

Diallyl Trisulfide Suppresses Growth of PC-3 Human Prostate Cancer Xenograft *In vivo* in Association with Bax and Bak Induction

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Abstract Purpose: The present study was undertaken to determine the effect of garlic constituent diallyl trisulfide (DATS) on growth of PC-3 human prostate cancer xenograft *in vivo*.

Experimental Design: DATS was given orally (6 μmol , thrice weekly) to male athymic mice s.c. implanted with PC-3 cells. Tumor sections from control and DATS-treated mice were examined for apoptotic bodies by terminal deoxynucleotidyl transferase – mediated dUTP nick end labeling assay. Protein levels of apoptosis and cell cycle regulating proteins in tumor tissues of control and DATS-treated mice were determined by immunoblotting. The effect of DATS treatment on *in vivo* angiogenesis was determined by immunohistochemical analysis of CD31 in tumors.

Results: Oral gavage of DATS significantly retarded growth of PC-3 xenografts in athymic mice without causing weight loss. For instance, 20 days after starting therapy, the average tumor volume in control mice was ~3-fold higher compared with DATS-treated mice. Tumors from DATS-treated mice exhibited a markedly higher count of apoptotic bodies compared with control tumors. Consistent with the results in cultured PC-3 cells, the DATS-mediated suppression of PC-3 xenograft growth correlated with induction of proapoptotic proteins Bax and Bak. Although DATS treatment inhibited migration of cultured PC-3 cells in association with down-regulation of vascular endothelial growth factor receptor-2 protein, formation of new blood vessels was comparable in tumors of control and DATS-treated mice as judged by CD31 immunostaining.

Conclusions: The present study indicates that DATS administration inhibits growth of PC-3 xenografts *in vivo* in association with induction of Bax and Bak.

Epidemiologic studies continue to support the premise that dietary intake of *Allium* vegetables, such as garlic, may reduce the risk of various types of malignancies, including cancer of the prostate (1–4). For example, the risk of prostate cancer was significantly lower in men consuming >10 g/d of total *Allium* vegetables than in men with total *Allium* vegetable intake of <2.2 g/d in a population-based case-control study (4). Laboratory studies indicate that the anticarcinogenic effect of *Allium* vegetables is due to organosulfur compounds (OSC), which are generated on processing (e.g., cutting or chewing) of these vegetables (5). Garlic-derived OSCs, including diallyl sulfide, diallyl disulfide (DADS), and diallyl trisulfide (DATS) have been shown to afford significant protection against cancer in animal models induced by a variety of chemical carcinogens (6–11). For example, prevention of chemically induced cancer

by naturally occurring OSC analogues has been observed against benzo[*a*]pyrene-induced forestomach and pulmonary carcinogenesis in mice (8), *N*-nitrosomethylbenzylamine-induced esophageal cancer in rats (9), and azoxymethane-induced colon carcinogenesis in rats (10). Induction of phase 2 carcinogen-detoxifying enzymes, such as glutathione transferases and quinone reductase, and inhibition of phase 1 carcinogen-activating enzymes are believed to be responsible for prevention of chemically induced cancers by OSCs (12–14). We have also shown previously that DADS administration significantly inhibits membrane association of oncogenic p21^{H-ras} *in vivo* (15).

Recent studies have revealed that certain garlic-derived OSCs can suppress proliferation of cultured cancer cells by causing apoptosis and/or cell cycle arrest (16–27). Apoptosis induction and cell cycle arrest was first documented for DADS in human colon cancer cells (16, 17). The DADS-induced apoptosis in colon cancer cells correlated positively with an increase in intracellular free calcium level (16). The DADS-induced cell cycle arrest was accompanied by an increase in cyclin B1 protein level, a decrease in complex formation between cyclin B1 and cyclin-dependent kinase (CDK) 1, and hyperphosphorylation of CDK1 (17). We have shown recently that DATS is a much more potent suppressor of PC-3 human prostate cancer cell proliferation compared with either diallyl sulfide or DADS (22). We also found that the proliferation of a normal prostate epithelial cell line is minimally affected by DATS even at concentrations that are highly cytotoxic to the prostate cancer cells (22). The DATS-mediated inhibition of PC-3 cell proliferation is associated with G₂ phase as well as mitotic

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arrest and apoptosis induction (22, 23, 25–27). The DATS-induced G₂ phase cell cycle arrest is caused by reactive oxygen species-dependent destruction and hyperphosphorylation of Cdc25C (23), whereas activation of checkpoint kinase 1 regulates the mitotic arrest executed by DATS (25). The DATS-induced apoptosis in PC-3 cells is associated with activation of caspase-3 and partially attenuated by ectopic expression of Bcl-2 as well as overexpression of constitutively active Akt (22, 26).

Cultured cancer cells are valuable not only for rapid screening of potential anticancer agents but also for gaining mechanistic insights not accessible otherwise. However, the observations made in cultured cancer cells need to be confirmed using appropriate animal models to test *in vivo* significance of the cellular findings. Moreover, *in vivo* efficacy testing of potential anticancer agents is a prerequisite for their further development as clinically useful agents. The present study was designed to test whether DATS administration affects growth of PC-3 cells *in vivo*. We now show that oral administration of DATS significantly retards growth of PC-3 xenografts in athymic mice without causing weight loss or any other side effects. The present study also reveals that the DATS-mediated inhibition of PC-3 xenograft growth correlates with induction of multidomain proapoptotic proteins Bax and Bak.

