

Reversion of P-glycoprotein-mediated multidrug resistance by diallyl trisulfide in a human osteosarcoma cell line

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Abstract. Diallyl trisulfide (DATS), the main sulfuric compound in garlic, has been shown to have antitumor effects. The present study aimed to ascertain whether DATS reverses the drug resistance of human osteosarcoma cells *in vitro* and to investigate its potential mechanisms. Human osteosarcoma U2-OS cells were treated with different concentrations of DATS. Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, while P-glycoprotein (P-gp) expression and the proportion of apoptotic cells were measured by flow cytometry. Morphological changes were observed under an optical microscope. Nuclear factor- κ B (NF- κ B) and inhibitor of NF- κ B (I κ B) activities were measured by PCR and western blot analysis. Results showed that the proliferation of U2-OS cells treated with different concentrations of DATS was significantly decreased in a concentration- and time-dependent manner. DATS increased the toxic effect of adriamycin on U2-OS cells. Moreover, P-gp expression was decreased and the apoptosis rate was increased in a concentration-dependent manner following treatment of DATS. Additionally, NF- κ B activity was inhibited by DATS while expression of I κ B was increased. Our data clearly suggest that DATS has significant anticancer effects on human osteosarcoma cells. The potential mechanisms include reducing the multidrug resistance and inducing apoptosis. NF- κ B suppression may be involved in DATS-induced inhibition of cell proliferation.

Introduction

Osteosarcoma is the most common primary bone tumor in children and adolescents, with a 5-year disease-free survival rate of 70%. The clinical features of osteosarcoma include its strong local infiltration and rapid metastasis to the lungs. Neoadjuvant chemotherapy accompanied by large doses of doxorubicin (DXR) has greatly improved the survival rate, yet 20-40% of the patients still die of metastasis and recurrence. The primary cause of treatment failure is the resistance of tumor cells to chemotherapeutic medicine. DXR is commonly used in clinical chemotherapy and is important in the chemotherapeutic treatment of osteosarcomas. However, clinical practice has shown that drug resistance to DXR is easily acquired. The clinical applications of anthracyclines such as adriamycin (ADM) have been limited by their unacceptable toxicity to the heart at the required treatment doses.

Multidrug resistance (MDR) is mainly attributed to the increased drug efflux mediated by the P-glycoprotein (P-gp) product of the multidrug resistance protein 1 (MDR1) gene. P-gp is a 170-kDa ATP-dependent transmembrane transporter that acts as a drug efflux pump to decrease intracellular drug accumulation and consequently reduce intracellular drug efficacy (1,2). Compounds such as verapamil have been reported to overcome MDR *in vitro* by decreasing the expression of MDR1 (3). However, these compounds have side effects that hinder their clinical applications. Therefore, low toxicity and high activity reversal agents are needed to be identified.

Recently, several researchers have focused on screening for natural product-derived drugs to reverse MDR (4-6). Epidemiologic data support the premise that the dietary intake of *Allium* vegetables, including garlic, may protect against the risk of various malignancies (7,8). Diallyl trisulfide (DATS) is the main sulfur-containing compound in garlic. Engdal *et al* (9) proposed that garlic compounds can inhibit the expression of P-gp *in vitro* and *in vivo*. The capacity of DATS to reverse the drug resistance of osteosarcoma cells is still unknown. Similarly, the signaling pathway for the inhibition of osteosarcoma cell proliferation by DATS remains unclear.

In the present study, we aimed to determine the effect of DATS on the reversal of drug resistance in human osteosarcoma cells *in vitro* and to investigate its potential mechanisms

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Table I. Primer sequences used for PCR analysis.

Genes		Primer (5'-3')	Product length (bp)
β -actin	Forward	5'-GTGGGGCGCCCCAGGCACCA-3'	540
	Reverse	5'-CTCCTTAATGTCACGCACGATTTTC-3'	
NF- κ B/p65	Forward	5'-TGCACCTAGCTGCCAAAGAAGGA-3'	293
	Reverse	5'-TCTGCTCCTGCTGCTTTGAGAA-3'	
I κ B α	Forward	5'-GCAGAGGATTACGAGCAGAT-3'	634
	Reverse	5'-CCTGGTAGGTTACTCTGTTG-3'	

of action. The study also explored whether the suppression of the nuclear factor κ light-chain enhancer of activated B cells (NF- κ B) is involved in the DATS-induced inhibition of osteosarcoma cell proliferation.

