Garlic Constituent Diallyl Trisulfide Prevents Development of Poorly Differentiated Prostate Cancer and Pulmonary Metastasis Multiplicity in TRAMP Mice

Shivendra V. Singh,[1,2] Anna A. Powolny,[1] Silvia D. Stan,[1] Dong Xiao,[1] Julie A. Arlotti,[2] Renaud Warin,[1] Eun-Ryeong Hahm,[1] Stanley W. Marynowski,[2] Ajay Bommareddy,[1] Douglas M. Potter,[3] and Rajiv Dhir[2]

[1]Department of Pharmacology and Chemical Biology, and [2]University of Pittsburgh Cancer Institute, University of Pittsburgh School of Medicine, and [3]Biostatistics Department, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania

Abstract

Identification of agents that are nontoxic but can delay onset and/or progression of prostate cancer, which is the second leading cause of cancer-related deaths among men in the United States, is highly desirable. We now show that p.o. gavage of garlic constituent diallyl trisulfide (DATS; 1 and 2 mg/day, thrice/week for 13 weeks beginning at age 8 weeks) significantly inhibits progression to poorly differentiated prostate carcinoma and pulmonary metastasis multiplicity in transgenic adenocarcinoma of mouse prostate (TRAMP) mice without any side effects. There was a trend of a decrease in average wet weights of the urogenital tract and prostate gland in 1 and 2 mg DATS–treated mice compared with controls (~ 25–46% decrease in DATS-treated mice compared with controls). The incidence and the area of the dorsolateral prostate occupied by the poorly differentiated carcinoma were significantly lower in both 1 and 2 mg DATS–treated mice compared with control mice. In addition, DATS administration resulted in a statistically significant decrease in pulmonary metastasis multiplicity compared with controls (P = 0.002). The dorsolateral prostate from DATS-treated TRAMP mice exhibited decreased cellular proliferation in association with induction of cyclinB1 and securin protein levels, and suppression of the expression of neuroendocrine marker synaptophysin. However, DATS administration did not have any appreciable effect on apoptosis induction, angiogenesis, or natural killer and dendritic cell function. In conclusion, the results of the present study show, for the first time, that DATS administration prevents progression to invasive carcinoma and lung metastasis in TRAMP mice. [Cancer Res 2008;68(22):9503–11]

Introduction

Prostate cancer is one of the most commonly diagnosed visceral malignancies and the second leading cause of cancer-related deaths among men in the United States (1). Prostate carcinogenesis is characterized by gradual transformation of normal prostate epithelium to prostatic intraepithelial neoplasia (PIN), localized tumor to advanced and metastatic disease (2). Prostate cancer is usually diagnosed in the sixth or seventh decade of life providing a large window of opportunity for intervention to prevent or slow progression of the disease. Consequently, identification of novel agents that are nontoxic but can delay onset and/or progression of human prostate cancer is highly desirable. Natural products have received increasing attention in recent years for the discovery of novel cancer preventive and therapeutic agents (3).

Epidemiologic studies continue to support the premise that dietary intake of *Allium* vegetables (e.g., garlic) may lower the risk of various types of malignancies including cancer of the prostate (4–6). For example, the risk of prostate cancer was shown to be significantly lower in men consuming >10 g/day of total *Allium* vegetables than in men with total *Allium* vegetable intake of <2.2 g/day in a population-based, case-control study (6). The anticarcinogenic effect of *Allium* vegetables is attributable to organosulfur compounds (OSC) including diallyl sulfide, diallyl disulfide (DADS), and diallyl trisulfide (DATS), which are generated upon processing (e.g., cutting or chewing) of these vegetables (7). The OSCs have been shown to afford significant protection against chemically induced cancers in animal models, including benzo(*a*)pyrene-induced forestomach and pulmonary carcinogenesis in mice, *N*-nitrosomethylbenzylamine–induced esophageal cancer in rats, and azoxymethane-induced colon carcinogenesis in rats (8–11). Initial studies indicated that the OSC-mediated prevention of chemically induced cancer correlated with induction of phase 2 carcinogen inactivating enzymes and inhibition of cytochrome P450-dependent monooxygenases (12–14).

More recent studies including those from our laboratory have revealed that certain garlic-derived OSCs are effective in suppressing proliferation of human cancer cells (15–25). The OSC-mediated suppression of cancer cell growth in association with G_2-M phase cell cycle arrest and/or apoptosis induction was documented for DADS and DATS in human colon, neuroblastoma, and prostate cancer cells (15–20). Studies from our laboratory have revealed that DATS is a much more potent suppressor of proliferation of human prostate cancer cells compared with either diallyl sulfide or DADS (18). Interestingly, a normal prostate epithelial cell line is significantly more resistant to G_2-M phase cell cycle arrest by DATS compared with prostate cancer cells (19), a feature highly desirable for cancer chemopreventive agents. Mechanistic studies have revealed that DATS treatment not only negatively regulates signaling pathways implicated in cell proliferation (e.g., Akt) and angiogenesis (vascular endothelial growth factor signaling axis) but also causes G_2 and prometaphase arrest and mitochondria-mediated apoptotic cell death (18, 19, 21–26). Additionally, the growth of PC-3 human prostate cancer cells s.c.

implanted in athymic mice was significantly retarded by p.o. administration of DATS (27).

Demonstration of *in vivo* efficacy of potential cancer chemopreventive agents in suitable animal models is a prerequisite for their further clinical development. The present study was undertaken to test whether DATS administration offers protection against prostate carcinogenesis in a transgenic mouse model of prostate cancer (transgenic adenocarcinoma of mouse prostate; hereafter abbreviated as TRAMP). We now show that p.o. gavage of DATS significantly prevents development of invasive prostate carcinoma and pulmonary metastasis multiplicity in TRAMP[C57BL/6×FVB]F1 mice without causing weight loss or affecting T-antigen expression.

Materials and Methods

Reagents. DATS (purity ~99%) was purchased from LKT Laboratories. Antibodies against CD31 (platelet/endothelial cell adhesion molecule 1; PECAM-1) and cyclinB1 were purchased from Santa Cruz Biotechnology; the anti–E-cadherin antibody was from BD Transduction Laboratories; the antibodies against proliferating cell nuclear antigen (PCNA) and Ki-67 were from DakoCytomation; the anti-securin antibody was purchased from MBL; and the antibodies against T-antigen and synaptophysin were from BD Biosciences. The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed using the *In Situ* Apoptosis Detection kit from Chemicon International.