Materials and Methods

Reagents and cell culture. DATS was procured from LKT Laboratories (St. Paul, MN). Tissue culture medium, fetal bovine serum, and antibiotics were from Life Technologies (Grand Island, NY). Antibodies against Bak, Bcl-xL, cyclin B1, and vascular endothelial growth factor receptor-2 (VEGFR-2) were from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies against Bax, Bid, and Cdc25B were from PharMingen (San Diego, CA); anti-Cdc25C antibody was from Cell Signaling (Beverly, MA); anti-Bcl-2 antibody was from DakoCytomation (Carpinteria, CA); antibody specific for detection of phosphorylated (Ser¹⁰) histone H3 was from Upstate (Lake Placid, NY); and the antibodies against CDK1 and actin were from Sigma (St. Louis, MO). Monolayer cultures of PC-3 cells were maintained in F-12K Nutrient Mixture (Kaighn's modification) supplemented with 7% nonheat inactivated fetal bovine serum and antibiotics.

Xenograft assay. Male athymic mice (6 weeks old) were purchased from Taconic (Germantown, NY) and housed in accordance with the Institutional Animal Care and Use Committee guidelines. The use of mice for studies described herein was approved by the Institutional Animal Care and Use Committee. Exponentially growing PC-3 cells were mixed in a 1:1 ratio with Matrigel (Becton Dickinson, Bedford, MA) and a 0.1 mL suspension containing 1×10^6 cells was injected s.c. on both left and right flank of each mouse. Mice were randomized into two groups of five mice per group with tumors implanted on both left and right flanks ($n = 10$). Power analysis indicated that a sample size of 10 (five animals per group with tumors implanted on both left and right flanks) would provide at least 80% power to detect a 50% reduction in tumor volume at $P = 0.05$. The experiment was terminated 20 days after tumor cell injection because PC-3 is a relatively fast-growing tumor. Experimental mice were treated by oral gavage with 6 μmol DATS in 0.1 mL PBS (Monday, Wednesday, and Friday) beginning the day of tumor cell implantation. Control mice received an equal volume of the vehicle. Tumor size was measured as described by us previously (28, 29). Body weights of the control and DATS-treated mice were recorded throughout the experiment. Mice of each group were also monitored for other symptoms of side effects, including food and water withdrawal and impaired posture or movement. At the termination of the experiment, the tumor tissues from control and

DATS-treated mice were harvested and used for immunohistochemistry or immunoblotting.

Histologic analysis of apoptotic bodies. The tumor tissues from control and DATS-treated mice were fixed in 10% neutral buffered formalin, dehydrated, embedded in paraffin, and sectioned at 4- μm thickness. Apoptosis in tumor sections of control and DATS-treated mice was assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay using ApopTag Peroxidase *In situ* Apoptosis Detection kit (Chemicon International, Temecula, CA) according to the manufacturer's recommendations. The tumor sections were also stained with H&E.

Immunoblotting. A portion of the tumor tissues harvested from control and DATS-treated mice at the termination of the experiment were stored at -80°C until used. A 10% (w/v) homogenate of tumor tissues from control and DATS-treated mice was prepared in PBS containing protease inhibitor (BD Biosciences, San Diego, CA) and phosphatase inhibitor cocktail (Sigma) at 4°C using a polytron. The homogenate was centrifuged at $21,000 \times g$ for 15 minutes. The supernatant proteins were resolved by sodium dodecyl sulfate PAGE and transferred onto polyvinylidene fluoride membrane (28, 29). After blocking with 5% nonfat dry milk in TBS containing 0.05% Tween 20, the membrane was incubated with the desired primary antibody for 1 hour at room temperature or overnight at 4°C. The membrane was then treated with appropriate secondary antibody and the immunoreactive bands were visualized using enhanced chemiluminescence method. Each membrane was stripped and reprobed with anti-actin antibody to normalize for differences in protein loading. Change in protein level was determined by densitometric scanning of the immunoreactive bands and corrected for actin loading control.

In vitro migration assay. The effect of DATS treatment on *in vitro* migration (invasion) by PC-3 cells was determined using Transwell Boyden chamber (Corning, Acton, MA) containing a polycarbonate filter with a pore size of 8 μm as described by us previously (30). Briefly, 0.2 mL PC-3 cell suspension containing 4×10^4 cells in complete medium was mixed with 10 or 20 $\mu\text{mol/L}$ DATS or DMSO (control) and the suspension was added to the upper compartment of the chamber. The lower compartment of the chamber contained 0.6 mL of complete medium containing the same concentrations of DATS or DMSO. Following incubation at 37°C for 24 hours, the nonmigrant cells from the upper face of the Transwell membrane were removed using a cotton swab. The membrane was washed with PBS and the migrated cells on the bottom face of the membrane were fixed with 90% ethanol and stained with eosin. Three randomly selected fields on each membrane were scored for migrated cells.