Materials and methods

Cell culture and experimental reagents. Human osteosarcoma U2-OS cells (The Institute of Basic Medicine of the Shandong Academy of Medical Sciences, China) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Los Angeles, CA, USA) supplemented with 10% (v/v) heat-inactivated newborn calf serum (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., China) and 100 U/ml penicillin and 100 μ g/ml streptomycin in a 5% CO₂ atmosphere at 37°C. DATS was purchased from Shandong Lukang Xin Chen Pharmaceutical Co. (Shandong, China), while ADM was obtained from Sigma (St. Louis, MO, USA). Annexin V-FITC/propidium iodide (PI) were purchased from JingMei Co. (Shanghai, China). Primary antibodies for P-gp, NF- κ B and I κ B α were obtained from Cell Signaling Technology (Beverly, MA, USA).

In vitro drug sensitivity assay. U2-OS cells were seeded at 1×10^4 cells/ml per well into a 96-well plate. After 24 h, the medium was replaced with DMEM supplemented with DATS at a dose of 10, 50 and 100 μ M. U2-OS cells were incubated in drug-free culture media as the blank controls. After treatment for 20, 44 and 68 h, 20 μ l 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added into each well, and the cells were further incubated for 4 h. The medium was then removed, and 150 μ l DMSO was added to each well to dissolve the formazan crystals. The absorbance (A) of each sample was measured using a spectrophotometer at a wavelength of 570 nm. IC₅₀ was calculated according to the results of the MTT assay. Inhibition of cell viability = [1 - (average A value of the experimental group/average A value of the control group)] \times 100%.

To determine the capacity of DATS to sensitize U2-OS cells to ADM cytotoxicity, U2-OS cells were seeded into 96-well culture plates at a density of 1×10^4 cells/ml and incubated for 24 h. ADM was added to a final concentration of 1 μ g/ml. The experimental group was treated combined with 10 μ M DATS for 24, 48 and 72 h. The U2-OS cells incubated in DMEM supplemented with ADM alone were used as controls.

Expression of P-gp by flow cytometry (FCM). The U2-OS cells were cocultured with 10, 50 and 100 μ M DATS for 48 h. The cells were then washed twice with PBS and suspended in phosphate-buffered saline (PBS). The cell suspensions were incubated with phycoerythrin-conjugated UIC2, mouse anti-human P-gp monoclonal antibody (P-gp-PE), and the homotype control IgG2a-PE. The mixture was incubated at room temperature and away from light for 30 min, washed twice, and then detected by FCM. The results were analyzed using Cell Quest software (BD Pharmingen Co., USA).

Morphological changes by light microscopy. U2-OS cells were grown in complete DMEM for 24 h on 24-well plates that had a coverslip at the bottom of each well. When a cell density of $\sim 1 \times 10^4$ cells/ml was reached, the cells were treated with either 50 μ M DATS alone or 10 μ M DATS combined with 1 μ g/ml ADM and incubated for another 48 h. Treatment with 1 μ g/ml ADM alone was applied to the control group. The coverslips were removed from each well, and stained with hematoxylin and eosin (H&E). The contents of the coverslips were fixed in 95% ethanol for 20 min, and then a series of washing steps was performed. The coverslips were air-dried, mounted on slides with neutral gum, and observed under a light microscope.

Apoptosis assay by statistical FCM. After incubation at 37°C for 24 h in DMEM with the different drug doses (i.e., 10, 50 and 100 μ M DATS or 10 μ M DATS with 1 μ g/ml ADM or 1 μ g/ml ADM alone), the cell suspensions were washed twice with PBS and centrifuged at 550 \times g for 5 min. The cells were resuspended in 500 μ l of binding buffer, 5 μ l of Annexin V-FITC, and 10 μ l of PI (20 μ g/ml). The samples were then incubated at room temperature for 15 min in the dark. The fluorescence intensities of the samples were measured by a flow cytometer with the FACS software (BD Pharmingen Co.).

Semi-quantitative RT-PCR assay. The total mRNA was extracted from the cells with the TRIzol reagent (Invitrogen Co., Carlsbad, CA, USA) according to the manufacturer's instructions. Single-stranded cDNA was synthesized by the reverse transcription of 1 μ g total RNA using RNase-free M-MLV reverse transcriptase (Invitrogen Co.) and the oligo-dT primer. Amplification was carried out in a thermal cycler. The following cycling conditions were used for each PCR run: initial denaturation at 94°C for 1 min, followed by 26 cycles of 58°C for 1 min and 72°C for 1 min, and then the final