Animal model and DATS treatment. Various mouse models of prostate cancer have been generated in recent years [e.g., TRAMP, LADY (12T-10) transgenic mice, PTEN conditional knockout mice, etc.], which develop lesions analogous to those observed in human prostate cancer (reviewed in ref. 28). Our choice to use TRAMP mice for the present study was based on the following considerations: (*a*) a rather well-defined course of disease progression analogous to the human prostate carcinogenesis from histologic PIN to invasive carcinoma with distant site metastasis coupled with the overall high tumor incidence renders TRAMP mice suitable for chemoprevention studies (29, 30), (*b*) the chemoprevention studies in TRAMP mice can be completed in a reasonable time frame (29, 30), and (*c*) TRAMP model has been used successfully to test chemopreventive efficacy of several synthetic and natural agents (31–33). We opted to use TRAMP[C57BL/6×FVB]F1 progeny because these mice tend to form well-defined tumor nodules and the frequency of phylloides-like structures, which are rare in human prostate cancers, is relatively higher in C57BL/6 pure transgenic mice (28–30). Male TRAMP[C57BL/6×FVB]F1 hybrid mice were generated by breeding female TRAMP in C57BL/6 background with nontransgenic FVB male mice. Transgene verification was performed using DNA obtained from tail clippings at 14 to 17 d of life as described by Greenberg and colleagues (29). Use of mice for the studies described herein was approved by the Institutional Animal Care and Use Committee. After transgene verification, male TRAMP mice were maintained in a climate-controlled environment with a 12-h light/12-h dark cycle. The mice were fed food and water *ad libitum*. At age 8 wk, mice were randomized into three groups. Mice in the control group (*n* = 16) received 0.1 mL PBS by p.o. gavage thrice per week (Monday, Wednesday, and Friday), whereas the experimental groups of mice (*n* = 19) received either 1 mg DATS/d or 2 mg DATS/d in 0.1 mL PBS by p.o. gavage thrice per week (Monday, Wednesday, and Friday). Body weights of the control and DATS-treated mice were recorded once each week beginning at the onset of the study. After 13 wk of treatment, the animals were sacrificed 24 h after the last administration of the vehicle or DATS by CO_2 inhalation followed by cervical dislocation. The weights of the vital organs, urogenital tract, and prostate were determined. A portion of the prostate/tumor tissue was placed in 10% neutral buffered formalin and paraffin embedded. Tissues were sectioned at 4- to 5-μm thickness for H&E staining, TUNEL assay, and immunohistochemical analyses.

Pathologic evaluation and scoring of tumor stage and metastasis. Ten randomly selected fields on H&E-stained sections of the dorsolateral prostate of individual mouse of control and DATS-treated groups were independently scored by two investigators for incidence and percentage of the area corresponding to each pathologic stage. Pathologic grading was performed as described by Greenberg and colleagues (29, 30). Tissue histology was classified as normal prostate gland with open ducts lined with tall secretory epithelial cells surrounded by a closely associated thin sheet of smooth muscle cells; PIN with piling up of the epithelium, changes in the nuclear to cytoplasmic ratio, elongation of the nucleus and an increase in epithelial stratification, and formation of cribriform structures; well-differentiated (WD) carcinoma with clear invasion of the epithelial cells into the stroma; and poorly differentiated (PD) carcinoma with sheets of anaplastic cells with little or no glandular structures. Lung, kidney, liver, and pelvic lymph nodes were evaluated for the presence of metastasis by two independent investigators. The incidence of metastasis (percentage of mice with metastatic lesions) in each tissue and pulmonary metastasis multiplicity (number of pulmonary metastatic lesions/mouse) were computed for the control and DATS-treated groups.

Immunohistochemical analyses. Deparaffinized and rehydrated sections were quenched with 3% hydrogen peroxide and blocked with normal serum. The sections were then incubated with the desired primary antibody (anti–T-antigen, anti-PCNA, anti–Ki-67, anti–E-cadherin, anti-synaptophysin, anti-CD31, anti-cyclinB1, or anti-securin antibody) and washed with TBS followed by incubation with appropriate biotinylated secondary antibody. Characteristic brown color was developed by incubation with 3,3-diaminobenzidine. The sections were counterstained with Meyers Hematoxylin (Sigma) and examined under a Leica microscope. At least three nonoverlapping representative images of each tissue were captured from each section using a camera mounted onto the microscope. The images were analyzed using Image ProPlus 5.0 software (Media Cybernetics) for quantitation of PCNA, Ki-67, E-cadherin, T-antigen, cyclinB1, and securin expression and analysis of microvessel number and vessel diameter (CD31 staining). Immunoblotting for T-antigen expression using prostate/tumor tissue supernatants from control and DATS-treated mice was performed as previously described by us for other proteins (27).

Detection of apoptotic bodies by TUNEL staining. The paraffin-embedded tissue sections were deparaffinized, rehydrated, and then used to visualize apoptotic bodies by TUNEL staining using the ApopTag Plus Peroxidase *In Situ* Apoptosis kit and following the manufacturer's protocol. Apoptosis was quantified by counting the number of TUNEL-positive cells in at least three randomly selected, nonoverlapping high-power fields.

Cytotoxicity of natural killer and dendritic cells. The natural killer (NK) cells were isolated from the spleen of control and DATS-treated mice using MACS beads (Myltenyi Biotech) as described by Giezeman-Smith and colleagues (34) and cultured in interleukin-2–supplemented (1,000 units/mL) medium. The DCs were isolated from the bone marrow of control and DATS-treated mice using the adhesion selection method (35) and cultured in medium supplemented with interleukin-4 (500 ng/mL), granulocyte macrophage colony-stimulating factor (500 ng/mL), and Flt-3 (25 ng/mL). After 5 d of culture, NK cells were plated in 96-well plates alone or cocultured with dendritic cells (DC; day 5) at different ratios (10:0, 10:4, or 10:8). After 24 h, TRAMP-C1 target cells were added to the wells at specified ratio. After 24 h, plates were spun down and supernatant was collected for analysis of cytotoxicity using the Cytotox 96 nonradioactive assay (Promega), which determines release of lactate dehydrogenase. Monolayer cultures of TRAMP-C1 cells, a generous gift from Dr. Barbara Foster (Roswell Park Cancer Institute, Buffalo, NY), were maintained as described by us previously (36).

Statistical analysis. Statistical significance of difference in mean urogenital and prostate weights and metastasis multiplicity between groups was assessed by Wilcoxon test. The difference in metastasis incidence between groups was assessed by Fisher's exact test. Differences were considered significant at *P* value of ≤0.05.

Results

P.o. administration of DATS prevented development of PD prostate carcinoma in TRAMP mice. A preliminary dose-finding study involving 8-week-old nontransgenic male [C57BL/6×FVB]F1 littermates (5 mice per group) revealed that p.o. gavage of 1 and 2 mg DATS, thrice per week, was well-tolerated by the mice. The nontransgenic mice treated with 1 and 2 mg DATS seemed healthy and did not exhibit weight loss or signs of distress. All vital organs (liver, lung, kidney, heart, and spleen) and prostate glands of 1 and 2 mg DATS-treated nontransgenic mice were also normal as judged by histology and wet organ weight measurements (data not shown). The DATS concentrations used in the present study (1 and 2 mg DATS) are within the range that can be generated through dietary intake of garlic (37). As can be seen in Fig. 1*A*, the average wet weight of the urogenital tract in mice given 1 and 2 mg DATS was ∼26% and 35% lower, respectively, compared with control mice. The average wet weight of the prostate gland in 1 and 2 mg DATS-treated mice was also lower by ∼32% and 46%, respectively, compared with control mice. The differences in urogenital and prostate weights between control and DATS-treated mice did not reach statistical significance due to large data scatter. However, there was a trend of a dose-dependent decrease in average wet weights of the urogenital tract and prostate in DATS-treated mice compared with controls (Fig. 1*A*). As shown in Fig. 1*B*, the initial and final body weights of the control and DATS-treated mice did not differ significantly.