Immunohistochemistry for CD31. The effect of DATS administration on formation of new blood vessels *in vivo* in PC-3 tumor xenograft was assessed by immunohistochemical analysis of CD31. Tumor sections (4 μm) were fixed in acetone for 10 minutes at 4°C. After endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 15 minutes, the sections were treated with normal rabbit serum for 20 minutes. The sections were then incubated with the primary anti-CD31 goat polyclonal antibody (1:750 dilution; Santa Cruz Biotechnology) for 1 hour at room temperature and washed with TBS. Biotinylated anti-goat IgG was applied for 30 minutes at room temperature. Color was developed by 10 minutes of incubation with the 3,3'-diaminobenzidine (DakoCytomation).

Results

DATS inhibits growth of PC-3 xenografts in athymic mice. We have shown previously that DATS inhibits growth of cultured PC-3 cells, but not a normal prostate epithelial cell line, by causing cell cycle arrest and apoptosis induction (22, 23, 25). To test *in vivo* relevance of these cellular findings, we determined the effect of DATS administration by oral gavage (6 μmol , thrice weekly) on PC-3 xenograft growth in athymic

mice. The DATS concentration used in the present study was within the range (up to 20 μmol) used in chemically induced carcinogenesis models (8, 10). The average tumor volume in mice treated with 6 μmol DATS was significantly lower compared with vehicle-treated control mice throughout the experimental protocol (Fig. 1A). For example, 20 days after starting therapy, the average tumor volume in control mice ($565 \pm 112 \text{ mm}^3$) was ~ 3 -fold higher compared with DATS-treated mice. Similarly, on day 15, the average tumor volume in 6 μmol DATS-treated mice was lower by $\sim 55\%$ compared with control mice. The DATS-mediated retardation of PC-3 xenograft growth *in vivo* was also evident in a separate study (data not shown).

The body weights of the control and experimental groups of mice were recorded periodically to determine whether DATS administration caused weight loss. As can be seen in Fig. 1B, the average body weights of the control and DATS-treated mice did not differ significantly throughout the experimental protocol. Moreover, the DATS-treated mice appeared healthy and did not exhibit impaired movement and posture, indigestion, and areas of redness or swelling. These results indicated that DATS

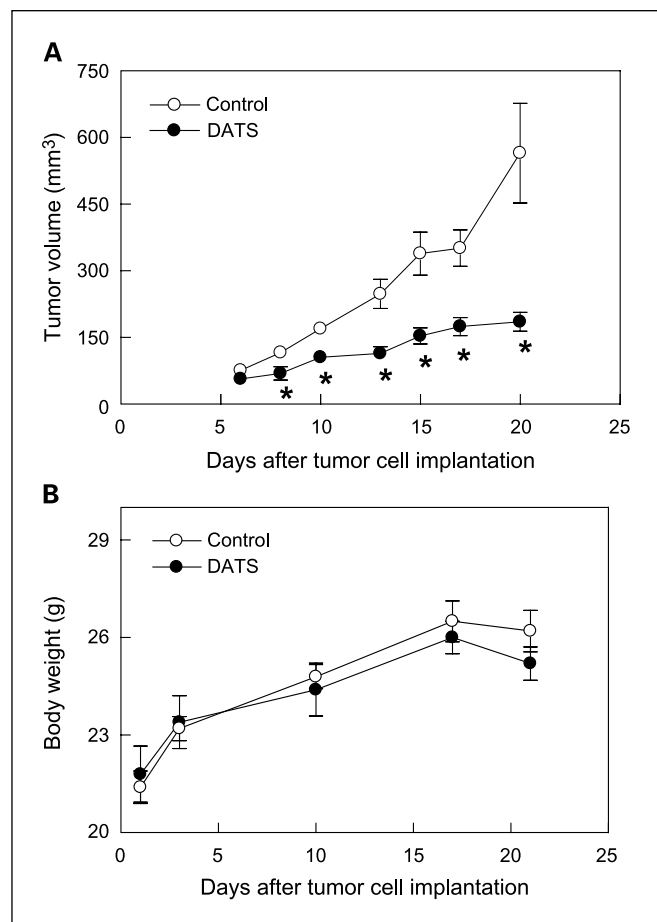


Fig. 1. A, average tumor volume in vehicle-treated control mice and mice treated with 6 μmol DATS (Monday, Wednesday, and Friday). The DATS administration was started on the day of tumor cell injection. Points, mean ($n = 10$; five mice per group with tumor cells injected on both left and right flank of each mouse); bars, SE. *, $P < 0.05$, significantly different compared with control by two-way ANOVA. B, average body weights of control and 6 μmol DATS-treated mice. The body weights of the control and DATS-treated mice did not differ significantly throughout the experimental protocol. Points, mean ($n = 5$); bars, SE.

administration significantly inhibited PC-3 xenograft growth without causing any side effects to the mice.

