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Diallyl trisulfide regulates cell apoptosis and invasion in human Osteosarcoma U2OS cells through regulating PI3K/AKT/GSK3β signaling pathway

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Running title:

DATS regulates U2OS cell apoptosis and invasion
Abstract:

Aims: To investigate the effects and the mechanisms of action of Diallyl trisulfide (DATS) on the proliferation and metastasis of human osteosarcoma (OS) U2OS.

Methods: U2OS cells were treated by different concentrations of DATS at different time points. Cell proliferations were measured by MTT assay. DATS induced cell cycle distribution and apoptosis were evaluated by flow cytometry (FCM) with Annexin-V. Cell migration and invasion were detected by wound healing assay and transwell assay. The effects of DATS in U2OS cell growth and metastasis were also detected in a mouse OS xenograft model.

Results: A time- and concentration-dependent cytotoxic effect of DATS was observed in U2OS cells. FCM with PI staining and Annexin-V-FITC indicated that DATS induces apoptosis and a G0/G1 cell cycle arrest of U2OS cells at all concentrations from 25 μmol/l to 100 μmol/l. DATS also inhibits the migration and invasion of U2OS cells. Western blot showed that the expression levels of p-AKT, p-GSK3β, Bcl-2, Vimentin and β-catenin were decreased, while the expression levels of Bad, Bax and E-cadherin were significantly increased in DATS treated U2OS cells. Analysis using a mouse xenograft model indicated that xenografts of DATS treatment group had a significant decrease in tumor volume and weight compared to the control group. Lung metastasis models in mice demonstrated that treatment of DATS inhibits
lung metastasis of OS \textit{in vivo}.

**Conclusions:** These data suggested that DATS inhibits OS development and progression through the regulation of PI3K/AKT/GSK3β signaling pathways, accompanied by downregulation of Bcl-2, Vimentin and β-catenin, as well as upregulation of Bad, Bax and E-cadherin. Therefore, our data demonstrated that DATS exerted its anticancer effects by inhibiting cell proliferation, migration and invasion \textit{in vitro} and \textit{in vivo}. These results provide evidence for the use of the natural product DATS either alone or in combination with standard therapy for OS.

**Keywords:** Osteosarcoma, Diallyl trisulfide, p-AKT, p-GSK3β

**Key Points:**

A time- and concentration-dependent cytotoxic effect of DATS was observed in U2OS cells. DATS induces apoptosis and cell cycle arrest of U2OS cells, and also inhibits the metastasis and invasive in U2OS cells. The expression levels of Bad, Bax and E-cadherin were increased, and the expression levels of Bcl-2, Vimentin and β-catenin were decreased in U2OS cells by DATS.
Introduction

Osteosarcoma (OS), an aggressive and highly malignant cancer, is the most common bone tumor type prevalent in children and young adults (Mackall et al., 2014; Cheng et al., 2015; Wagle et al., 2015). The cancer can be found on the bone surface, within the bone, or in extraosseous sites, including the lung (Klein and Siegal, 2006). The incidence rate in all people has been reported to be 6 - 8/1 million (Friebele et al., 2015; Bielack et al., 2016; Chen et al., 2017; Liu et al., 2017; Taran et al., 2017), accounting for 2.4% of all malignancies and 60% of all malignant bone tumors. Despite intensive application of combined neoadjuvant chemotherapy and surgery, only 30% patients with metastatic OS achieve a 5-year event free survival (Anderson, 2016). Therefore, it is necessary to explore the pathogenesis of osteosarcoma further and to develop novel effective drugs with reduced side effects for these patients.

Diallyl trisulfide (DATS), one of the main active compounds derived from allium vegetables, has a variety of biological effects including lipid lowering, stimulation of the immune system, antiaging, reduction in the risk of cardiovascular disease and diabetes (Rivlin, 2001). Meanwhile, considerable research and epidemiologic studies have revealed that DATS has exhibited strong anticancer effects, it is widely used as an anticancer and chemopreventive agent for certain tumors. It can induce apoptosis of multiple cancer cells, such as gastric, colon, breast and prostate cancer (Lai et al., 2015; Zhang et al., 2015b). Studies also indicated that DATS inhibits cell migration and invasion by downregulating matrix metalloproteinases (MMPs) (Shin et
al., 2013; Liu et al., 2015). In addition, several studies suggested that reactive oxygen species (ROS) plays an important role in DATS-induced cancer cell death (Chandra-Kuntal et al., 2013). However, the effects of DATS on OS and the underlying mechanism are largely unknown. A previous study demonstrated that DATS reversed drug resistance and lowered the ratio of CD133+ cells in conjunction with methotrexate in OS cells (Li et al., 2009; Zhang et al., 2009). Studies also found that DATS treatment concomitantly attenuated Notch-1 expression and upregulated miR-34a in OS cells (Li et al., 2013). Recently, various studies have found that DATS-induced apoptosis involves the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways in bladder cancer cells (Shin et al., 2014). PI3K/Akt signaling is a frequently hyperactivated pathway in cancer and is important for tumor cell growth and survival. Zhou et al. confirmed that the PI3K/Akt signaling pathway is deregulated in the vast majority of localized disease and 100% of advanced-stage disease in OS (Zhou et al., 2014). It implies that alterations in this pathway may be a prerequisite for OS progression.