The histologic grades (normal prostate, PIN, WD carcinoma, and PD carcinoma) in dorsolateral prostate of control and DATS-treated mice with prostate weight of <1g were independently scored by two investigators. Prostate sections from control as well

Figure 1. Effect of p.o. administration of 1 and 2 mg DATS, thrice weekly (Monday, Wednesday, and Friday), on (*A*) urogenital tract and prostate weights and (*B*) initial and final body weights. *Columns*, mean (*n* = 16 for control group and *n* = 19 for DATS treatment groups); *bars*, SE.

as DATS-treated mice with prostate weight of >1 g were excluded because the majority of the prostate gland in these mice was occupied by PD carcinomas. Representative H&E staining in dorsolateral prostate sections from two different control and DATS-treated mice are depicted in Fig. 2*A*. The dorsolateral prostate of the vehicle-treated control TRAMP mice exhibited low- and high-grade PIN, WD carcinoma, and PD carcinomas along with areas consistent with normal prostate. The incidence of the PD carcinoma in the dorsolateral prostate of mice treated with 1 and 2 mg DATS was lower by 34% (*P* = 0.0147) and 41% (*P* = 0.035), respectively, in comparison with control mice (Fig. 2*B*). Moreover, the area occupied by the PD carcinoma in the dorsolateral prostate of DATS-treated mice was statistically significantly lower compared with that in vehicle-treated control mice. For example, the percentage of the area occupied by the PD carcinoma in dorsolateral prostate of mice given 1 and 2 mg DATS was lower by ∼64% (*P* = 0.0484) and 76% (*P* = 0.0189), respectively, compared with control mice (Fig. 2*C*). The DATS administration also resulted in a modest increase in the area occupied by the PIN and WD carcinomas compared with control mice (Fig. 2*C*). For example, the area occupied by the PIN in dorsolateral prostate of mice treated with 1 mg DATS and 2 mg DATS was higher by ∼57% (*P* = 0.0692 by Wilcoxon rank-sum test) and 60% (*P* = 0.0277 by Wilcoxon rank-sum test), respectively, compared with vehicle-treated control mice (Fig. 2*C*). Likewise, the area occupied by the WD carcinoma in the dorsolateral prostate of mice treated with 2 mg DATS was higher by ∼45% compared with control mice (*P* = 0.0986; Fig. 2*C*). These results indicated that DATS administration significantly inhibited progression from PIN/WD to PD carcinoma in TRAMP mice, which was independently verified by two investigators. These results are significant because the mortality in prostate cancer patients is mainly attributable to advanced and metastatic disease (2).

Prostate carcinogenesis in TRAMP mice is driven by the expression of the viral large T and small t antigen in the secretory epithelial cells of the prostate under the control of the minimal rat probasin promoter (29). We considered the possibility that the DATS-mediated prevention of PD carcinoma incidence/burden in TRAMP mice was due to the suppression of the transgene expression. We tested this possibility by determining the expression of T-antigen in the prostate of control and 2 mg DATS–treated mice by immunohistochemistry and immunoblotting. Fig. 3*A* depicts immunohistochemical staining for the T-antigen expression in the prostate of a representative mouse of both control and DATS-treated group. As can be seen in Fig. 3*B* and *C*, the DATS-mediated inhibition of prostate cancer development in TRAMP mice was not due to the suppression of the T-antigen expression.

DATS administration reduced pulmonary metastasis multiplicity. Because DATS administration significantly retarded the development of PD carcinoma, we proceeded to determine the incidence and multiplicity of pulmonary and pelvic lymph node metastasis. Figure 4*A* depicts H&E staining in a lung section of a representative mouse of both the control group and 2 mg DATS–treated group. The incidence of pulmonary metastasis was ∼88% in vehicle-treated control mice, which was reduced to ∼79% and 68% in mice given with 1 and 2 mg DATS, respectively (Fig. 4*B*). Likewise, the incidence of metastasis to the pelvic lymph nodes was ∼1.5- to 1.9-fold higher in the vehicle-treated control mice compared with the DATS-treated mice (Fig. 4*B*). Majority of the control mice exhibited multiple pulmonary metastatic lesions. The multiplicity of the pulmonary metastasis in mice given 1 and 2 mg
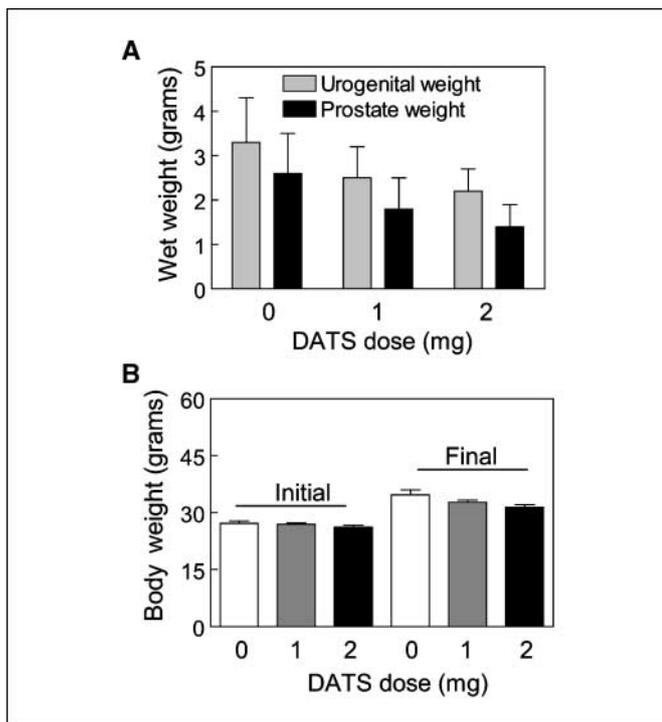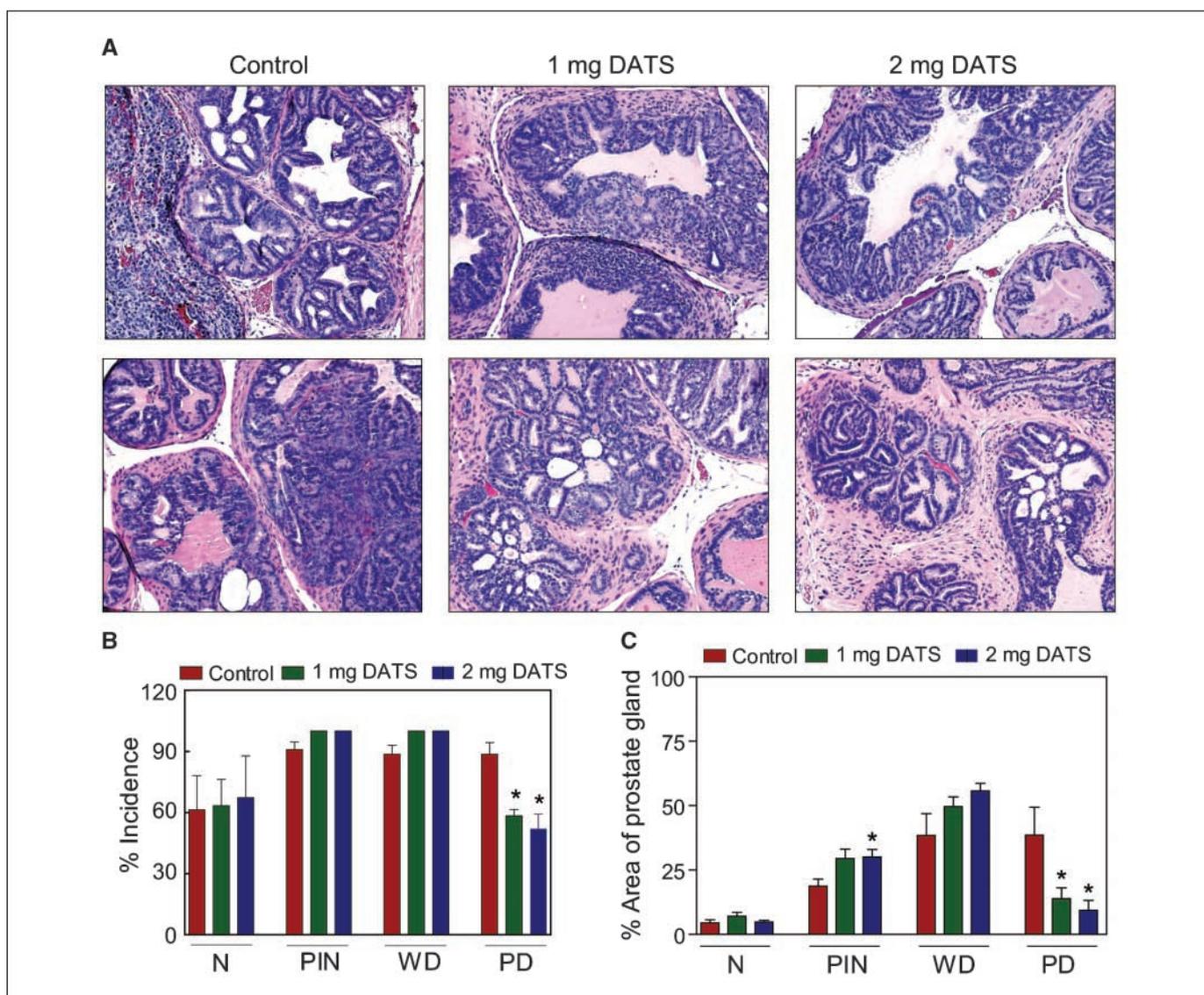
Figure 2. *A,* H&E staining in the dorsolateral prostate of representative vehicle-treated control TRAMP mice and TRAMP mice given 1 or 2 mg DATS, thrice weekly (Monday, Wednesday, and Friday) for 13 wk beginning at age 8 wk (magnification, ×200). *B,* incidence of histologic grades classified as normal prostate gland (*N*), low- and high-grade PIN, WD carcinoma, and PD carcinoma in the dorsolateral prostate of vehicle-treated control TRAMP mice and TRAMP mice given 1 or 2 mg DATS, thrice weekly (Monday, Wednesday, and Friday) for 13 wk. Tissue sections from mice with wet prostate weight of <1 gram were used because the majority of the area of the prostate gland larger than 1 gram was occupied by the PD carcinoma. Ten representative fields of each section were independently scored by two investigators. *C,* percentage of the area corresponding to the normal prostate gland, PIN, WD carcinoma, and PD carcinoma. *Columns,* mean (*n* = 11 for the control group, and *n* = 15 and 13 for the 1 and 2 mg DATS treatment group, respectively); *bars,* variability of the results in histologic grading between two independent investigators. *, significantly different compared with vehicle-treated control by Wilcoxon rank-sum test.