DATS administration increases apoptosis in tumors. We have shown previously that DATS suppresses proliferation of PC-3 cells in culture by causing apoptosis induction (22). To test whether DATS-mediated inhibition of PC-3 xenograft growth *in vivo* was due to increased apoptosis, tumor tissues from control and DATS-treated mice were examined for histologic evidence of apoptosis. The apoptotic bodies in tumor sections of control and DATS-treated mice were visualized by TUNEL staining and representative micrographs are shown in Fig. 2A. The tumors from DATS-treated mice exhibited a markedly higher count of brown-color apoptotic bodies compared with control tumors. The tumor sections from control and DATS-treated mice were also stained with H&E, and the results are shown in Fig. 2B. Eosin is an acidic stain, which interacts with cellular proteins rich in basic amino acids and commonly used for cytoplasmic staining. The eosin-protein complex is characterized by a vivid pink cytoplasmic staining. Hematoxylin is a nuclear stain that interacts with negatively charged phosphate groups of nuclear DNA forming a blue-purple color. The staining for hematoxylin was relatively brighter in tumors of DATS-treated mice compared with control mice (Fig. 2B). We have shown previously that DATS treatment causes mitotic arrest in cultured PC-3 cells that is characterized by chromatin condensation and Ser¹⁰ phosphorylation of histone H3 (25). The Ser¹⁰ phosphorylation of histone H3 has emerged as a sensitive marker for mitotic cells (31). We therefore determined the level of phosphorylated (Ser¹⁰) histone H3 using tumor supernatants from control and DATS-treated mice by immunoblotting to test whether DATS treatment resulted in mitotic arrest *in vivo*. The level of Ser¹⁰-phosphorylated histone H3 was comparable in tumors of control and DATS-treated mice (data not shown). These results indicated that the DATS-mediated suppression of PC-3 xenograft growth *in vivo* was associated with increased apoptosis.

DATS administration increases protein levels of Bax and Bak in tumors. The Bcl-2 family proteins have emerged as critical regulators of apoptosis by functioning as either inhibitors (Bcl-2 and Bcl-xL) or promoters (Bax, Bak, and Bid) of the cell death process (32–34). To gain insights into the mechanism of DATS-mediated suppression of PC-3 xenograft growth, we compared the levels of Bcl-2 family proteins in tumors from control and DATS-treated mice. Representative immunoblots for Bax, Bak, Bcl-xL, Bid, and Bcl-2 proteins using tumor supernatants from control and DATS-treated mice are shown in Fig. 3A. Change in protein level was quantified by densitometric scanning of the immunoreactive bands and corrected for actin loading control. As can be seen Fig. 3B, the protein levels of Bax and Bak were statistically significantly higher in tumors from DATS-treated mice compared with control tumors. For instance, the protein level of Bax was ~ 2.1 -fold higher in tumors of DATS-treated mice compared with control tumors (Fig. 3B). Similarly, the tumors from DATS-treated mice exhibited an ~ 3.7 -fold increase in Bak protein level compared with control tumors. The tumors from DATS-treated mice also exhibited an increasing trend in the protein level of Bcl-xL and a decreasing trend in the level of Bid protein compared with control tumors, although the differences did not reach statistical significance ($P > 0.05$, paired *t* test). The protein level of Bcl-2 was comparable in tumors of control and

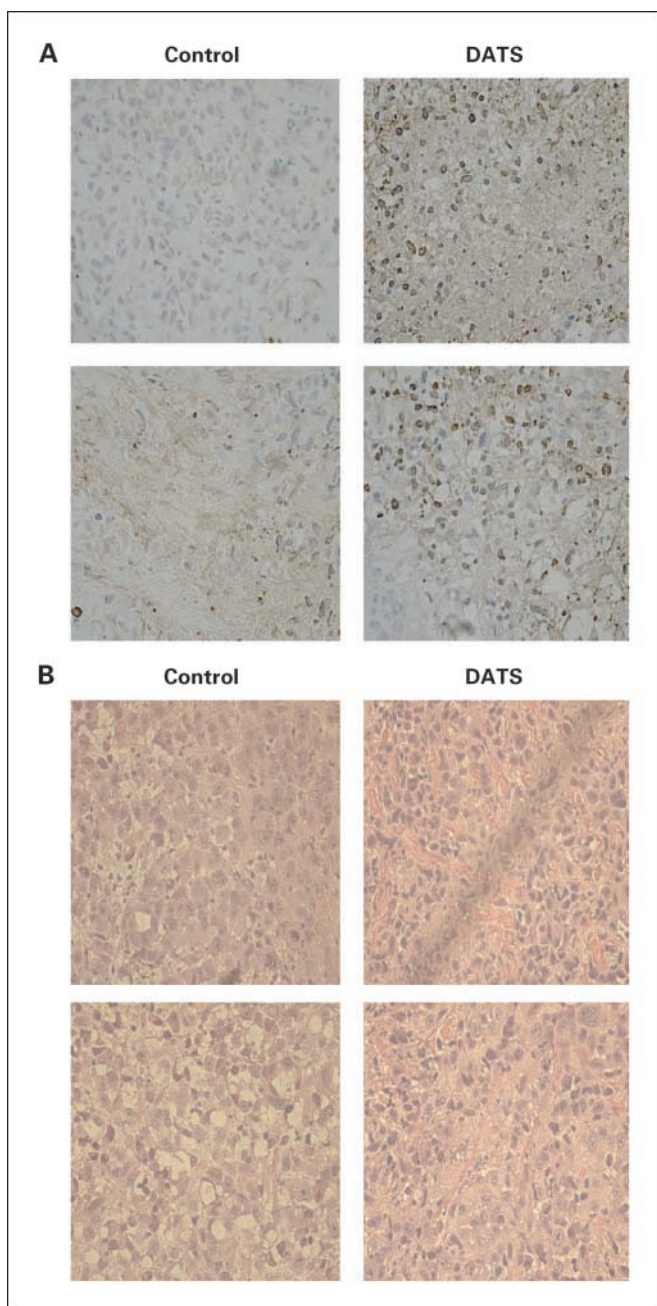


Fig. 2. *A*, TUNEL staining for apoptotic bodies in tumor sections from control and DATS-treated mice. Representative TUNEL staining in tumor sections from two different control and DATS-treated mice is shown. The tumors from DATS-treated mice exhibited a markedly higher count of brown-color apoptotic bodies compared with control tumors. *B*, histologic analysis of tumors from control and DATS-treated mice by H&E staining. Representative H&E staining in tumor sections from two different control and DATS-treated mice is shown.

