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

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Abstract

Identification of agents that are non-toxic 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 demonstrate that oral gavage of garlic constituent diallyl trisulfide (1 and 2 mg/d, thrice/week for thirteen weeks beginning at eight weeks of age) 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 demonstrate, for the first time, that DATS administration prevents progression to invasive carcinoma and lung metastasis in TRAMP mice.

Keywords

Diallyl Trisulfide; Prostate Cancer; Metastasis; Chemoprevention

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 non-toxic 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).

Epidemiological 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 <2.2g/day in a population-based, case-control study (6). The anticarcinogenic effect of *Allium* vegetables is attributable to organosulfur compounds (OSCs) 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 G2-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 (PrEC) is significantly more resistant to G2-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 G2 and prometaphase arrest and mitochondria-mediated apoptotic cell death (18,19,21-26). Additionally, the growth of PC-3 human prostate cancer cells subcutaneously implanted in athymic mice was significantly retarded by oral 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 demonstrate that oral 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 (St. Paul, MN). Antibodies against CD31 (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 (Woburn, MA); and the antibodies against T-antigen and synaptophysin were from BD Biosciences. The 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 histological 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 non-transgenic FVB male mice. Transgene verification was performed using DNA obtained from tail clippings at 14-17 days of life as described by Greenberg *et al.* (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 eight weeks of age, mice were randomized into three groups. Mice in the control group (n= 16) received 0.1 mL phosphate buffered saline (PBS) by oral gavage three times per week (Monday, Wednesday, and Friday) while the experimental groups of mice (n= 19) received either 1 mg DATS/d or 2 mg DATS/d in 0.1 mL PBS by oral gavage three times 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. Following thirteen weeks of treatment, the animals were sacrificed 24 h after the last administration of the vehicle or DATS by CO₂ 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 4-5 µm thickness for hematoxylin and eosin (H&E) staining, TUNEL assay, and immunohistochemical analyses.

Pathological 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 pathological stage. Pathological 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 Tris-buffered saline 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 non-overlapping 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 terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining

The paraffin-embedded tissue sections were deparaffinized, rehydrated, and then used to visualize apoptotic bodies by TUNEL staining employing 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, non-overlapping high-power fields.

Cytotoxicity of natural killer (NK) and dendritic cells (DC)

The NK cells were isolated from the spleen of control and DATS-treated mice using MACS beads (Myltenyi Biotech) as described by Giezeman-Smith et al. (34) and cultured in interleukin-2 supplemented (1000 units/mL) media. 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), GM-CSF (500 ng/mL), and Flt-3 (25 ng/mL). After 5 days of culture, NK cells were plated in 96-well plates alone or co-cultured with DCs (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 non-radioactive 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 $P \leq 0.05$.

Results

Oral administration of DATS prevented development of poorly-differentiated prostate carcinoma in TRAMP mice

A preliminary dose-finding study involving 8-week old non-transgenic male [C57BL/6×FVB] F1 littermates (5 mice/group) revealed that oral gavage of 1 and 2 mg DATS, thrice per week, was well-tolerated by the mice. The non-transgenic mice treated with 1 and 2 mg DATS appeared 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 non-transgenic mice were also normal as judged by histology and wet organ weight measurements (results 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. 1A, the average wet weight of the urogenital tract in mice administered with 1 and 2 mg DATS was about 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 about 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. 1A). As shown in Fig. 1B, the initial and final body weights of the control and DATS-treated mice did not differ significantly.