DATS was lower by ∼49% and 55% (*P* = 0.002) compared with control mice (Fig. 4*C*). Together, these results indicated that DATS administration delayed development of metastatic lesions especially pulmonary metastasis multiplicity in comparison with the vehicle-treated control mice.

DATS administration decreased cellular proliferation and synaptophysin expression in the dorsolateral prostate. Because studies in cultured human prostate cancer cells (18, 25) and PC-3 xenografts (27) have shown that DATS treatment reduces cell proliferation, we performed immunohistochemistry for well-known proliferation marker PCNA (38) to determine the effect of DATS treatment on proliferation index in the dorsolateral prostate of TRAMP mice. Immunohistochemical comparisons for PCNA expression in prostate of the control and DATS-treated mice were

carried out using size-matched tissues. Immunohistochemical staining for PCNA expression in representative prostate of a vehicle-treated control mouse and a 2 mg DATS–treated mouse is shown in Fig. 5*A*. The PCNA expression was ∼46% lower in the dorsolateral prostate of mice given 2 mg DATS compared with that of control mice (*P* = 0.035). Inhibitory effect of DATS administration on proliferation index was confirmed by immunohistochemical analysis of Ki-67 expression (Fig. 5*B*), which is another widely used marker for cellular proliferation (39). The PD carcinomas in TRAMP mice exhibit neuroendocrine (NE) differentiation (40, 41). Because DATS administration inhibited incidence and burden of PD carcinoma (Fig. 2*B* and *C*), we raised the question of whether DATS treatment affected fraction of NE cells. We addressed this question by determining the expression of synaptophysin in the
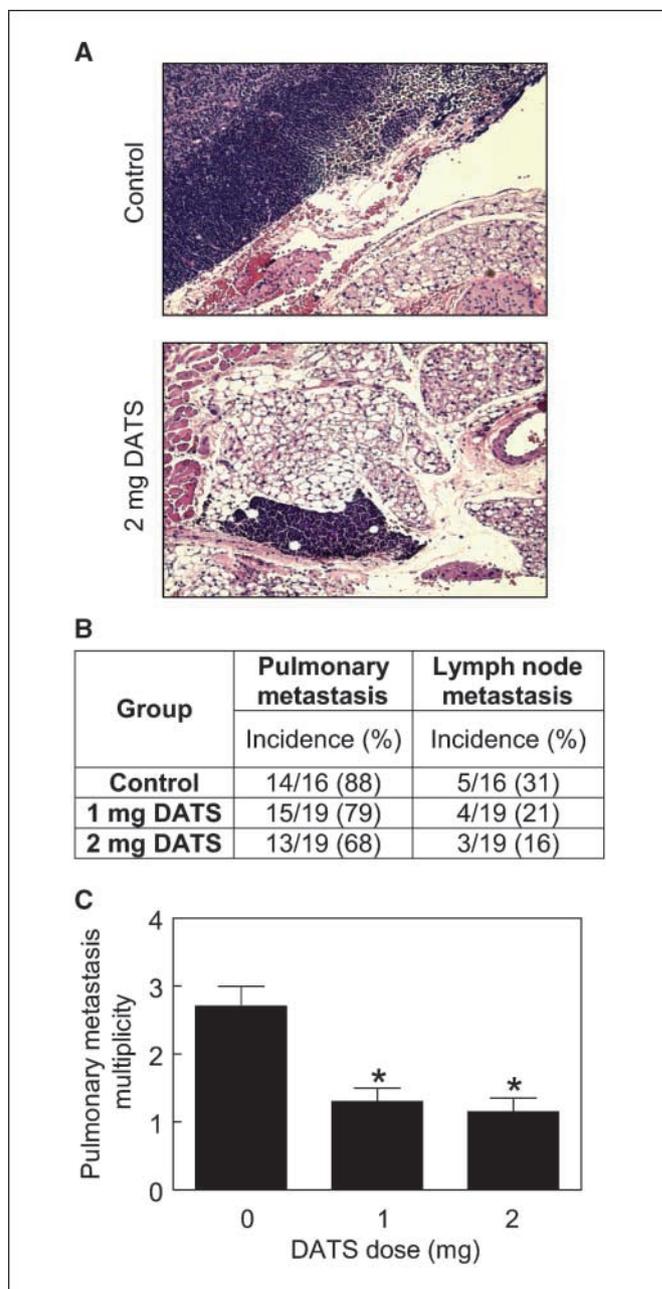
prostate of control and 2 mg DATS–treated mice. Synaptophysin is a membrane-associated glycoprotein and well-accepted marker of NE cells (41). The expression of synaptophysin in tissue sections from control mice was predominant in the membrane (Fig. 5C). The fraction of synaptophysin-expressing NE cells was significantly lower in the prostate of 2 mg DATS–treated mice compared with that of control mice. Collectively, these results indicated that the DATS-mediated prevention of PD carcinoma development in TRAMP mice correlated with reduced cellular proliferation and suppression of NE differentiation.

Our previous studies in cultured human prostate cancer cells have revealed that DATS treatment causes prometaphase arrest that is characterized by accumulation of cyclinB1 and securin proteins (21, 24). Consistent with the cellular results (21, 24), the dorsolateral prostate of 2 mg DATS–treated mice exhibited increased expression of cyclinB1 and securin proteins compared with the control mice (Fig. 5D).