In this study, we hypothesized that DATS can inhibit the proliferation and invasiveness of OS cells by inactivating the PI3K/AKT/GSK3β signaling pathway. We attempted to characterize the anti-tumor activities of DATS and to find the potential of DATS for clinical applications in OS.
Materials and Methods

DATS and Cell line

DATS (98% pure) was purchased from Sigma (St Louis, MO, USA). The human OS cell line U2OS was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS), 1% penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA) at 37 °C in a humidified atmosphere containing 5% CO2.

MTT assay

U2OS cell proliferation was measured by MTT assay. Briefly, The U2OS cells (2×10³) were seeded in 96-well plate then treated with different concentrations of DATS (0, 25, 50 and 100 μmol/l) for 24 h, and with 50 μmol/l DATS for different time periods (0, 24, 48 and 72h) (Xiao et al., 2004, Na et al., 2012). MTT (10 μL of 5 mg/mL, Sigma) was added to each well and incubated for 4 h. The media were then discarded and 150 μL dimethyl sulfoxide were added to each well to solubilize formazan crystals. Absorbances at 490 nm were measured on a microplate reader.

Flow cytometric analysis of the cell apoptosis

The cells treated with the indicated concentration of DATS (0, 25, 50 and 100 μmol/l)
for 48 h were harvested and washed three times with PBS. Viable cells were double
stained with PI and FITC-conjugated Annexin V (Beyotime Institute of Biotechnology,
Jiangsu, China). The Annexin V FITC was highlighted in green, and PI was
highlighted in red. Apoptosis was analyzed by quadrant statistics.

**Wound-healing assay**

Cells were seeded in $1 \times 10^5$ cells/ml and grown to 80–90% confluence in a 12-well
plate. After starvation for 24 h, monolayers of cells were wounded by manually
scraping with a 10 μl pipette tip. Cells were rinsed twice with PBS and then treated
with DATS (0, 25, 50 and 100 μmol/l) in serum free medium, cells were allowed to
migrate for 24 h. Images were taken at 0 h and 24 h after wounding under the
inverted microscope.

**Cell invasion assay**

For the transwell invasion assay, the cells were cultured in serum free medium for 24
h, and then same numbers of cells ($1 \times 10^5$) were placed on upper chambers (24 well
inserts, pore size 8 µm, BD Biosciences, MA, USA) coated with Matrigel. 20% FBS
was added to the lower chamber as a chemoattractant. The cells were treated with
DATS (0, 25, 50 and 100 μmol/l). After 24 h, the non-invading cells and the EC matrix
were gently removed with a cotton swab. The invasive cells located on the lower side
of the chamber were stained with 0.1% Crystal Violet, air-dried and imaged. Cell
migration was quantified by direct microscopic visualization and counting. The values for invasion were obtained by counting three fields per membrane, and the results are presented as the average of six independent experiments performed over multiple days.

**Western blot analysis**

After treating with DATS for 24 h, the proteins were isolated using cell lysis buffer. Protein concentrations were determined using a BCA protein assay kit (Pierce, USA). 30 μg of protein samples were run on a 10% SDS-polyacrylamide gel, and then transferred to PVDF transfer membranes (Millipore, Billerica, Massachusetts, USA). Membranes were blocked in blocking buffer for 1 h at room temperature, followed by incubating overnight at 4°C with primary antibodies. Antibodies against AKT, p-AKT, GSK3β, p-GSK3β, Bcl-2, Bad, Bax, E-cadherin, β-catenin, Vimentin and β-actin were purchased from Cell Signaling Technology (Supplement table 1). Then, membranes were incubated for 1 h at room temperature with the secondary antibodies anti-mouse IgG or anti-rabbit IgG. The immunoreactive proteins were detected using an ECL detection reagent and were captured on X-ray film. β-actin was used as a control for protein loading. Densitometric analyses of Western blots were analyzed by ImageJ (Public domain, BSD-2, NIH, USA).
Xenograft Tumor Model and Lung Metastasis Model in Vivo