The histological 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 as DATS-treated mice with prostate weight of >1g 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. 2A. 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. 2B). 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 administered with 1 and 2 mg DATS was lower by about 64% ($P = 0.0484$) and 76% ($P = 0.0189$), respectively, compared with control mice (Fig. 2C). The DATS administration also resulted in a modest increase in the area occupied by the PIN and WD carcinomas compared with control mice (Fig. 2C). 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 about 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. 2C). Likewise, the area occupied by the WD carcinoma in the dorsolateral prostate of mice treated with 2 mg DATS was higher by about 45% compared with control mice ($P = 0.0986$) (Fig. 2C). 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 since 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. The Fig. 3A 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. 3B and Fig. 3C, 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. Fig. 4A 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 about 88% in vehicle-treated control mice, which was reduced to about 79% and 68% in mice administered with 1 and 2 mg DATS, respectively (Fig. 4B). 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. 4B). Majority of the control mice exhibited multiple pulmonary metastatic lesions. The multiplicity of the pulmonary metastasis in mice administered with 1 and 2 mg DATS was lower by about 49% and 55% ($P=0.002$) compared with control mice (Fig. 4C). 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. 5A. The PCNA expression was about 46% lower in the dorsolateral prostate of mice administered with 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. 5B), 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. 2B,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 mice was due to increased apoptosis. Even though 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. 6B). 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 platelet endothelial adhesion molecule or 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 (results 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 employed in the present study.

Cytotoxicity of natural killer (NK) and dendritic cells (DC) 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 co-cultures against TRAMP-C1 cells (results not shown).

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 impact 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 towards 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 demonstrates that DATS administration prevents development of poorly-differentiated 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 poorly-differentiated 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 poorly-differentiated 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 one month without any harmful side effects (46).

We have shown previously that DATS treatment suppresses growth of cultured human prostate cancer cells by causing G2 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 G2 and prometaphase cells (19,21). The DATS-mediated G2 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 and unpublished results). 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 oral 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 G1 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 (G1, S, G2, 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 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 4 weeks of age) leads to increased apoptosis as well as even greater protection against prostate carcinogenesis in TRAMP mice can't 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 neovascularization, 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 metalloproteinases, 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 neuroendocrine differentiation (48). The number of NE cells correlates with stage, Gleason Grade, and survival in castration-recurrent prostate cancers (48-50). The poorly-differentiated 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 oral administration of DATS prevents development of poorly-differentiated 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.

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Figure 1. Effect of oral 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.

Figure 2.

A, H&E staining in the dorsolateral prostate of representative vehicle-treated control TRAMP mice and TRAMP mice administered with 1 or 2 mg DATS, thrice weekly (Monday, Wednesday, and Friday) for thirteen weeks beginning at eight weeks of age (200 $\times$ magnification). B, incidence of histological grades classified as normal prostate gland (N), low- and high-grade prostatic intraepithelial neoplasia (PIN), well-differentiated (WD) carcinoma, and poorly-differentiated (PD) carcinoma in the dorsolateral prostate of vehicle-treated control TRAMP mice and TRAMP mice administered with 1 or 2 mg DATS, thrice weekly (Monday, Wednesday, and Friday) for thirteen weeks. Tissue sections from mice with wet prostate weight of <1g were used because the majority of the area of the prostate gland larger than 1g 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 (N), PIN, WD carcinoma, and PD carcinoma. Columns, mean ($n=11$ for the control group, and $n=15$ and 13 for the 1 mg and 2 mg DATS treatment group, respectively); bars, variability of the results in histological grading between two independent investigators.

*Significantly different compared with vehicle-treated control by Wilcoxon rank-sum test.

Figure 3.

A, immunohistochemical analysis for T-antigen expression in a representative dorsolateral prostate of both control and 2 mg DATS-treated mouse (400× magnification). *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 re-probed with anti-actin antibody to correct for differences in protein loading.

Figure 4.

A, H&E staining depicting metastatic lesion in the lung of a representative control mouse and a 2 mg DATS-treated mouse (200× magnification). **B**, incidence of metastasis in the lungs and pelvic lymph nodes of vehicle-treated control TRAMP mice and TRAMP mice administered with 1 or 2 mg DATS. **C**, pulmonary metastasis multiplicity in vehicle-treated control TRAMP mice and TRAMP mice administered with 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.

Figure 5. Immunohistochemical analysis for the expression of (A) proliferating cell nuclear antigen (PCNA; 200× magnification), (B) Ki-67 (400× magnification), 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 (200× magnification). 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 (400× magnification). The bar graph represents quantitative analysis of E-cadherin expression. *Columns*, mean ($n=9-10$); *bars*, SE.