DATS administration failed to cause apoptosis or alter E-cadherin expression. We have shown previously that DATS treatment causes apoptosis in cultured human prostate cancer cells (18, 22, 25). We performed TUNEL assay using prostate sections from control and treated mice to test whether DATS-mediated inhibition of prostate cancer development in TRAMP
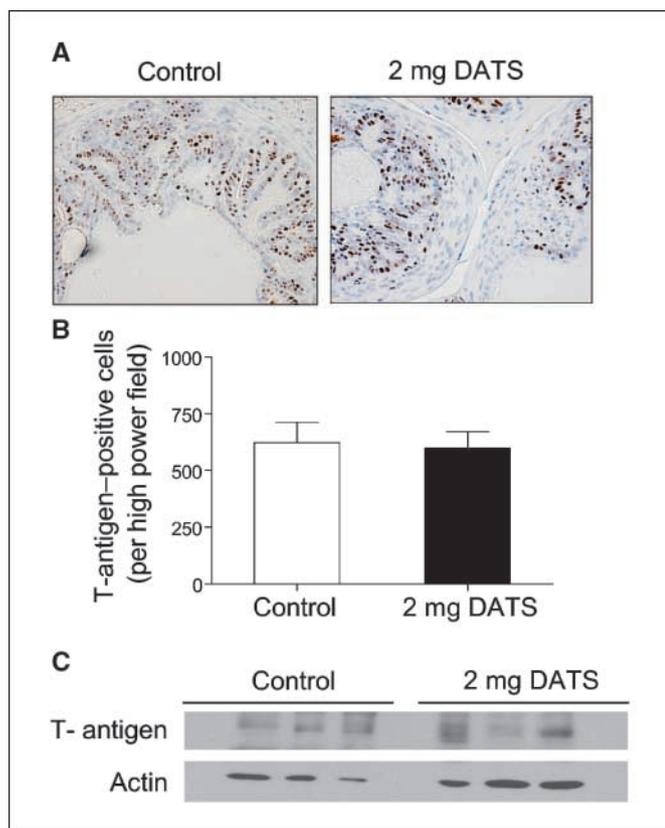
Figure 3. *A,* immunohistochemical analysis for T-antigen expression in a representative dorsolateral prostate of both control and 2 mg DATS–treated mouse (magnification, ×400). *B,* quantitation of T-antigen expression from immunohistochemical analyses. *Columns,* mean (*n* = 4); *bars,* SE. *C,* immunoblotting for T-antigen expression using lysates from prostate tissues of three individual mice from both control and 2 mg DATS–treated groups. The blot was stripped and reprobed with anti-actin antibody to correct for differences in protein loading.

Group	Pulmonary metastasis Incidence (%)	Lymph node metastasis Incidence (%)
Control	14/16 (88)	5/16 (31)
1 mg DATS	15/19 (79)	4/19 (21)
2 mg DATS	13/19 (68)	3/19 (16)

Figure 4. *A,* H&E staining depicting metastatic lesion in the lung of a representative control mouse and a 2 mg DATS–treated mouse (magnification, ×200). *B,* incidence of metastasis in the lungs and pelvic lymph nodes of vehicle-treated control TRAMP mice and TRAMP mice given 1 or 2 mg DATS. *C,* pulmonary metastasis multiplicity in vehicle-treated control TRAMP mice and TRAMP mice given 1 or 2 mg DATS. *Columns,* mean (*n* = 16 for the control group and *n* = 19 for the DATS treatment groups); *bars,* SE. *, significantly different (*P* < 0.05) compared with the vehicle-treated control group.

mice was due to increased apoptosis. Although the average number of TUNEL-positive apoptotic bodies was slightly higher in the prostate of 2 mg DATS–treated mice compared with control mice, the difference did not reach statistical significance (Fig. 6A).

E-cadherin is considered to be a suppressor of invasion and growth of many epithelial cancers (42). Some anticancer agents function by causing up-regulation of E-cadherin expression (32). We therefore compared expression of E-cadherin in prostate of the

control and 2 mg DATS–treated mice. Expression of E-cadherin did not differ in the dorsolateral prostate between control and 2 mg DATS groups (Fig. 6*B*). Collectively, these results indicated that the DATS-mediated prevention of PD carcinoma development in TRAMP mice was not due to increased apoptosis or altered expression of E-cadherin.

DATS administration failed to inhibit neovascularization. We have shown previously that DATS treatment inhibits *in vitro* angiogenic features (e.g., formation of capillary-like tube structures and/or migration) in human umbilical vein endothelial cells and prostate cancer cells (26). To test whether DATS administration caused suppression of neovascularization *in vivo*, the dorsolateral prostate sections from the vehicle-treated control and 2 mg DATS–treated mice were stained for angiogenic marker CD31 (also known as PECAM-1). The average number of vessels as well as the mean vessel diameter did not differ significantly in the dorsolateral prostate between the vehicle-treated control and the 2 mg DATS–

treated mice (data not shown). These results indicated that the DATS-mediated suppression of angiogenesis *in vitro* was not translated into inhibition of neovascularization *in vivo*, at least with the DATS dosing regimen used in the present study.