Male BALB/c nude mice were obtained from Center for Animal Experiment of Wuhan University. All animals were maintained in standard vinyl cages with air filter tops in a filtered laminar air flow room at 25°C on a 12-hr light/dark cycle. Water and food were autoclaved and provided for all animals. Mice were injected subcutaneously (s.c.) into the right flank with $4 \times 10^6$ U2OS cells in 0.1 ml DMEM. When the xenografts solid tumors reached a volume of about 100 mm$^3$, tumor-bearing mice were randomly divided into 4 groups with 3 mice each group. Each mouse received either 100 μl of control vehicle (olive oil), or DATS (25, 50 and 100 mg/kg) by oral administration daily for 4 weeks. All the mice were sacrificed three days after the last injection and the xenograft tumors were observed and measured. The lung metastasis model was established by injecting $5 \times 10^6$ U2OS cells into the BALB/c nude mice via the tail vein. Treatments were administered 5 days a week, for 3 weeks, starting from day 15 of tumor implantation. After 6 weeks, the lungs were excised under anesthesia and the numbers of macroscopically visible lung metastatic nodules were counted and validated by assessment of hematoxylin and eosin (HE) -stained sections by microscopy. All experiments were approved by the First Affiliated Hospital Ethics Committee of South China University.

Statistical analyses

The data from these experiments were assessed using SPSS 22.0. Differences
between groups were tested by Student’s t-test or one-way ANOVA. Values of p < 0.05 were considered to be statistically significant.

Results

DATS inhibits growth of U2OS cells

U2OS cell proliferation was assessed using MTT assay to determine the effect of DATS on osteosarcoma cell growth. U2OS cells were treated with 25, 50 or 100 μmol/l of DATS for 24 h. The results demonstrated that all three groups have an inhibitory effect on cell proliferation compared to control group (Figure 1A). After treating with 50 μmol/l of DATS for up to 3 days, U2OS cells showed a cumulative cell growth inhibition (Figure 1B). These results suggested that the inhibition of cell growth by DATS was found to be dose- and time- dependent in U2OS cells.

DATS causes a redistribution of the cell cycle and induced cell apoptosis

To further understand the mechanism by which DATS exerted these growth-regulatory effects, cells treated with different concentrations of DATS for 48 h were stained with PI and analyzed by flow cytometry. Challenging U2OS cells with rising concentrations of DATS (25, 50 and 100 μmol/l) dose dependently arrested U2OS cells in the G0/G1 phase of the cell cycle, thereby decreasing the proportion of cells in the S phase (Figure 2A and 2B).
To determine whether the growth inhibitory effect of DATS is associated with cell apoptosis, Annexin V- PI double staining of U2OS cells followed flow cytometry were performed. The total apoptotic rates were 11.5%±1.1%, 18.6%±1.7% and 32.6%±2.09% at DATS concentrations of 25, 50 and 100 μmol/l respectively (Figure 2C and 2D). These results demonstrated that the inhibition of cell growth by DATS was caused by the induction of apoptosis.

**DATS inhibits migration and invasion of U2OS cells**

To provide further support to the effects of DATS on the migration and invasion of U2OS, the wound-healing assay was performed by scratching the cell layer. The migration assay showed that DATS strongly inhibited cell migration activity when U2OS cells were treated with 25, 50, and 100 μmol/l DATS for 24h, respectively (Figure 3A and 3B).

Moreover, the invasion assay demonstrated that the number of cells invading the lower chamber was significantly reduced by DATS compared to control group (Figure 3C). The percentage of invasion relative to control was 84 ± 8.31, 71 ± 5.11, and 42 ± 3.12% (Figure 3D) when cells were exposed to 25, 50, and 100 μmol/l DATS for 24 h, respectively. These results indicated that DATS is an important regulator of migration and invasion in U2OS cells.
DATS regulates cell proliferation and metastasis via the AKT signaling pathway.

