

DATS suppresses invasion of oral squamous cell carcinoma cell lines by reducing matrix metalloproteinase-9 via PI3K/AKT

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Received: 04.07.2011 • Accepted: 02.04.2012

Abstract: Oral squamous cell carcinomas (OSCCs) are the most common type of oral cancers, and high morbidity and mortality are largely attributable to late-stage diagnosis. Despite significant advances in therapeutic strategies, the 5-year survival rate still remains low. Diallyl trisulfide (DATS), an oil-soluble organosulfur compound of garlic, was recently reported for its anticancer effect on several types of cancer cells *in vitro*; however, these anticancer effects are still unknown in human OSCC cells. In the present study, the effects of DATS on OSCC cell invasion *in vitro* were investigated. Human OSCC cell lines (HSC-2 and Ca9-22 cells) were treated with DATS and examined for cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay; the suppression of cell invasion was demonstrated by transwell assay. Western blot was performed to detect the effect of DATS on the expression of matrix metalloproteinase-9 (MMP-9) and the activation of v-akt murine thymoma viral oncogene homolog 1 (AKT1), respectively. The results showed that DATS (at 40 μ M, a nontoxic dose) and GM6001 inhibited ($P < 0.05$) the invasion of OSCC cells, respectively, when compared to the DMSO control. DATS (at 40 μ M) significantly decreased ($P < 0.05$) the expression of MMP-9 and time-dependently inhibited ($P < 0.05$) the activation of AKT1, respectively. Moreover, LY294002 also decreased ($P < 0.05$) the expression of MMP-9 in a dose-dependent manner. It was concluded that DATS inhibits the invasion of OSCC cells by decreasing MMP-9 expression via the PI3K/AKT pathway.

Key words: DATS, oral squamous cell carcinomas, invasion, MMP-9, AKT

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer and a major cause of morbidity and mortality globally. Over 500,000 cases of HNSCC are diagnosed each year, and about 90% of HNSCCs are oral squamous cell carcinomas (OSCCs) (1,2). The associated high morbidity and mortality are largely attributable to late-stage diagnosis and the occurrence of second primary tumors. Although diagnosis and treatment of OSCC have improved, the survival rate has not increased substantially in recent

years. The treatment of these types of oral cancers includes surgery and/or radiotherapy, which are often associated with loss of function, disfigurement, and reduced quality of life (3). The development of novel therapeutic agents targeting the malignant behavior of these cancers is important to improve the prognosis of treatment.

Garlic, which has a high content of organosulfur compounds such as diallyl sulfide (DAS), diallyl disulfide (DADS), and diallyl trisulfide (DATS) (4,5), is considered to have medicinal properties in many

areas. Among the garlic-derived allyl compounds, DATS has been demonstrated to have an anticancer effect on different types of human tumor cells, including prostate cancer cells (6), lung cancer cells (7), and skin cancer cells (8). However, little is known about the functional role of DATS in the human OSCC cell metastasis and the related molecular mechanisms.

Metastasis is one of the major causes of mortality in cancer patients and occurs as a highly coordinated multistep process involving cell proliferation, invasion, cell-cell and cell-matrix adhesion, and remodeling of the extracellular matrix (ECM) (9). Although a number of proteinases are involved in the degradation of the ECM by cancer cells, including matrix metalloproteinases (MMPs), serine proteinase, and cathepsins (10), the secretion of MMPs is crucial in cancer cell metastasis and is deeply involved in cancer cell invasion (11). Among human MMPs, MMP-9 is abundantly expressed in various malignant tumors, including OSCC, and is closely correlated with the invasive behavior of these cancers (12-15).