Cytotoxicity of NK and dendritic cells isolated from control and DATS-treated mice against TRAMP-C1 target cells. Experimental data exist to support the hypothesis that NK and DC play an important role in immune surveillance during tumorigenesis (43, 44). Moreover, garlic compounds have been shown to modulate T-cell function (45). To test whether DATS-mediated suppression of PD carcinoma development in TRAMP mice was accompanied by boosting of NK/DC function, the cytotoxicity of these cells isolated from control and 2 mg DATS–treated mice was determined against TRAMP-C1 as a target cell line. The DATS administration did not have any appreciable effect on cytotoxic effects of NK cell alone or NK/DC cell cocultures against TRAMP-C1 cells (data not shown).
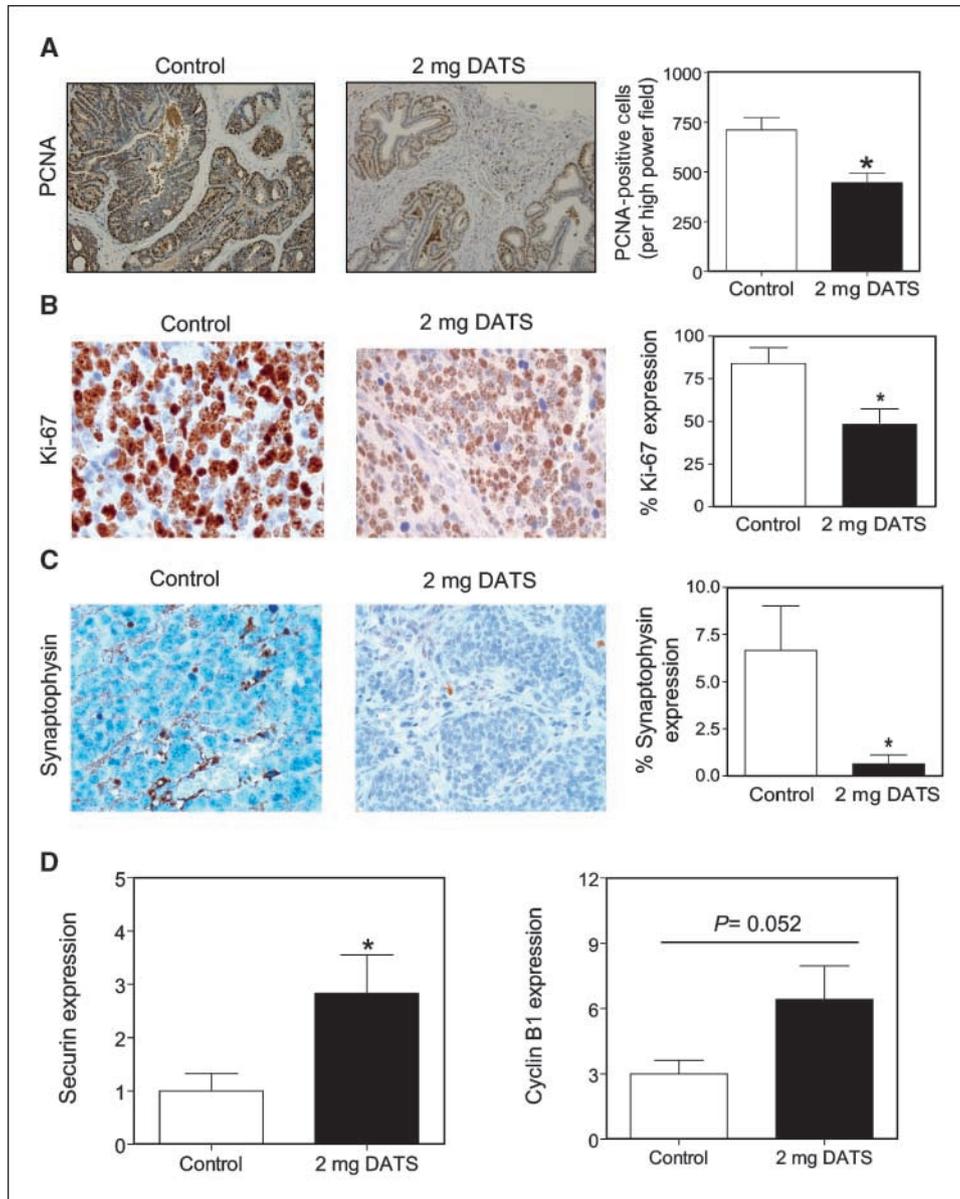
Figure 5. Immunohistochemical analysis for the expression of (*A*) PCNA (magnification, ×200), (*B*) Ki-67 (magnification, ×400), and (*C*) synaptophysin (×600 for control and ×400 magnification for 2 mg DATS–treated group) in the prostate of a representative mouse of both control and 2 mg DATS–treated groups. The bar graphs show quantitative data. *Columns*, mean (*n* = 9–11); *bars*, SE. *, significantly different (*P* < 0.05) compared with control. *D*, immunohistochemical analysis for cyclinB1 and securin protein expression in the dorsolateral prostate of control and 2 mg DATS-treated mice. *Columns*, mean (*n* = 3); *bars*, SE.
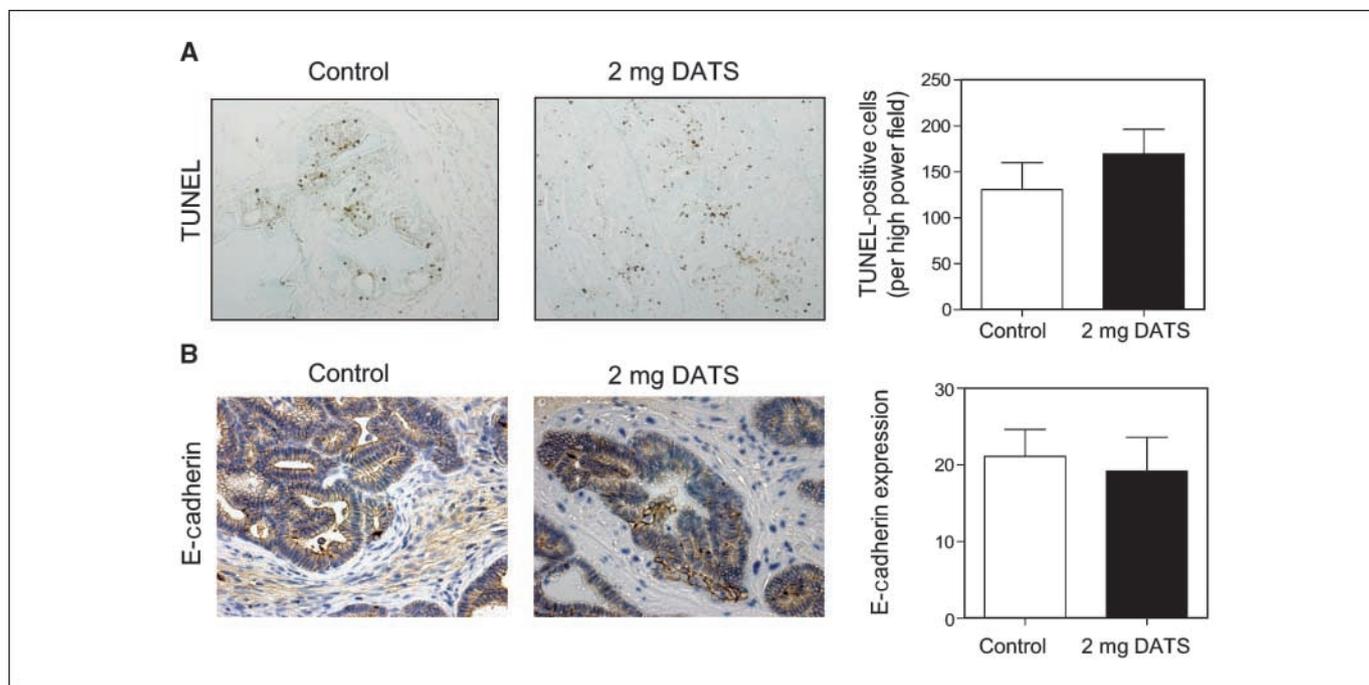
Figure 6. *A,* TUNEL staining in the dorsolateral prostate of a representative mouse of both control and 2 mg DATS-treated group (magnification, ×200). The bar graph represents quantitative analysis of TUNEL-positive apoptotic bodies/high power field. *Columns,* mean (n = 9–10); *bars,* SE. *B,* immunohistochemical analysis for E-cadherin expression in the dorsolateral prostate of a representative mouse of both control and 2 mg DATS–treated group (magnification, ×400). The bar graph represents quantitative analysis of E-cadherin expression. *Columns,* mean (n = 9–10); *bars,* SE.

Discussion

The long latency of prostate carcinogenesis renders this disease highly amenable to chemoprevention. Accordingly, identification and preclinical evaluation of novel agents potentially useful for chemoprevention of human prostate cancer is highly desirable and could have a significant effect on disease-related cost, morbidity, and mortality for a large segment of population. Guided by the results of population-based case-control studies (6), we have devoted considerable effort toward identification and preclinical evaluation of *Allium* vegetable–derived sulfur compounds for their efficacy against human prostate cancer cells (18, 19, 21–27). Our previous studies have revealed that garlic constituent DATS is highly effective in suppressing growth of human prostate cancer cells in culture as well as *in vivo* in xenograft model (18, 19, 21–27). The present study builds upon these observations and shows that DATS administration prevents development of PD carcinoma and multiplicity of lung metastasis in male TRAMP mice without causing weight loss or affecting the T-antigen expression. The incidence and the area occupied by the PD carcinoma were statistically significantly lower in the dorsolateral prostate of DATS-treated TRAMP mice compared with control mice. The DATS concentrations effective against development of PD carcinoma and pulmonary metastasis multiplicity are within the range that can be generated through dietary intake of garlic (37). It is important to point out that DATS has been administered to humans at a dose of 200 mg in combination with 100 μg selenium every other day for 1 month without any harmful side effects (46).