The PI3K/AKT pathway is crucial for many aspects of cell growth and survival. We further investigated whether DATS regulates the PI3K/AKT/GSK3β pathway. After treating with DATS on U2OS cells, the protein expression levels of p-AKT and its downstream target p-GSK3β (Figure 4A) were all decreased. These results indicated that DATS is a negative regulator of PI3K/AKT/GSK3β pathways.

We also measured the protein expression levels of the pro-apoptotic proteins Bax and Bad, and the anti-apoptosis protein Bcl-2. As shown in Figure 4B, the expression levels of Bax and Bad were increased at DATS concentrations of 25, 50 and 100μmol/l, and Bcl-2 was decreased when compared with control group.

As epithelial-mesenchymal transition (EMT) has been accepted as a potential mechanism underlying cancer metastasis and invasion, we explored the role of DATS in regulating EMT in U2OS cells. Western Blot analyses demonstrated that DATS was able to decrease the protein expression of Vimentin and β-catenin (mesenchymal marker) and increase epithelial markers (E-cadherin) in U2OS cells (Figure 4C).

DATS regulates cell proliferation and metastasis in mice xenograft model

On the basis of our in-vitro studies, we further examined the in-vivo anti-cancer activities of DATS in a BALB/c nude mouse U2OS xenograft model. As shown in Figure 5A, DATS (25, 50 and 100mg/kg) reduced tumor volume compared to control
groups. Representative tumor weight in the U2OS xenograft mice treated with or without DATS are shown in Figure 5B. DATS significantly decreased the tumor weight compared to control groups. Our results suggested that DATS causes anti-tumor activities in vivo.

To establish a metastatic model of OS cells, the U2OS were injected into nude mice via tail vein. The HE staining showed that the control group of OS cells metastasized in the lung, while the lung metastasis cells were significantly reduced after DATS treatment (Figure 5C). Statistic analysis showed that the visible tumor nodules were significantly reduced after DATS treatment (Figure 5D). Taken together, these results demonstrated that DATS decreased the invasion and metastasis of OS in mice model.

Discussion

OS continues to be a challenging cancer to treat, our ability to target cellular mediators involved in proliferation and invasion was impaired due to lack of understanding of those mediators. Nature is the best source of drugs, plants have been primary sources of natural product drug discovery. Accumulating evidence found that DATS could exert its antitumor effect in a variety of malignancies including prostate, stomach, lung, breast, cervix and colon cancer (Shukla and Kalra, 2007; Powolny and Singh, 2008; Seki et al., 2008), and it can suppress the proliferation in human prostate cancer cells by inducing apoptosis and cell cycle arrest (Xiao et al.,
2004; Xiao et al., 2005); however, the anti-metastatic function and potential mechanisms of DATS are not fully understood in human OS cancer cells. In this study, we demonstrated that DATS effectively inhibited proliferation and accelerated apoptosis in U2OS cells. Moreover, our results indicated that DATS inhibited OS cell migration and invasion.

The AKT signaling pathway is involved in various physiological and pathological processes including cell proliferation, migration, invasion and apoptosis in all kinds of cancers (Shen et al., 2014; Daniele et al., 2015; Zhou et al., 2015; Zhang et al., 2016). Evidence also proved that the AKT signaling pathway plays a very important role in OS (Zhang et al., 2015a). Abnormal activation of AKT signaling contributes to oncogenesis by affecting the regulation of multiple downstream molecules (Chen et al., 2013; Cui et al., 2014). The state of cell survival or apoptosis is determined by the pro-apoptotic molecule Bax, Bad and the anti-apoptotic molecule Bcl-2, which are important regulators in the AKT signaling pathway. To elucidate the molecular mechanisms of proliferation and aggressiveness inhibition by DATS, we investigated the activity of the AKT signaling pathway. Our results demonstrated that after treating with DATS, the protein expression level of Bcl-2 was significantly decreased, while Bax and Bad were increased, which indicated that DATS affects apoptosis in OS cells by regulating the protein expression of apoptosis related genes. Other researchers also proved traditional Chinese herbs can suppress proliferation and induce apoptosis by altering the ratio of Bax/Bcl-2 (Yu et al., 2015; Yao et al., 2017; Wan and Ouyang, 2018). However, this is the first time DATS has been confirmed to
suppress proliferation and induce apoptosis by decreasing the expression Bcl-2 and increasing the expression of Bax and Bad.