It is well demonstrated that the expression of MMP-9 in various tumor cells is tightly controlled, and many extracellular stimuli trigger multiple signaling cascades that are involved in the regulation of MMP-9 expression and activity. Among these pathways, the phosphatidylinositol 3-kinase (PI3K)/v-akt murine thymoma viral oncogene homolog 1 (AKT1) is the best studied (16,17). Activated AKT has been shown to be a frequent event in several cancer types, such as breast, colon, and ovarian cancer (18,19). Furthermore, it is reported that kalopanax saponin A inhibits phorbol 12-myristate 13-acetate (PMA)-induced cell invasion by reducing MMP-9 expression, mainly via the PI3K/AKT signaling pathways in MCF-7 cells (20). However, it is still unknown whether this signaling pathway is involved in DATS-regulated MMP-9 expression in OSCC cells.

In the present study, we used human OSCC cell lines (HSC-2 and Ca9-22 cells) to investigate the effects of the organosulfur compound of garlic, DATS, on OSCC cell invasion during the metastasis process. Our findings demonstrated that DATS inhibits the invasion of OSCC cells by reducing MMP-9 expression via the PI3K/AKT pathway.

Materials and methods

Materials

DATS was purchased from Fluka (Ronkonkoma, NY, USA); LY294002 (PI3K-specific inhibitor) and GM6001 (broad-spectrum matrix metalloproteinase inhibitor) were purchased from Calbiochem-Novabiochem (San Diego, CA, USA); and the primary antibodies for MMP-9, AKT1, and phospho-AKT1 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

Cell culture

OSCC cell lines (HSC-2 and Ca9-22 cells), derived from human OSCCs, were purchased from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All of the cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10 mM L-glutamine. All of the cells were cultured in a humidified atmosphere of 5% CO₂ at 37 °C.

MTT assay

The 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) viability assay was performed as described. Briefly, the cells were seeded into a 96-well plate with 0.5×10^4 cells/well. After 16 h of attachment, various concentrations of DATS (0-320 µM) were applied to the cells in serum-free DMEM and incubation was extended for another 24, 48, and 72 h, respectively. The cells were washed with phosphate buffered saline, 200 µL of MTT (0.5 mg/mL) was added to each well, and the cells were further incubated for 4 h. The MTT solution was carefully removed by aspiration and the formazan product was dissolved in 150 µL of dimethyl sulfoxide (DMSO). Absorbance was measured at 570 nm on a microplate reader (BioTek Instruments, Winooski, VT, USA). For each treatment concentration, 6 wells were used, and the experiment was repeated 3 times.

Invasion assay

Cell invasion was evaluated using a 24-Multiwell BD Falcon FluoroBlok Insert System with 8.0-µm pore polycarbonate filter inserts (BD Biosciences, San Jose, CA, USA). The cells (8×10^4) were suspended in 0.5 mL of serum-free medium with or without 40 µM DATS and were seeded into the upper compartment of the invasion chamber coated with Matrigel.

The lower compartment was filled with complete medium in the absence or presence of 40 μ M DATS. After 16 h, the cells that had been invaded on the bottom of the inserts were fixed, stained, and counted under a microscope. The numbers of invaded cells were counted using the MetaMorph image analysis program (Molecular Devices Inc., Sunnyvale, CA, USA). Triplicate independent experiments were done. To determine the role of MMP-9 on DATS-regulated cell invasion, the specific inhibitor GM6001 (40 μ M) was applied in this invasion assay.

Western immunoblot analysis

Western blot analysis was carried out as described. The collected cells were lysed by sonication in a buffer (50 mM HEPES, 0.1 M NaCl, 10 mM EDTA, 4 mM sodium pyrophosphate, 10 mM sodium fluoride, 1% Triton X-100, and protease inhibitor cocktail). After centrifugation, protein concentrations of the supernatant were determined with bovine serum albumin (fraction V; Sigma, St. Louis, MO, USA) as standards. The protein samples were separated on 10% SDS-PAGE gels and electrically transferred to polyvinylidene fluoride membranes. Proteins on the membrane were visualized using electrochemiluminescence reagents. The immunoreactive signals were analyzed by densitometry. Changes in the total and phosphorylated AKT1 were quantified by scanning densitometry (model GS 670; Bio-Rad, Hercules, CA, USA). Data on MMP-9 expression were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Statistical procedures

Values were expressed as the mean \pm SEM of at least 3 independent experiments. Data were analyzed using one-way ANOVA (SigmaStat; Jandel Co., San Rafael, CA, USA). When an F-test was significant, the data were compared with their respective controls by the Bonferroni multiple comparison test or Student's t-test. $P < 0.05$ was considered to be statistically significant.