We have shown previously that DATS treatment suppresses growth of cultured human prostate cancer cells by causing G_2 and M phase cell cycle arrest (19, 21, 23, 24). Exposure of PC-3, DU145, and/or LNCaP human prostate cancer cells to growth suppressive concentrations of DATS results in accumulation of G_2 and prometaphase cells (19, 21). The DATS-mediated G_2 phase cell cycle arrest in human prostate cancer cells is transient and correlates with down-regulation and increased Ser216 phosphorylation of cell division cycle 25C (19).[4] On the other hand, the prometaphase arrest resulting from DATS exposure seems irreversible and persists for several hours even after removal of the drug (24). The DATS-mediated prometaphase arrest is not unique to the prostate cancer cells but accompanied by inhibition of anaphase promoting complex/cyclosome as evidenced by accumulation of its substrates cyclinB1 and securin (24). The present study reveals that p.o. administration of DATS causes growth arrest of cancerous cells in the dorsolateral prostate of TRAMP mice. This conclusion is supported by the following observations: (*a*) the dorsolateral prostates from DATS-treated mice exhibit significantly lower protein levels of the proliferation marker PCNA, a 36 kDa protein synthesized in early G_1 and S phases of the cell cycle and implicated in cell cycle progression, DNA replication, and DNA repair (38); (*b*) DATS administration causes a marked decrease in the protein levels of Ki-67, which is a large nuclear protein preferentially expressed during all active phases of the cell cycle (G_1, S, G_2, and M phase; ref. 39), in the dorsolateral prostate; and (*c*) consistent with the results in cultured human prostate cancer cells (24), the dorsolateral prostates from DATS-treated mice display increased levels of cyclinB1 and securin proteins. Thus, it is reasonable to conclude that reduced cellular proliferation is an important mechanism in DATS-mediated prevention of prostate cancer development in TRAMP mice.

We have shown previously that apoptosis induction is an equally important mechanism in antiproliferative effect of DATS against

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[4] S.V. Singh, unpublished results.

human prostate cancer cells (18, 25). It is interesting to note that the number of apoptotic bodies is comparable in the dorsolateral prostate of control and DATS-treated mice. Several possibilities exist to explain discrepancies in the results between cultured human prostate cancer cells and TRAMP model *in vivo*. One possibility relates to the frequency and dose of DATS administration. A more intensive dosing regimen, such as higher dose and/or daily administration of DATS, may be required to elicit apoptotic response in the dorsolateral prostate of TRAMP mice *in vivo*. Likewise, the possibility that earlier treatment with DATS (e.g., starting at age 4 weeks) leads to increased apoptosis as well as even greater protection against prostate carcinogenesis in TRAMP mice cannot be ignored. Additional work is needed to systematically explore these possibilities.

Metastasis is the major cause of death in prostate cancer patients (2). The pathogenesis of metastasis is dynamic and complex involving a series of molecular events including synthesis and secretion of several angiogenic factors to promote neo-vascularization, motility and local invasion of the host stroma, entry into the circulation, detachment, and extravasation (47). Proliferation of the tumor cells within vasculature or the organ parenchyma is necessary for the completion of metastasis process (47). Steps leading to metastasis are complex and regulated by multiple molecules including growth factors, matrix metallopro-teinases, and cell adhesion molecules (47). For example, loss of expression of cell adhesion molecules especially E-cadherin is believed to be important for development of metastatic lesions (42). Inhibition of prostate carcinogenesis and metastasis by plant flavonoids apigenin in TRAMP mice was shown to correlate with retained expression of E-cadherin (32). The present study reveals that the DATS administration inhibits pulmonary metastasis multiplicity in TRAMP mice. However, the inhibitory effect of DATS administration against pulmonary metastasis multiplicity seems independent of changes in E-cadherin expression or

inhibition of angiogenesis. Determination of the precise mechanisms by which DATS administration inhibits pulmonary metastatic multiplicity requires additional work.

The TRAMP model shares many features important in human prostate cancer progression, including metastasis to distant sites, progression to androgen independence, and NE differentiation (48). The number of NE cells correlates with stage, Gleason Grade, and survival in castration-recurrent prostate cancers (48–50). The PD tumors and lymph node metastases in C57/BL6×FVB TRAMP mice express NE marker synaptophysin (40). We found that the fraction of synaptophysin-expressing NE cells is significantly lower in the prostate of 2 mg DATS–treated mice compared with control mice. These results indicate that DATS administration suppresses NE cells in TRAMP mice, which is consistent with inhibition of PD carcinoma development.

In conclusion, the results of the present study indicate that p.o. administration of DATS prevents development of PD carcinoma and multiplicity of pulmonary metastatic lesions in TRAMP mice without causing weight loss or affecting T-antigen expression. The DATS-mediated prevention of prostate cancer development correlates with reduced cell proliferation as evidenced by suppression of PCNA and Ki-67 expression and accumulation of cyclinB1 and securin proteins.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. CA Cancer J Clin 2007;57:43–66.
2. Nelson WG, DeMarzo A, Isaacs WB. Prostate cancer. N Engl J Med 2003;349:366–81.
3. Newman DJ, Cragg GM, Snader KM. Natural products as sources of new drugs over the period 1981–2002. J Nat Prod 2003;66:1022–37.
4. You WC, Blot WJ, Chang YS, et al. Allium vegetables and reduced risk of stomach cancer. J Natl Cancer Inst 1989;18:162–4.
5. Gao CM, Takezaki T, Ding JH, Li MS, Tajima K. Protective effect of allium vegetables against both esophageal and stomach cancer: a simultaneous case-referent study of a high-epidemic area in Jiangsu Province, China. Jpn J Cancer Res 1999;90:614–21.
6. Hsing AW, Chokkalingam AP, Gao YT, et al. Allium vegetables and risk of prostate cancer: a population-based study. J Natl Cancer Inst 2002;94:1648–51.
7. Block E. The organosulfur chemistry of the genus *Allium*- implications for the organic chemistry of sulfur. Angew Chem Int Ed Engl 1992;31:1135–78.
8. Sparnins VL, Barany G, Wattenberg LW. Effects of organosulfur compounds from garlic and onions on benzo[*a*]pyrene-induced neoplasia and glutathione S-transferase activity in the mouse. Carcinogenesis 1988;9:131–4.
9. Wargovich MJ, Woods C, Eng VWS, Stephens LC, Gray K. Chemoprevention of *N*-nitrosomethylbenzylamine-induced esophageal cancer in rats by the naturally

occurring thioether, diallyl sulfide. Cancer Res 1988;48:6872–5.
10. Reddy BS, Rao CV, Rivenson A, Kelloff G. Chemo-prevention of colon carcinogenesis by organosulfur compounds. Cancer Res 1993;53:3493–8.
11. Hu X, Benson PJ, Srivastava SK, et al. Induction of glutathione S-transferase π as a bioassay for the evaluation of potency of inhibitors of benzo(a)pyrene-induced cancer in a murine model. Int J Cancer 1997;73:897–902.
12. Hu X, Benson PJ, Srivastava SK, et al. Glutathione S-transferases of female A/J mouse liver and forestomach and their differential induction by anti-carcinogenic organosulfides from garlic. Arch Biochem Biophys 1996;336:199–214.
13. Singh SV, Pan SS, Srivastava SK, et al. Differential induction of NAD(P)H:quinone oxidoreductase by anti-carcinogenic organosulfides from garlic. Biochem Biophys Res Commun 1998;244:917–20.
14. Brady JF, Ishizaki H, Fukuto JM, et al. Inhibition of cytochrome P-450 2E1 by diallyl sulfide and its metabolites. Chem Res Toxicol 1991;4:642–7.
15. Sundaram SG, Milner JA. Diallyl disulfide induces apoptosis of human colon tumor cells. Carcinogenesis 1996;17:669–73.
16. Knowles LM, Milner JA. Diallyl disulfide inhibits p34(cdc2) kinase activity through changes in complex formation and phosphorylation. Carcinogenesis 2000;21:1129–34.
17. Filomeni G, Aquilano K, Rotilio G, Ciriolo MR. Reactive oxygen species-dependent c-Jun NH$_2$-terminal kinase/c-Jun signaling cascade mediates neuroblastoma