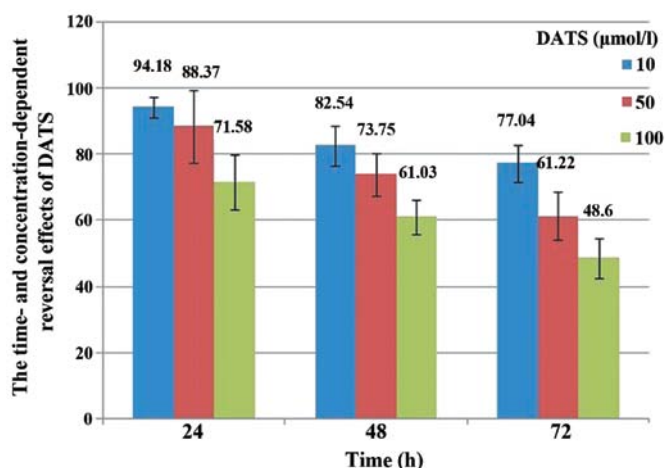


Figure 1. The time- and concentration-dependent reversal effects of DATS. The MTT assay was used to determine the cytotoxic effect of DATS. U2-OS cells were treated with DATS at a dose of 10, 50 and 100 μM for varying time intervals (24, 48 and 72 h). DATS, diallyl trisulfide; U2-OS, human osteosarcoma cells.

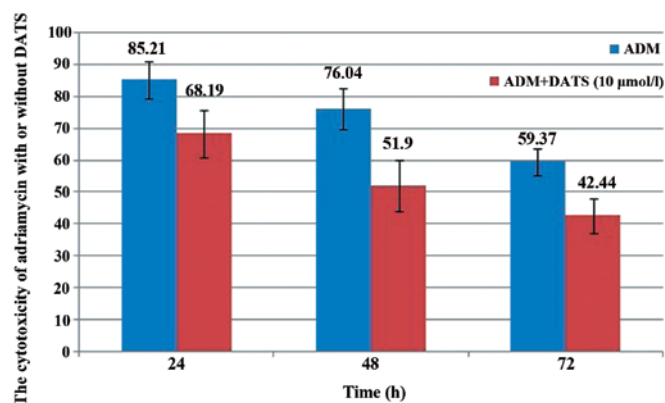


Figure 2. The cytotoxicity of adriamycin with or without DATS. The MTT assay was used to study the ability of DATS to enhance ADM cytotoxicity. U2-OS cells were treated with 10 μM DATS and 1 $\mu\text{g/ml}$ ADM for 24, 48 and 72 h. U2-OS cells treated with 1 $\mu\text{g/ml}$ ADM alone were used as controls. DATS, diallyl trisulfide; ADM, adriamycin; U2-OS, human osteosarcoma cells.

extension at 72°C for 7 min. The PCR products were separated by electrophoresis on 1.5% agarose gels. The products were further analyzed using the UVP bioimaging system (UVP, Upland, CA, USA), with β -actin as the internal reference. The PCR primers are listed in Table I.

Protein expression by western blot analysis. The total protein content was isolated and subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The blots were incubated overnight at 4°C with the rabbit anti-human NF- κB (p65) or the mouse anti-human I $\kappa\text{B}\alpha$ primary antibodies (1:1,000 dilution), and then further incubated for 1 h at room temperature with the HRP-conjugated goat anti-rabbit IgG secondary antibody (1:5,000 dilution). The fluorescent signals were detected with an ECL western blotting detection kit (Zhongshan Co., Beijing, China). After normalization according to the corresponding level of β -actin expression, the