Results

DATS reduces OSCC cell viability at high concentrations

A cell viability assay was performed in response to different concentrations of DATS (0-320 μ M). The

MTT assay showed that at lower concentrations (0-40 μ M), no clear effect was observed after 24-h and 48-h treatments of DATS. However, viability of the cells was significantly ($P < 0.05$) decreased at lower concentrations (20 μ M) after 72 h of treatment (Figure 1). Thus, for the following experiments, the concentration of 40 μ M DATS was chosen.

DATS suppresses the invasion of OSCC cell lines

To investigate the inhibitory effect of DATS on the invasion of OSCC cells, the cells that invaded through the Matrigel-coated polycarbonate filter in the Boyden chamber were analyzed. After 16 h of incubation, the cells showed a significantly ($P <$

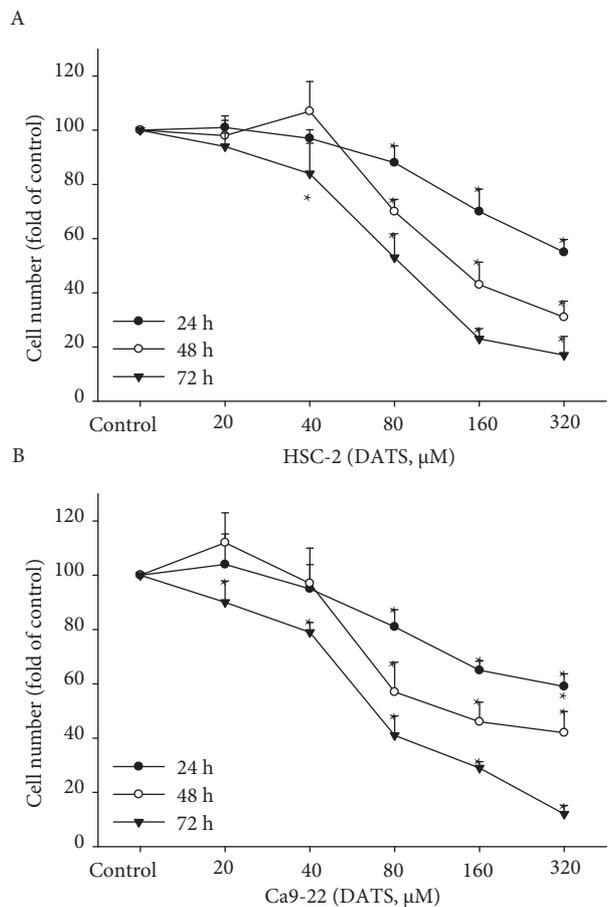


Figure 1. Cell viability response to DATS treatment. HSC-2 (A) and Ca9-22 (B) cells were treated with various concentrations of DATS (0-320 μ M) for 24, 48, and 72 h, respectively. Cell viability was determined with MTT assay, and the graphs show the results of 3 independent experiments. The results are represented as a percentage of absorbance relative to the control cells (100%). *Different ($P < 0.05$) from their controls, respectively.

0.05) decreased invasiveness response to 40 μM DATS compared with the DMSO treatment in both the HSC-2 and Ca9-22 cells (Figure 2). These results demonstrated that DATS markedly suppressed the invasion of oral squamous cell carcinoma cells.

Given the fact that MMP is deeply involved in the cancer invasion process, we further observed the role of MMPs on OSCC cell invasion through treatment with its specific inhibitor, GM6001. The results showed that the treatment of GM6001 at 40 μM sharply suppressed the invasion of both HSC-2 and Ca9-22 cells ($P < 0.05$) (Figure 2). The results suggested that the decreased ability of OSCC cell invasion might be due to the proteolytic activities of MMPs.

