice without causing weight loss or any other side effects compared with the control group. Angiogenesis, involving the growth of new blood vessels that come from preexisting vessels, is required for tumor growth and metastasis [33]. Formation of metastases is a key factor in cancer progression and causes major cancer deaths. In our nude mice bearing A549 cells, neither remarkable inhibitory of angiogenesis nor obvious metastasis could be judged by nude eyes. Relevant research is still undergoing in our lab. But the present study found that DATS-mediated inhibition of A549 xenograft growth in vivo was associated with a decrease of Bcl-2, which was consistent with cellular studies of treating A549 cells with DATS resulting in a concentration- and time-dependent apoptosis induction. So it is reasonable to draw the conclusion that apoptosis induction is a key factor in DATS-mediated suppression of A549 cell growth in vivo. The present study revealed that the DATS-mediated suppression of A549 xenograft growth in vivo was accompanied by decrease of Bcl-2, whose function is to promote cell death. These results strongly suggested that DATS suppressed the A549 cell growth in vivo by the mechanisms observed in vitro. The results both in vitro and in vivo provided strong preclinical evidence that DATS could be used to prevent and/or treat pulmonary adenocarcinoma.

Large, double-blinded, carefully designed, and well-controlled research is still necessary. This will draw a reliable conclusion of DATS’ effect on pulmonary adenocarcinoma and, more importantly, on the end points of all kinds of malignant tumors.

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