DATS-treated mice. These results indicated that DATS-mediated suppression of PC-3 xenograft growth *in vivo* was accompanied by induction of multidomain proapoptotic proteins Bax and Bak.

We have shown previously that the DATS-induced G₂-M phase cell cycle arrest in PC-3 cells is associated with a reduction in protein levels of CDK1 and Cdc25C and an increase in cyclin B1 protein level (23, 25). These proteins play an important role in regulation of G₂-M transition (35). We

therefore compared the levels of CDK1, cyclin B1, Cdc25B, and Cdc25C proteins by immunoblotting using tumor supernatants from control and DATS-treated mice (Fig. 4A) to test *in vivo* relevance of the cellular findings. The levels of the above cell cycle regulatory proteins did not differ significantly between tumors of control and DATS-treated mice (Fig. 4B). These

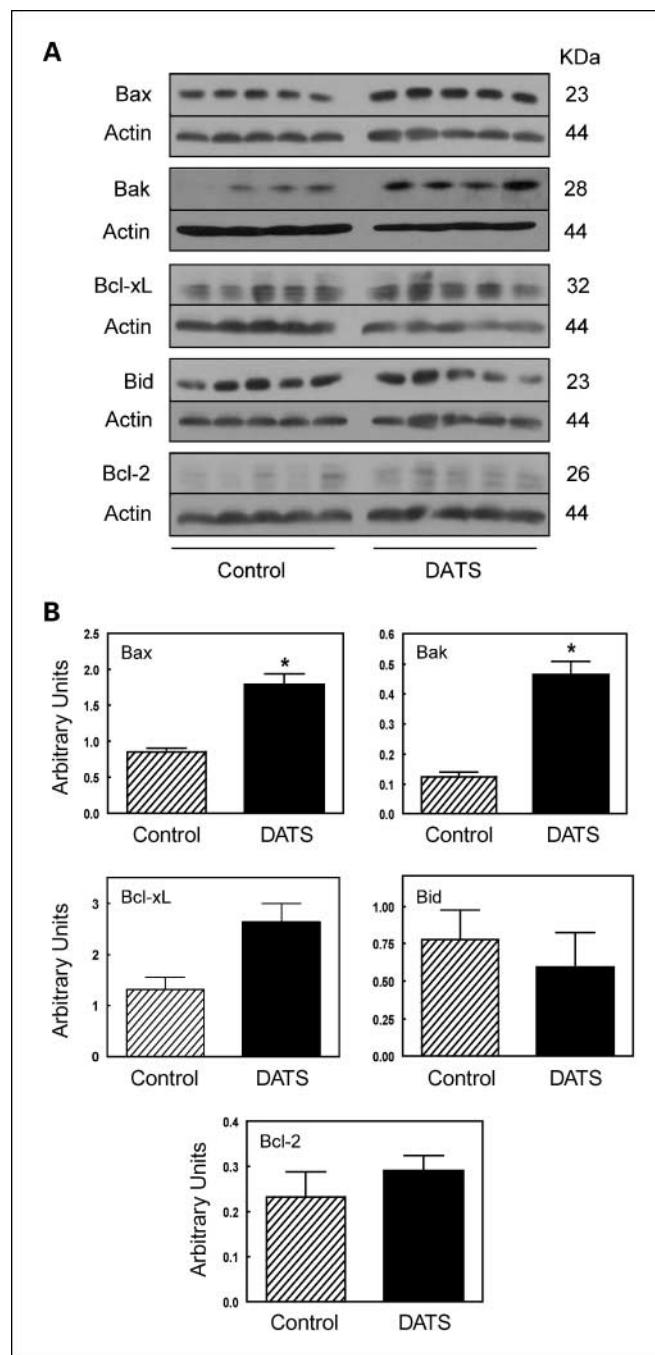


Fig. 3. *A*, immunoblotting for Bcl-2 family proteins using tumor supernatants from control and DATS-treated mice. The blots were stripped and reprobed with anti-actin antibody to correct for differences in protein loading. *B*, densitometric scanning data for Bcl-2 family protein levels in tumors from control and DATS-treated mice. Tumor tissues from four or five mice of each group were used for immunoblotting. Columns, mean ($n = 4-5$); bars, SE. *, $P < 0.05$, significantly different compared with control by paired t test.