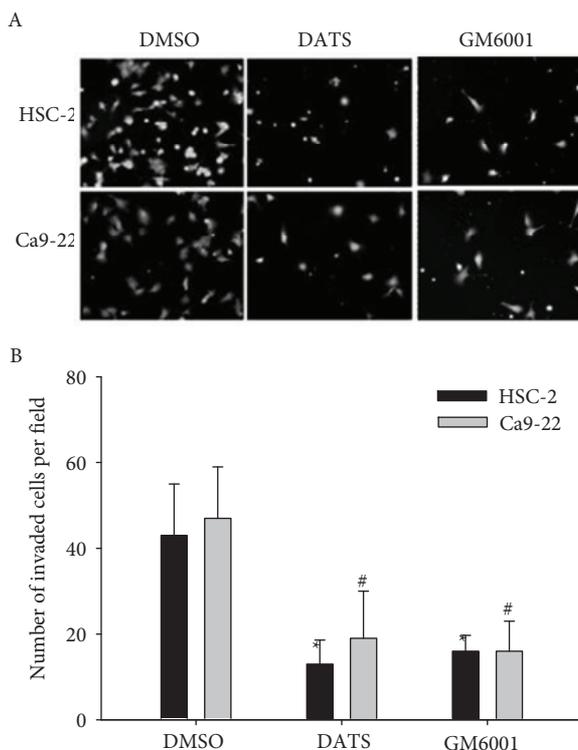


Figure 2. Effects of DATS on OSCC cell invasion using a transwell assay. The cells suspended in serum-free DMEM were overlaid in the upper chamber of each transwell. After incubation with DATS (40 μM) or GM6001 (40 μM) for 16 h, the penetrating cells were stained with calcein AM and recorded under a microscope mounted with a charge-coupled device camera. A) Photographs depict the invasion of HSC-2 and Ca9-22 cells; B) quantified data are expressed as means \pm SEM from 3 independent experiments. * and # show difference ($P < 0.05$) from their controls, respectively.

DATS decreases the expression of MMP-9 and inhibits the activation of AKT1 in OSCC cells

To further confirm the effects of DATS on the expression of MMP-9, western blot analysis was performed for both the MMP-9 and GAPDH gene. As shown in Figure 3, after 24 h of treatment, DATS significantly ($P < 0.05$) decreased the protein expression of MMP-9 at 20 and 40 μM , respectively. This decreased effect was also observed in the GM6001-treated group, suggesting that GM6001 inhibited the expression of MMP-9 in these 2 cell lines. Moreover, we detected the phosphorylation of AKT1 after treatment with 40 μM of DATS. As shown in Figure 4, DATS time-dependently ($P < 0.05$) inhibited the activation of AKT1 in comparison with time 0. This inactivation was maintained for at least 60 min. However, DATS did not alter the total AKT1 levels at any time point during the DATS treatments studied (Figure 4).