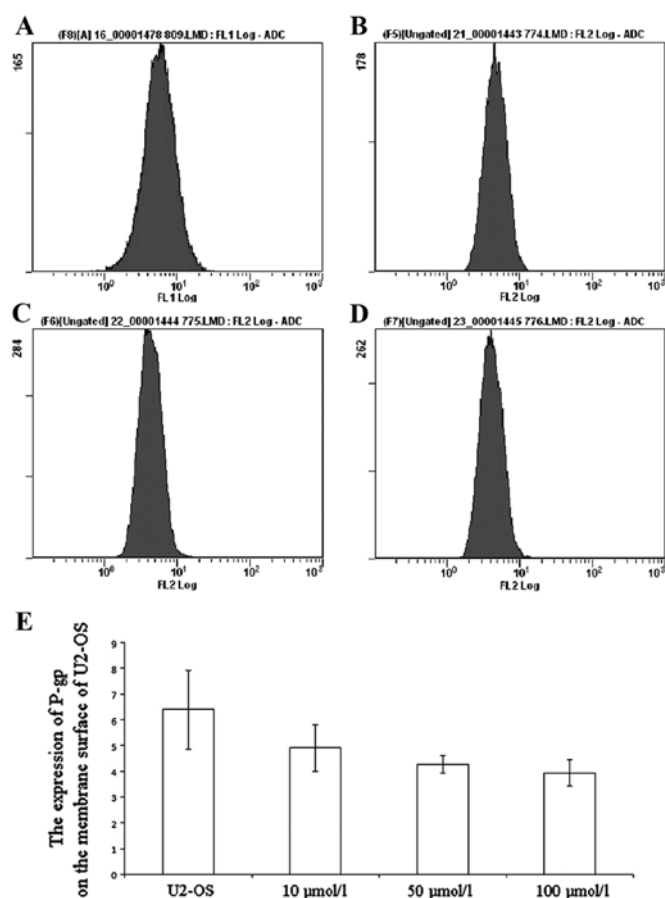


Figure 3. The expression of P-gp on the membrane surface of U2-OS cells. The DATS-treated U2-OS cells were incubated with phycoerythrin-conjugated UIC2 and were then detected by FCM. (A) U2-OS cells; (B) U2-OS cells + DATS (10 μM); (C) U2-OS cells + DATS (50 μM); (D) U2-OS cells + DATS (100 μM). (E) Results are plotted as the relative expression of P-gp in each group. Data are presented as means \pm SD of three independent experiments. P-gp, P-glycoprotein; U2-OS, human osteosarcoma cells; DATS, diallyl trisulfide; FCM, flow cytometry.

protein expression levels were determined by densitometry scans and calculated using Quantity One software (Bio-Rad Co., USA).

Statistical analysis. The results are expressed as the means \pm standard deviation (SD) of three independent experiments. The Student's t-test was used for the statistical analyses, and $P < 0.05$ was considered to be significant. Statistical calculations were carried out using the Student's t-test with SPSS 13.0 for Windows software package.

Results

Drug sensitivity. We determined the cytotoxic effect of DATS on osteosarcoma cells by MTT assay. Treatment of U2-OS cells with DATS resulted in the inhibition of cell viability in a dose- and time-dependent manner (Fig. 1). As shown in Fig. 2, the survival rate of the U2-OS cells significantly decreased to 68.19, 51.9 and 42.44% at 24, 48 and 72 h, respectively, after treatment using 10 μM DATS with 1 $\mu\text{g/ml}$ ADM as compared with treatment using ADM alone (85.21, 76.04 and 59.37% at 24, 48 and 72 h, respectively; $P < 0.05$).

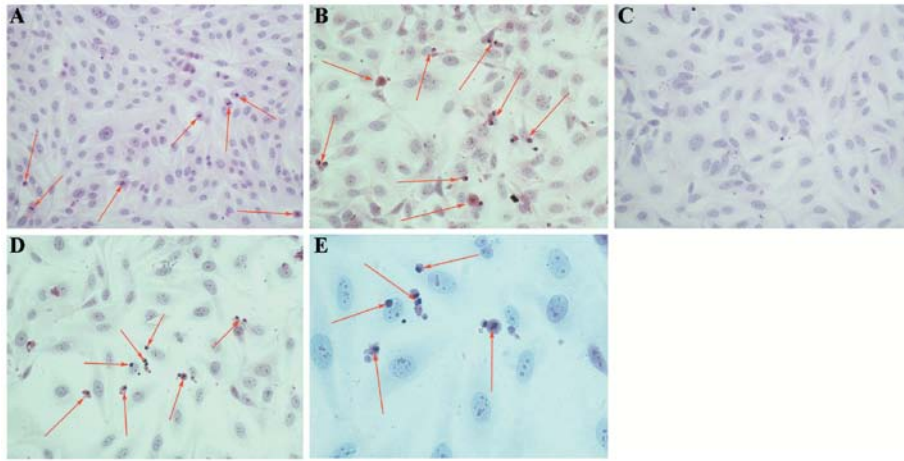


Figure 4. Observation of morphologic changes by light microscopy. (A) U2-OS cells grown for 48 h without drugs (x200 magnification). Cells proliferated actively; mitotic cells were easily observed (shown with †). (B) U2-OS cells grown for 48 h with DATS (50 μ M) (x200 magnification). Proliferation of U2-OS cells slowed down, apoptotic bodies were easily observed (shown with †). (C) U2-OS cells grown for 48 h with adriamycin (1 μ g/ml) alone (x200 magnification). Proliferation of U2-OS cells slowed down slightly; apoptotic bodies were rarely noted. (D) U2-OS cells grown for 48 h with adriamycin (1 μ g/ml) and DATS (10 μ M) (x200 magnification). U2-OS cells showed cell shrinkage, chromatin condensation, margination, nuclear fragmentation; apoptotic bodies were easily observed (shown with †). (E) U2-OS cells grown for 48 h with adriamycin (1 μ g/ml) and DATS (10 μ M) (x400 magnification). Apoptotic morphology was more clearly observed (shown with †). U2-OS, human osteosarcoma cells; DATS, diallyl trisulfide.