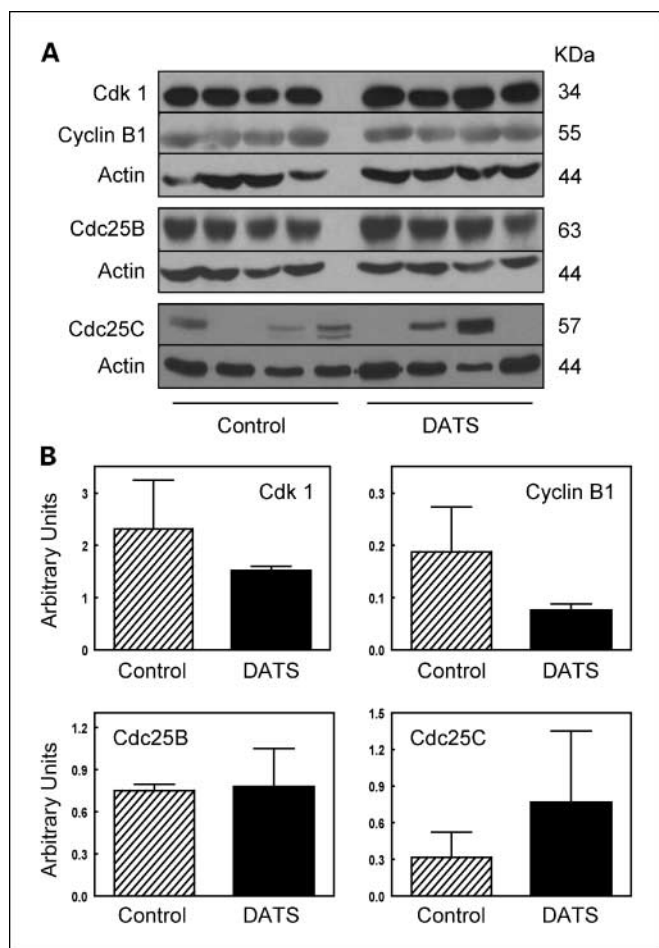


Fig. 4. A, immunoblotting for CDK1, cyclin B1, Cdc25B, and Cdc25C using tumor supernatants from control and DATS-treated mice. The blots were stripped and reprobed with anti-actin antibody to correct for differences in protein loading. B, densitometric scanning data for levels of cell cycle regulatory proteins in tumors from control and DATS-treated mice. Tumor tissues from four mice of each group were used for immunoblotting. Columns, mean ($n = 4$); bars, SE.

results indicated that the DATS-mediated changes in protein levels of the cell cycle regulators observed in cultured PC-3 cells (23) were not translated *in vivo*.

DATS inhibits *in vitro* migration by PC-3 cells. We have shown previously that DATS treatment significantly inhibits *in vitro* capillary-like tube formation and migration (invasion) by human umbilical vein endothelial cells (30), suggesting that this agent may be antiangiogenic. In the present study, we tested this possibility by determining the effect of DATS treatment on *in vitro* migration by PC-3 cells. As can be seen in Fig. 5A, in DMSO-treated control samples, a large fraction of PC-3 cells migrated (red staining) to the bottom face of the membrane (Fig. 5A, a), which was decreased significantly in the presence of DATS (Fig. 5A, b and c). As shown in Fig. 5A (d), the migration of PC-3 cells was inhibited by roughly 50% and 70% in the presence of 10 and 20 $\mu\text{mol/L}$ DATS, respectively, compared with control. Similar to human umbilical vein endothelial cells (30), DATS treatment caused a marked reduction in the protein level of VEGFR-2, which plays an important role in angiogenesis (36), as revealed by immunoblotting using lysates from PC-3 cells treated with 20 $\mu\text{mol/L}$ DATS for the indicated times (Fig. 5B). To test