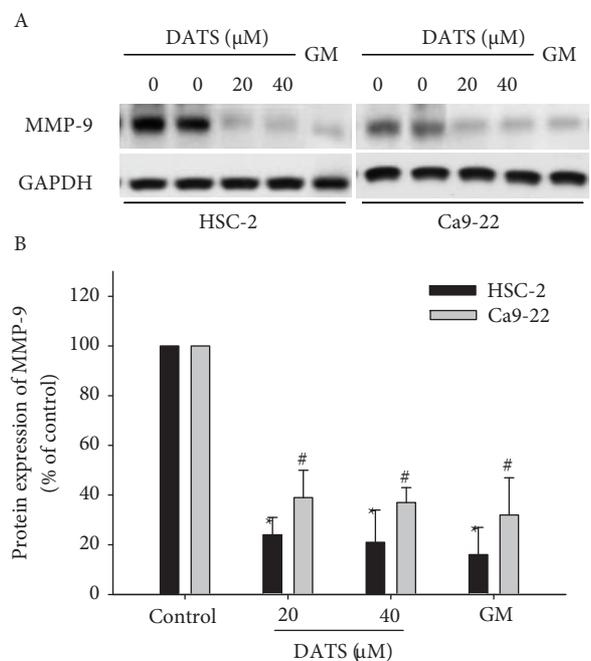


Figure 3. Effects of DATS on MMP-9 protein expression in OSCC cells. The cells were cultured in culture dishes until reaching 60%-70% confluence. They were then treated with DATS (20 and 40 μM) and GM6001 (40 μM) for 24 h, respectively. A) Proteins were subjected to western blot analysis and detected using an antibody against MMP-9 and GAPDH; B) data normalized to GAPDH are expressed as means \pm SEM fold of the control from 3 individual experiments. * and # show difference ($P < 0.05$) from their controls, respectively.

Inactivation of AKT1 is closely associated with decreased MMP-9 expression

Several studies have indicated that signaling proteins, including AKT1, are involved in the expression of MMP-9. In order to evaluate whether the inhibition of DATS on MMP-9 expression was through the inactivation of AKT1, OSCC cells were treated with a PI3K inhibitor (LY294002; 5 μ M, 10 μ M) for 24 h. Western blot results (Figure 5) showed that LY294002 significantly ($P < 0.05$) reduced the MMP-9 expression. The quantitative results showed that this inhibitory effect has a dose-dependent manner. The results revealed that the inhibition of MMP-9 by DATS could partly occur through suppression of the activation of AKT1.

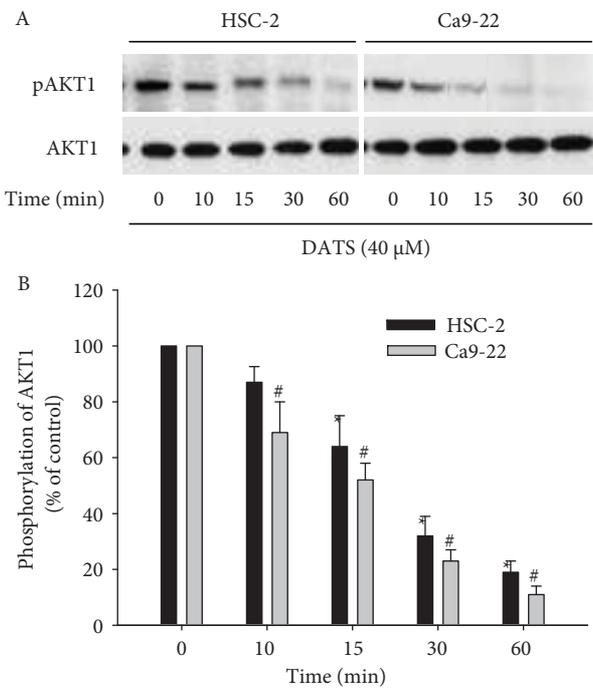


Figure 4. Effects of DATS on AKT1 phosphorylation in OSCC cells. The cells were cultured in culture dishes until reaching 60%-70% confluence. They were then treated with 40 μ M of DATS for 0-60 min. A) Proteins were subjected to western blot analysis and detected using an antibody against phospho-specific and total AKT1; B) data normalized to total AKT1 are expressed as means \pm SEM fold of the control from 4 individual experiments. * and # show difference ($P < 0.05$) from their controls, respectively.

Discussion

Within the last couple of years, interest in natural compounds has grown greatly, especially in relation to anticancer properties (21,22). Garlic has been found to contain a large number of organosulfur compounds, which possess anticancer properties. Among the compounds, DATS has the most potent chemopreventive and anticarcinogenic activities (5-8). In the present study, we have provided evidence that DATS was able to inhibit invasion in human OSCC cells through AKT1-dependent downregulation of MMP-9 expression, suggesting that DATS might possess antimetastatic potential.