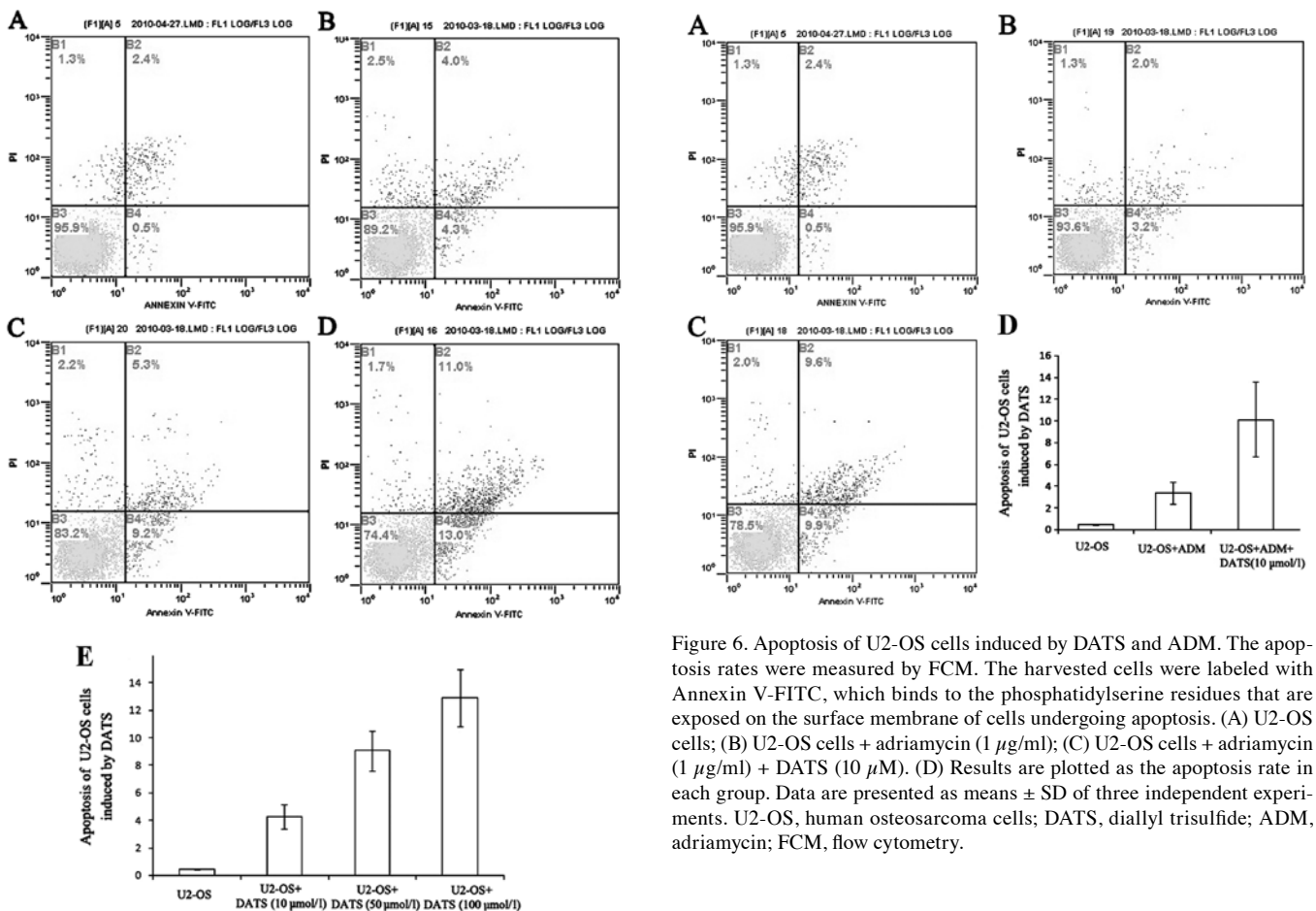


Figure 5. Apoptosis of U2-OS cells induced by DATS. The apoptosis rates were measured by FCM. The harvested cells were labeled with Annexin V-FITC, which binds to the phosphatidylserine residues that are exposed on the surface membrane of cells undergoing apoptosis. (A) U2-OS cells; (B) U2-OS cells + DATS (10 μ M); (C) U2-OS cells + DATS (50 μ M); (D) U2-OS cells + DATS (100 μ M). (E) Results are plotted as the apoptosis rate in each group. Data are presented as means \pm SD of three independent experiments. U2-OS, human osteosarcoma cells; DATS, diallyl trisulfide; FCM, flow cytometry.