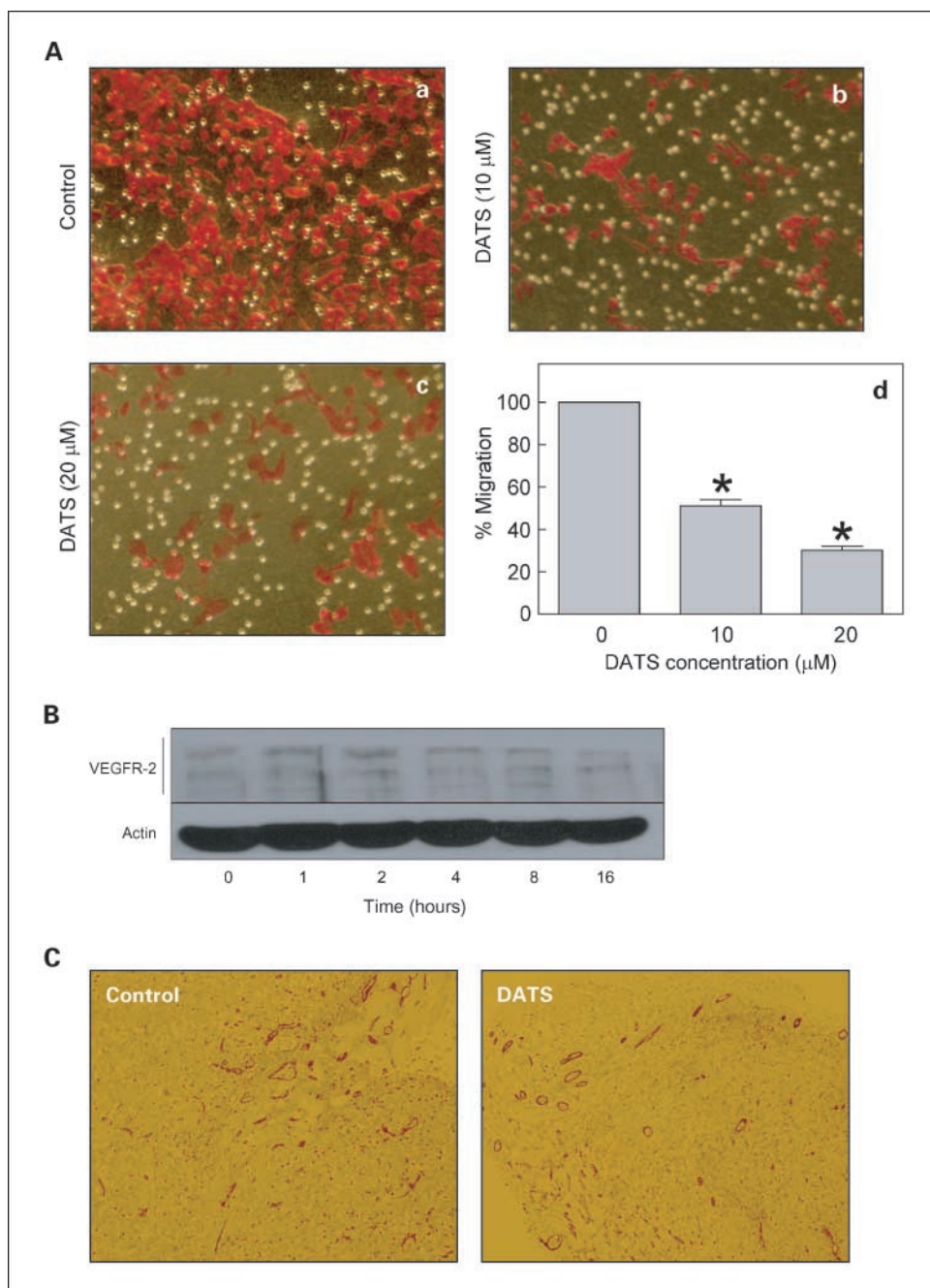
whether DATS administration affected angiogenesis *in vivo*, we did immunohistochemistry for CD31 to visualize blood vessels using tumor sections from control and DATS-treated mice. As can be seen in Fig. 5C, the CD31 staining was comparable in tumors of control and DATS-treated mice. The CD31-positive blood vessels were counted in tumor sections of control and DATS-treated mice, which also revealed that DATS administration did not have any appreciable effect on formation of blood vessels (data not shown). These results indicated that DATS administration did not inhibit angiogenesis in PC-3 xenografts *in vivo*.

Discussion

According to the American Cancer Society, prostate cancer is the most commonly diagnosed malignancy in American men after skin cancer. The society estimates diagnosis of $\sim 234,460$ new cases of prostate cancer in the United States in 2006. About 27,350 men are expected to die of this disease in the year 2006. Prostate cancer is the third leading cause of cancer-related deaths in men, after lung cancer and colorectal cancer. Molecular mechanism underlying onset or progression of prostate cancer is not fully defined, but age, race, diet, and androgen secretion and metabolism are the identifiable risk factors associated with this malignancy (37, 38). Therapeutic options exist for localized disease, including surgery, radiation therapy, and hormonal therapy. Androgen ablation is a frequently prescribed treatment option for prostate cancer (39). This treatment modality, however, is palliative and has limited scope for hormone-refractory prostate cancers (39). Moreover, chemotherapy and radiation therapy are largely ineffective against advanced prostate cancer (40, 41). Prostate cancer is usually diagnosed in the 6th and 7th decades of life, which allows a large window of opportunity for intervention to prevent or slow progression of the disease. Therefore, clinical development of agents that are nontoxic to normal cells but can delay onset and/or progression of human prostate cancer, especially hormone-independent prostate cancers, could have a significant effect on disease-related cost, morbidity, and mortality for a large segment of population. The present study provides experimental evidence to indicate that DATS, which is a naturally occurring constituent of processed garlic, significantly inhibits growth of androgen-independent PC-3 xenografts in athymic mice without causing weight loss or any other side effects. We also found that the DATS-mediated inhibition of PC-3 xenograft growth *in vivo* is associated with an increase in apoptotic bodies. These observations are consistent with cellular studies where treatment of PC-3 cells with DATS results in a concentration-dependent apoptosis induction (22). Thus, it is reasonable to conclude that apoptosis induction is a critical event in DATS-mediated suppression of PC-3 cell growth *in vivo*.