cell death induced by diallyl disulfide. Cancer Res 2003;63:5940–9.
18. Xiao D, Choi S, Johnson DE, et al. Diallyl trisulfide-induced apoptosis in human prostate cancer cells involves c-Jun N-terminal kinase and extracellular-signal regulated kinase-mediated phosphorylation of Bcl-2. Oncogene 2004;23:5594–606.
19. Xiao D, Herman-Antosiewicz A, Antosiewicz J, et al. Diallyl trisulfide-induced G$_2$-M phase cell cycle arrest in human prostate cancer cells is caused by reactive oxygen species-dependent destruction and hyperphos-phorylation of Cdc25C. Oncogene 2005;24:6256–68.
20. Hosono T, Fukao T, Ogihara J, et al. Diallyl trisulfide suppresses the proliferation and induces apoptosis of human colon cancer cells through oxidative modifica-tion of β-tubulin. J Biol Chem 2005;280:41487–93.
21. Herman-Antosiewicz A, Singh SV. Checkpoint kinase 1 regulates diallyl trisulfide-induced mitotic arrest in human prostate cancer cells. J Biol Chem 2005;280:28519–28.
22. Xiao D, Singh SV. Diallyl trisulfide, a constituent of processed garlic, inactivates Akt to trigger mitochon-drial translocation of BAD and caspase-mediated apoptosis in human prostate cancer cells. Carcinogen-esis 2006;27:533–40.
23. Antosiewicz J, Herman-Antosiewicz A, Marynowski SW, Singh SV. c-Jun NH$_2$-terminal kinase signaling axis regulates diallyl trisulfide-induced generation of reactive oxygen species and cell cycle arrest in human prostate cancer cells. Cancer Res 2006;66:5379–86.
24. Herman-Antosiewicz A, Stan SD, Hahm ER, Xiao D, Singh SV. Activation of a novel ataxia-telangiectasia

mutated and Rad3 related/checkpoint kinase 1-dependent prometaphase checkpoint in cancer cells by diallyl trisulfide, a promising cancer chemopreventive constituent of processed garlic. Mol Cancer Ther 2007; 6:1249–61.

25. Kim YA, Xiao D, Xiao H, et al. Mitochondria-mediated apoptosis by diallyl trisulfide in human prostate cancer cells is associated with generation of reactive oxygen species and regulated by Bax/Bak. Mol Cancer Ther 2007;6:1599–609.

26. Xiao D, Li M, Herman-Antosiewicz A, et al. Diallyl trisulfide inhibits angiogenic features of human umbilical vein endothelial cells by causing Akt inactivation and down-regulation of VEGF and VEGF-R2. Nutr Cancer 2006;55:94–107.

27. Xiao D, Lew KL, Kim Y, et al. Diallyl trisulfide suppresses growth of PC-3 human prostate cancer xenograft *in vivo* in association with Bax and Bak induction. Clin Cancer Res 2006;15:6836–43.

28. Klein RD. The use of genetically engineered mouse models of prostate cancer for nutrition and cancer chemoprevention research. Mutat Res 2005; 576:111–9.

29. Greenberg NM, DeMayo F, Finegold MJ, et al. Prostate cancer in a transgenic mouse. Proc Natl Acad Sci U S A 1995;92:3439–43.

30. Kaplan-Lefko PJ, Chen TM, Ittmann MM, et al. Pathobiology of autochthonous prostate cancer in a pre-clinical transgenic mouse model. Prostate 2003;55: 219–37.

31. Gupta S, Ahmad N, Marengo SR, et al. Chemoprevention of prostate carcinogenesis by α-difluoromethylornithine in TRAMP mice. Cancer Res 2000;60: 5125–33.

32. Shukla S, MacLennan GT, Flask CA, et al. Blockade of β-catenin signaling by plant flavonoid apigenin suppresses prostate carcinogenesis in TRAMP mice. Cancer Res 2007;67:6925–35.

33. Raina K, Singh RP, Agarwal R, Agarwal C. Oral grape seed extract inhibits prostate tumor growth and progression in TRAMP mice. Cancer Res 2007;67: 5976–82.

34. Giezeman-Smits KM, Okada H, Brissette-Storkus CS, et al.Cytokine gene therapy of gliomas: induction of reactive CD4+ T cells by interleukin-4-transfected 9L gliosarcoma is essential for protective immunity. Cancer Res 2000;60:2449–57.

35. Brissette-Storkus CS, Kettel JC, Whitham TF, et al. Flt-3 ligand (FL) drives differentiation of rat bone marrow-derived dendritic cells expressing OX62 and/or CD161 (NKR-P1). J Leukoc Biol 2002;71:941–9.

36. Xiao D, Zeng Y, Choi S, Lew KL, Nelson JB, Singh SV. Caspase dependent apoptosis induction by phenethyl isothiocyanate, a cruciferous vegetable derived cancer chemopreventive agent, is mediated by Bak and Bax. Clin Cancer Res 2005;11:2670–9.

37. Shukla Y, Kalra N. Cancer chemoprevention with garlic and its constituents. Cancer Lett 2007;247:167–81.

38. Chuang LC, Yew PR. Proliferating cell nuclear antigen recruits cyclin-dependent kinase inhibitor Xic1 to DNA and couples its proteolysis to DNA polymerase switching. J Biol Chem 2005;280:35299–309.

39. Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. J Cell Physiol 2000;182:311–22.

40. Huss WJ, Gray DR, Tavakoli K, et al. Origin of androgen-insensitive poorly differentiated tumors in the transgenic adenocarcinoma of mouse prostate model. Neoplasia 2007;9:938–50.

41. Chiaverotti T, Couto SS, Donjacour A, et al. Dissociation of epithelial and neuroendocrine carcinoma lineage in the transgenic adenocarcinoma of mouse prostate model of prostate cancer. Am J Pathol 2008;172: 236–46.

42. Wheelock MJ, Johnson KR. Cadherins as modulators of cellular phenotype. Annu Rev Cell Dev Biol 2003;19: 207–35.

43. Terabe M, Berzofsky JA. NKT cells in immunoregulation of tumor immunity: a new immunoregulatory axis. Trends Immunol 2007;28:491–6.

44. Ljunggren HG, Malmberg KJ. Prospects for the use of NK cells in immunotherapy of human cancer. Nat Rev Immunol 2007;7:329–39.

45. Lau BH, Yamasaki T, Gridley DS. Garlic compounds modulate macrophage and T-lymphocyte functions. Mol Biother 1991;3:103–7.

46. Li H, Li H, Wang Y, et al. An intervention study to prevent gastric cancer by micro-selenium and large dose of allitridum. Chinese Med J 2004;117:1155–60.

47. Fidler IJ, Kim SJ, Langley RR. The role of the organ microenvironment in the biology and therapy of cancer metastasis. J Cell Biochem 2007;101:927–36.

48. Segawa N, Mori I, Utsunomiya H, et al. Prognostic significance of neuroendocrine differentiation, proliferation activity and androgen receptor expression in prostate cancer. Pathol Int 2001;51:452–9.

49. Aprikian AG, Cordon-Cardo C, Fair WR, et al. Neuroendocrine differentiation in metastatic prostatic adenocarcinoma. J Urol 1994;151:914–9.

50. Ahlgren G, Pedersen K, Lundberg S, Aus G, Hugosson J, Abrahamsson PA. Regressive changes and neuroendocrine differentiation in prostate cancer after neoadjuvant hormonal treatment. Prostate 2000;42:274–9.