Figure 6. Apoptosis of U2-OS cells induced by DATS and ADM. The apoptosis rates were measured by FCM. The harvested cells were labeled with Annexin V-FITC, which binds to the phosphatidylserine residues that are exposed on the surface membrane of cells undergoing apoptosis. (A) U2-OS cells; (B) U2-OS cells + adriamycin (1 μ g/ml); (C) U2-OS cells + adriamycin (1 μ g/ml) + DATS (10 μ M). (D) Results are plotted as the apoptosis rate in each group. Data are presented as means \pm SD of three independent experiments. U2-OS, human osteosarcoma cells; DATS, diallyl trisulfide; ADM, adriamycin; FCM, flow cytometry.

Alteration of P-gp expression. The DATS-treated U2-OS cells were incubated with phycoerythrin-conjugated UIC2 and then P-gp expression was detected by FCM. After treatment with different concentrations of DATS (10, 50 and 100 μ M), the P-gp expression in the U2-OS cells was significantly decreased to 4.91, 4.28 and 3.94, as compared with the untreated group (6.4; $P < 0.01$; Fig. 3).

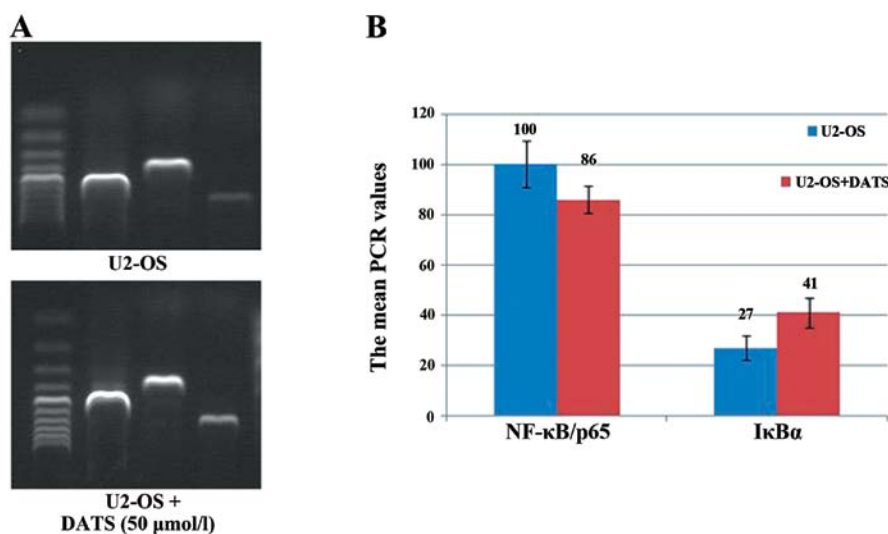


Figure 7. The mRNA expression of NF-κB/p65 and IκBα genes. The expression levels of NF-κB/p65 and IκBα in the U2-OS cells treated with 50 μM of DATS were detected by semi-quantitative RT-PCR. The mean PCR values of the ratio were relative to the β-actin gene. NF-κB, nuclear factor-κB; DATS, diallyl trisulfide; U2-OS, human osteosarcoma cells.