Cellular systems are valuable in obtaining mechanistic insights not accessible otherwise. The observations made in cells, however, need to be confirmed in animal models to establish *in vivo* relevance of the cellular findings. In addition, identification of an appropriate biomarker(s) of *in vivo* response is an equally important consideration in clinical development of potential chemopreventive and therapeutic agents. Identification of biomarker(s) is especially desirable for cancer chemoprevention trials because reduction of cancer incidence is too rigorous endpoint for malignancies with a

Fig. 5. A, representative images depicting effect of DATS treatment (24 hours) on PC-3 migration as determined by Boyden chamber assay. The migration by PC-3 cells was inhibited significantly in the presence of DATS in a concentration-dependent manner. Columns, mean ($n = 3$); bars, SE (d). *, $P < 0.05$, significantly different compared with DMSO-treated control by one-way ANOVA followed by Dunnett's test. B, immunoblotting for VEGFR-2 using lysates from PC-3 cells treated with 20 $\mu\text{mol/L}$ DATS for the indicated times. The blot was stripped and reprobbed with anti-actin antibody to ensure equal protein loading. C, immunohistochemical analysis of CD31 using tumor sections from control and DATS-treated mice. The CD31 staining was comparable in tumor sections from control and 6 μmol DATS-treated mice, indicating lack of an inhibitory effect of DATS treatment on formation of blood vessels.



long latency, such as prostate cancer. The present study reveals that the DATS-mediated suppression of PC-3 xenograft growth *in vivo* is accompanied by induction of Bak and Bax, which function to promote cell death by neutralizing antiapoptotic effects of Bcl-2 and Bcl-xL (42, 43). Mouse embryonic fibroblasts (MEF) derived from Bax and Bak double knockout mice are significantly more resistant to apoptosis induction by different agents, including dietary cancer chemopreventive agents sulforaphane and phenethyl isothiocyanate compared with wild-type MEFs (44–46). Recent unpublished studies from our laboratory have revealed that DATS treatment causes apoptotic cell death in association with an increase in the protein levels of Bax and Bak in SV40-immortalized MEFs derived from wild-type mice, but the immortalized MEFs

derived from Bax and Bak double knockout mice are significantly more resistant to DATS-induced cell death compared with wild-type MEFs.¹ These results suggest that Bax and Bak may serve as useful biomarkers of DATS efficacy, but validation of this hypothesis awaits clinical trials.

Eukaryotic cell cycle progression is regulated by sequential activation of CDKs whose association with regulatory cyclins is essential for their activation (35). A complex between CDK1 and cyclin B1 plays an important role in regulation of G₂-M transition (35). The CDK1/cyclin B1 complex is retained in an inactive state by reversible phosphorylations at Thr¹⁴ and Tyr¹⁵

¹ Y.-A. Kim and S.V. Singh, unpublished observation.

(35). Dephosphorylation and hence activation of CDK1/cyclin B1 is accomplished by Cdc25B and Cdc25C phosphatases (35). Analysis of the cell cycle regulatory proteins in tumors reveals that the DATS-mediated changes observed in cells may not be reflected *in vivo*. For example, we have shown previously that DATS-mediated G₂-M phase cell cycle arrest in cultured PC-3 cells is associated with a reduction in protein levels of CDK1 and Cdc25C (23). The levels of these proteins are comparable in tumors from control and DATS-treated mice. Several possibilities exist to explain inconsistencies in cellular and *in vivo* results about the effect of DATS on levels of cell cycle regulatory proteins. One possibility is that the concentration of DATS necessary for lowering of CDK1 and Cdc25C protein level is not achieved *in vivo*. Difference in metabolism of DATS between cultured cancer cells and *in vivo* is another likely possibility to explain inconsistencies in results. It is also possible that the timing of tumor tissue collection at the time of animal sacrifice is not optimum to detect DATS-mediated *in vivo* changes in levels of cell cycle regulatory proteins. Carefully designed experiments, including a time course analysis of effect of DATS administration on levels of cell cycle regulatory proteins in tumors *in vivo*, are needed to systematically explore these possibilities.

We have shown previously that DATS is a potent inhibitor of *in vitro* capillary-like tube formation and migration by human umbilical vein endothelial cells (30). The DATS-mediated inhibition of migration of human umbilical vein endothelial cells is associated with inactivation of Akt, suppression of VEGF

secretion, and down-regulation of VEGFR-2 protein (30). Consistent with these results, DATS treatment causes inhibition of migration by PC-3 cells, which is accompanied by a decrease in protein level of VEGFR-2 (Fig. 5). Despite these effects in cultured PC-3 cells, DATS administration does not inhibit angiogenesis in PC-3 xenograft *in vivo* as judged by immunohistochemical analysis of CD31.

The DATS-mediated growth arrest and apoptosis induction in prostate cancer cells, including PC-3 cell line, is observed at concentrations (10-40 $\mu\text{mol/L}$) pharmacologically achievable based on a recent pharmacokinetic study (47). The concentration of DATS in rat blood following treatment with 10 mg DATS is $\sim 31 \mu\text{mol/L}$ (47). Although the pharmacokinetic variables for DATS in humans have not yet been measured, oral administration of 200 mg of synthetic DATS (also known as allitridum) in combination with 100 μg selenium every other day for 1 month to humans did not cause any harmful side effects (48). It is therefore possible that the plasma concentrations of DATS required for cancer cell growth inhibition may be achievable in humans.

In conclusion, the present study indicates that (a) the DATS administration significantly retards growth of PC-3 xenografts in athymic mice without causing weight loss or any other side effects and (b) the DATS-mediated suppression of PC-3 xenograft growth *in vivo* correlates with induction of Bax and Bak proteins. We are tempted to speculate that Bax and Bak may serve as useful biomarkers of DATS response *in vivo*.

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