Growing evidence also suggested that activation of PI3K/AKT promotes EMT (Grille et al., 2003). GSK-3β, a downstream target of the PI3K/AKT signaling pathway, is necessary for maintenance of epithelial architecture. Functional inactivation and phosphorylation of GSK-3β is important for typical molecular changes during EMT (Lee et al., 2006; Tang et al., 2019). We detected the expression of P-GSK-3β, mesenchymal markers (vimentin and β-catenin) and epithelial markers (E-cadherin) after treating with DATS. Our results show that DATS can downregulate the expression level of P-GSK-3β. Meantime, DATS decreased expression of vimentin and β-catenin, while it increased expression of E-cadherin in U2OS cells. Accordingly, we propose that DATS inhibits the proliferation, migration and invasion of osteosarcoma cells via inactivation of the PI3K/AKT/GSK3β pathway.

Collectively, we presented the mechanism of the anti-metastatic effect of DATS in U2OS cells, which strongly suggests that DATS inhibits OS development and progression through the regulation of PI3K/AKT/GSK3β signaling pathways, accompanied by downregulation of Bcl-2, Vimentin and β-catenin, as well as upregulation of Bad, Bax and E-cadherin. Therefore, our data demonstrated that DATS exerted its anticancer effects by inhibiting cell proliferation, cell migration and invasion. These results provide evidence for the use of the natural product DATS either alone or in combination with standard therapy for OS.
Acknowledgments

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Conflict of interest

The authors declare that they have no conflict of interest.

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**Fig. 1.** The effect of DATS on cell proliferation. Values are given as a percentage of untreated control cells. The data are presented as the averages for triplicate results from a representative experiment; bars, SD. (A) The dose-dependent effect of DATS on U2OS cell proliferation. (B) The time-dependent effect of DATS on U2OS cell proliferation.

**Fig. 2.** The effect of DATS on cell cycle arrest and cell apoptosis. Cell cycle distribution of U2OS cells in the three phases of the cell cycle are represented by representative pictures (A) and percentages (B) under these treatment conditions. (C) The ratio of apoptotic cells was measured in U2OS cells treated with DATS, which are represented by representative pictures. (D) The percentage ratio of apoptotic cells was measured in U2OS cells treated with DATS. The data are presented as the mean ± SD (N=3, ** p<0.01).

**Fig. 3.** The effect of DATS on cell migration and invasion. (A) Wound healing assay. Upper panel: DATS groups at 0h points. The wound edge’s changes are illustrated with broken lines. Bottom panel: DATS groups at 24 h points. Original magnifications, × 100. Scale bar, 400 μm. (B) Wound closures were measured in U2OS cells treated with DATS. Wound closure data obtained after calculation in ImageJ. (C) The invasive cells per field were measured in U2OS cells treated with DATS, which are represented by representative pictures. Original magnifications, × 100. Scale bar, 400 μm. (C) The invasive cells per field were measured in U2OS cells treated with DATS. Data are presented as percentage of mean ± SD (N=3, ** p<0.01) relative to
control.

**Fig. 4.** The effect of DATS on AKT signaling pathway in U2OS cells. U2OS cells were treated with DATS. The cells were harvested, and the total proteins were extracted. (A) The effect of DATS on the protein levels of AKT, p-AKT and GSK3β, p-GSK3β was assessed by Western blot. Lane 1: Control; Lane 2: 25 μmol/l DATS; Lane 3: 50 μmol/l DATS; Lane 4: 100 μmol/l DATS. (B) The effect of DATS on the protein levels of Bad, Bax and Bcl-2 was assessed by Western blot. Lane 1: Control; Lane 2: 25 μmol/l DATS; Lane 3: 50 μmol/l DATS; Lane 4: 100 μmol/l DATS. (C) The effect of DATS on the protein levels of E-cadherin, Vimentin and β-catenin was assessed by Western blot. Lane 1: Control; Lane 2: 25 μmol/l DATS; Lane 3: 50 μmol/l DATS; Lane 4: 100 μmol/l DATS.

**Fig. 5.** DATS affected U2OS tumor in BALB/c nude mouse xenograft model *in vivo.* The U2OS cell xenografted mice (3 mice per group) were either untreated or treated with DATS (See Materials and methods for detail). (A) Xenograft models (3 mice per group) were established. (B) Summary of tumor weights. (C) Representative images of lung metastatic nodules and HE-stained sections. Black arrow indicates metastatic nodules. Original magnifications, × 40. Scale bar, 50 μm. (D) The number of metastatic nodules was quantified. **P < 0.01
**SUPPLEMENTARY INFORMATION**

**Table 1.** Detailed description of antibodies used for western blot.

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<td>GSK3β</td>
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