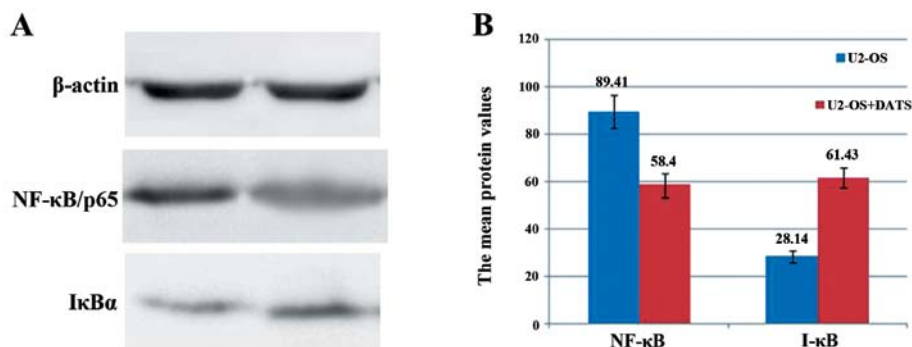


Figure 8. The protein expression of NF-κB/p65 and IκBα. The expression levels of NF-κB/p65 and IκBα in the U2-OS cells treated with 50 μM of DATS were detected by western blotting. The mean protein values of the ratio were relative to the β-actin. NF-κB, nuclear factor-κB; DATS, diallyl trisulfide; U2-OS, human osteosarcoma cells.

Apoptosis as observed by light microscopy. The untreated U2-OS cells proliferated actively, and had the typical morphological characteristics of malignant cells, such as uneven cell size, deeply stained nucleus, reduced cytoplasm and an enlarged nucleolus (Fig. 4A). After treatment with 50 μM DATS, proliferation of the U2-OS cells was reduced, and the cells shrank into rounded shapes with abundant cytoplasm and vacuoles, chromatin condensation and margination, nuclear fragmentation, apoptotic bodies, as well as other typical apoptotic cytomorphological features (Fig. 4B). However, the proliferation of the U2-OS cells treated with ADM (1 μg/ml) alone was slightly inhibited and apoptotic cells were rare (Fig. 4C). As shown in Fig. 4D and E, the apoptotic cytomorphological features appeared at 24 h after simultaneous treatment with ADM (1 μg/ml) and DATS (10 μM).

Apoptosis assay by statistical flow cytometry. Annexin V and PI were used to further discriminate apoptotic from necrotic cell death in the cell cycle. The percentages of early apoptotic cells were significantly increased at 24 h after treatment with 10, 50 and 100 μM of DATS as compared with that of the controls ($P < 0.01$; Fig. 5). Moreover, the percentage of apop-

totic U2-OS cells treated with both ADM and DATS was much higher than the percentage of apoptotic cells treated with ADM alone ($P < 0.01$; Fig. 6).

Detection of NF-κB and IκB expression. As demonstrated in Fig. 7, semi-quantitative RT-PCR revealed the gray value of NF-κB/p65 expression to be 100 ± 9.21 . After treatment with 50 μM DATS, an evident decrease in the NF-κB/p65 levels was observed (86 ± 5.57 , $P < 0.05$). Conversely, the IκBα mRNA expression in the U2-OS cells treatment with 50 μM DATS was increased as compared with that of the control group (27 ± 4.80 vs. 41 ± 5.94 , $P < 0.05$).

As shown in Fig. 8, western blot analysis revealed that the NF-κB/p65 protein expression was decreased by DATS (50 μM) in the U2-OS cells from 89.41 ± 6.98 to 58.40 ± 5.03 , whereas the IκBα protein expression was increased from 28.14 ± 2.58 to 61.43 ± 4.22 ($P < 0.05$).

Discussion

Alliin is the general term that refers to the main bioactive ingredient of garlic, which is actually a complex variety

of allyl organic sulfides that include the diallyl disulfides (20-50%) and the diallyl sulfides (DATS, 50-80%). A large number of studies (10-15) have confirmed that allicin functions against infection as well as it prevents and treats acute and chronic liver injury, atherosclerosis, reperfusion injury and hypoglycemia. DATS also lowers total cholesterol and blood pressure, inhibits platelet activity and regulates immune functions. Since the 1980s, research on the anticancer effects of allicin has increasingly attracted the interest of researchers. The *in vitro* and *in vivo* preclinical studies (16-23) have implicated DATS as an important mediator of cyclins and cell cycle arrest as well as apoptosis, cell adhesion and angiogenesis. In 2008, Shankar *et al* (23) confirmed that DATS improves the TNF-related apoptosis-inducing ligand (TRAIL) treatment effect in prostate cancer *in vitro* and in orthotopic androgen-independent prostate cancer PC-3 cells. These results implicate DATS as a promising reversal agent of drug resistance.

Our previous study (24) showed that DATS effectively reversed the P-gp-mediated MDR of K562/AO2 cells *in vitro*. Similarly, DATS inhibited the growth of osteosarcoma cell lines *in vitro*. Zhang *et al* (25) found that the expression of 27 proteins was significantly altered in osteosarcoma Saos-2 cells following DATS treatment. The expression levels of 18 proteins were increased, whereas those of 9 proteins were decreased. Approximately half of these proteins (13/27) were relevant to the cell cycle or to apoptosis. However, the capacity of DATS to reverse the drug resistance of osteosarcoma cells remains unknown.