Several recent studies have demonstrated that DATS is able to inhibit the growth of different types of cancer cells, including prostate (6), lung (7), and colon cancer cells (23), in culture. However, little is known about the functional role of DATS in OSCC

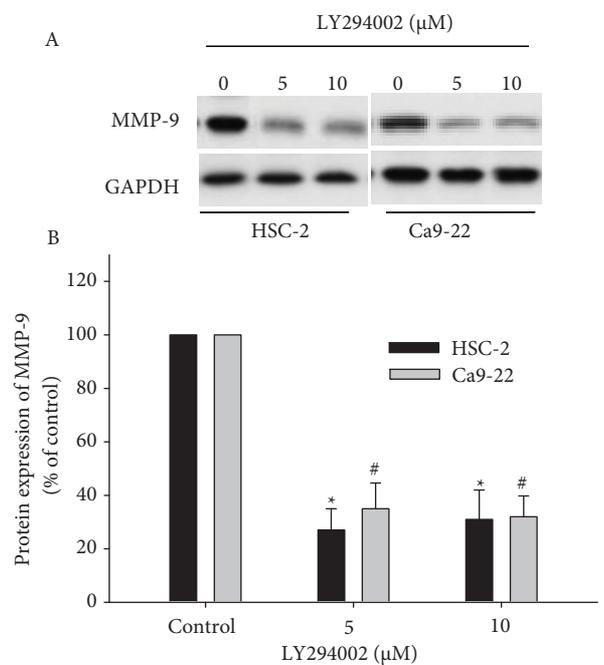


Figure 5. Effects of LY294002 on MMP-9 protein expression in OSCC cells. The cells were cultured in culture dishes until reaching 60%-70% confluence. They were then treated with 5 and 10 μ M of LY294002 for 24 h. A) Proteins were subjected to western blot analysis and detected using an antibody against MMP-9 and GAPDH; B) data normalized to GAPDH are expressed as means \pm SEM fold of the control from 4 individual experiments. * and # show difference ($P < 0.05$) from their controls, respectively.

metastatic progression. Our present results indicated that DATS significantly suppressed the viability of both HSC-2 and Ca9-22 cells at the relatively higher concentration of 80 μM . When these cells were treated with DATS at nontoxic doses (40 μM), invasion was significantly inhibited. These results implied that the inhibitory effects of DATS on OSCC cell invasion were not due to its cytotoxic effect.

Cancer metastasis is a complex multistep process, in which cancer cells invade the basement membrane and the ECM, and then migrate into new tissue. MMPs have been implicated for invasion and metastasis of cancer cells (24). It is well demonstrated that the expression of MMP-9 is an important phenotypic determinant of OSCC (25), and inhibition of MMP-9 has been shown to suppress cell migration and invasion by in vitro Matrigel invasion assay in a couple of cancer cell lines, including OSCC cells (26-28). Our current inhibition of MMP-9 using MMP's inhibitor, GM6001, significantly suppressed the invasion of OSCC cells. Treatment with DATS at 20 μM and 40 μM for 24 h both robustly decreased the expression of MMP-9. These results suggest that the decrease of MMP-9 expression might be attributed to the antiinvasion effect of DATS. The inhibitory effect of DATS on MMP-9 may be, at least in part, responsible for its antimetastatic potential.

The PI3K/AKT pathway has been suggested to be highly involved in tumor cell invasion (29). The induction of PI3K/AKT is involved in MMP-9 expression in different cell types (30,31). However,

the signaling pathway related to decreased MMP-9 expression induced by DATS in OSCC cells is still unknown. Therefore, we examined the effect of DATS on the activities of the PI3K/AKT signaling pathways. The results showed that treatment with DATS time-dependently inhibited AKT1 phosphorylation, suggesting that the signaling pathways mediated by PI3K/AKT were suppressed by DATS. Furthermore, inhibition of the PI3K/AKT signaling pathway using its specific inhibitor significantly decreased the expression of MMP-9, suggesting that the DATS-decreased MMP-9 expression is partly mediated by the PI3K/AKT pathway.

In conclusion, this preliminary investigation has shown that the decrease of MMP-9 expression by DATS is attributed to the inactivation of the PI3K/AKT signaling pathways, and such a suppressive effect might contribute to the inhibition of invasion in human OSCC cell by DATS. These findings reveal a new therapeutic potential for DATS in antimetastatic therapy.

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