The present study used the MTT assay to detect the survival rate of U2-OS cells after treatment with different DATS concentrations. The survival rates of the U2-OS cells were significantly reduced. The time- and concentration-dependent inhibition effects of DATS on the U2-OS cells were likewise observed. Moreover, the survival rate of U2-OS cells that were treated with a combination of both DATS and ADM was significantly lower than treatment with ADM alone. Subsequent research is needed to confirm the specific mechanism by which DATS inhibits the growth of U2-OS cells.

An extensively characterized MDR mechanism involves the overexpression of P-gp, which reduces the cellular accumulation of cytotoxic drugs in tumor cells. P-gp is a transmembrane glycoprotein that functions as an adenosine triphosphatase energy-dependent transporter (26,27). The drug intake capacity of cells with high *MDR1* gene expression levels is not significantly different as compared with those of sensitive cells, while the drug efflux capacity of the former is significantly increased (28,29). Therefore, the modulation of MDR transporters is a promising approach to overcome MDR. In the present study, FCM was used to detect the P-gp protein expression on the U2-OS cell surface, which subsequently showed whether DATS could reduce P-gp expression. After treatment of the U2-OS cells with different concentrations of DATS, the P-gp expression was decreased. The significant difference between the treated and untreated groups supported the hypothesis that DATS is a candidate for the development of a new MDR reversal agent.

In addition to P-gp overexpression, various other mechanisms could cause MDR. In recent years, studies have shown that apoptosis is closely related to MDR. Several anticancer drugs with different structures and different targets can induce

apoptosis in tumor cells. The mechanisms of apoptosis may probably be involved in MDR. Thus, apoptosis is the common pathway of various drugs. The inhibition of apoptosis makes the tumor cells simultaneously resistant to several drugs, which eventually leads to MDR. The results showed that the apoptosis rate of U2-OS cells was inherently very low but increased after treatment with different concentrations of DATS. The combined ADM and DATS treatment caused a significantly higher apoptosis rate in U2-OS cells as compared with ADM treatment alone. This effect of DATS was supported by observation of the morphology of the treated cells. Thus, the induction of apoptosis by DATS is one of the important mechanisms for inhibiting the proliferation of U2-OS cells and increasing the response of tumor cells to ADM.

The deregulated activation of NF- κ B has been causally linked to the development of several human pathologies, including cancers (30,31). The hyperactivation of NF- κ B signaling contributes to tumorigenicity and chemoresistance. Several other studies have reported that NF- κ B can inhibit apoptosis via various mechanisms (32-34). NF- κ B may participate in tumor chemoresistance, which is mediated by the expression of MDR proteins. As previously confirmed, NF- κ B can increase the expression of *MDR1* genes in tumor cells (35,36). A purified NF- κ B binding sequence (5'-CCTTTCGGGG-3') was identified in the first exon of the *MDR1* promoter region, which confirmed the presence of binding sites of NF- κ B in the *MDR1* gene. It was further confirmed that *MDR1* may be a downstream gene regulated by NF- κ B (37). Furthermore, previous literature has confirmed that anticancer drugs such as DXR can damage tumor cell DNA, which consequently leads to the activation of NF- κ B. The activated NF- κ B then promotes the transcription of *MDR1* via the NF- κ B binding sites. Therefore, if the expression of NF- κ B can be inhibited, the sensitivity of tumor cells to chemotherapy can be increased (38-41). The NF- κ B pathway has been associated with the proliferation and differentiation of osteosarcoma cells (42). However, the molecular mechanisms of NF- κ B in chemoresistance of osteosarcoma still remain poorly understood.

Semi-quantitative RT-PCR and western blot analysis in the present study demonstrated that the NF- κ B level was decreased by DATS treatment in U2-OS cells. Furthermore, the expression levels of I κ B mRNA in U2-OS cells were increased by DATS treatment as compared with those of the control group. Therefore, the inhibition of NF- κ B activation in U2-OS cells may be involved in the reversal of MDR by DATS.

In conclusion, the present study demonstrated that DATS can serve as a novel modulator of MDR *in vitro* by downregulating P-gp expression and inducing the apoptosis of U2-OS cells. For the first time, we demonstrated that DATS blocks NF- κ B activation, which subsequently produces downstream inhibitory effects on the sensitivity to chemotherapy and apoptosis of U2-OS cells. Thus, DATS could be a highly feasible candidate for the development of a new MDR reversal agent.

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