

Diallyl trisulfide increases the effectiveness of TRAIL and inhibits prostate cancer growth in an orthotopic model: molecular mechanisms

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Abstract

Recent studies have shown that naturally occurring compounds can enhance the efficacy of chemotherapeutic drugs. The objectives of this study were to investigate the molecular mechanisms by which diallyl trisulfide (DATS) enhanced the therapeutic potential of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in prostate cancer cells *in vitro* and on orthotopically transplanted PC-3 prostate carcinoma in nude mice. DATS inhibited cell viability and colony formation and induced apoptosis in PC-3 and LNCaP cells. DATS enhanced the apoptosis-inducing potential of TRAIL in PC-3 cells and sensitized TRAIL-resistant LNCaP cells. Dominant-negative FADD inhibited the synergistic interaction between DATS and TRAIL on apoptosis. DATS induced the expression of DR4, DR5, Bax, Bak, Bim, Noxa, and PUMA and inhibited expression of Mcl-1, Bcl-2, Bcl-X_L, survivin, XIAP, cIAP1, and cIAP2. Oral administration of DATS significantly inhibited growth of orthotopically implanted prostate carcinoma in BALB/c nude mice compared with the control group, without causing weight loss. Cotreatment of mice with DATS and TRAIL was more effective in inhibiting prostate tumor growth and inducing DR4 and DR5 expression, caspase-8 activity, and apoptosis than either agent alone. DATS inhibited angiogenesis (as measured by CD31-positive and factor VIII-positive blood vessels and hypoxia-inducible factor-1 α , vascular endothelial growth factor, and interleukin-6

expression) and metastasis [matrix metalloproteinase (MMP)-2, MMP-7, MMP-9, and MT-1 MMP expression], which were correlated with inhibition in AKT and nuclear factor- κ B activation. The combination of DATS and TRAIL was more effective in inhibiting markers of angiogenesis and metastasis than either agent alone. These data suggest that DATS can be combined with TRAIL for the prevention and/or treatment of prostate cancer. [Mol Cancer Ther 2008;7(8):2328–38]

Introduction

Prostate cancer is one of the major life-threatening diseases in most western countries. The incidence and mortality rates of prostate cancer have also rapidly increased in the past decade. Although patients with metastatic prostate cancer can benefit from androgen-ablation therapy at the initial stage, most patients die of hormone-refractory prostate cancer in only few years. Salvage cytotoxic therapy has been notoriously related to significant morbidity with little, if any, survival benefit. Therefore, new agents and approaches are needed to prevent prostate cancer.

Diallyl sulfide, diallyl disulfide, and diallyl trisulfide (DATS) are major organosulfur compounds of garlic, which is widely used as a food spice. Organosulfur compounds can modulate drug metabolism systems, especially various phase II detoxifying enzymes (1). Garlic has been used for centuries as a naturally occurring herbal remedy for lowering the blood pressure and cholesterol as well other diseases (2). DATS has been recognized as an antioxidant that has antiproliferative and anticarcinogenic properties (3). DATS can augment the activation of T cells and enhance the antitumor function of macrophage, suggesting that DATS may be potentially useful in tumor therapy (4). DATS inhibited cyclooxygenase-2 gene expression (5). DATS increases histone acetylation and p21^{WAF1/CIP1} expression in human colon tumor cell lines (6). However, there are no studies examining the effects of DATS on the regulation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptors in prostate cancer.

TRAIL induces apoptosis in a wide variety of transformed and cancer cells but not in normal cells (7). Therefore, TRAIL is considered to be a tumor-selective, apoptosis-inducing cytokine and a promising new candidate for cancer prevention and treatment. TRAIL binds to several distinct receptors: (a) TRAIL-R1/DR4, (b) TRAIL-R2/DR5, (c) TRAIL-R3/DcR1, and (d) TRAIL-R4/DcR2 (7). Both DR4 and DR5 contain the intracellular death domain essential for the induction of apoptosis following receptor ligation. In contrast, neither DcR1 nor DcR2 mediates apoptosis due to a complete or partial lack of

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the intracellular death domain, respectively (7). TRAIL receptors are expressed ubiquitously in cancer cells. The binding of TRAIL to DR4 and DR5 leads to the cleavage and activation of caspase-8 (8) that in turn activates downstream effector caspases, such as caspase-3 and caspase-7, leading to apoptosis (7). The activation of caspase-8 also links to mitochondrial pathway of apoptosis through Bid. We have shown recently that the activation of caspase-8 by TRAIL cleaves BID, whose cleavage product triggers mitochondrial depolarization and subsequent release of cytochrome *c* and Smac/DIABLO from mitochondria (9). The role of BH3-only proteins at the level of mitochondria in DATS-induced apoptosis has not been investigated. We have recently observed several TRAIL-resistant prostate, breast, and lung cancer cell lines (10–13). However, these resistant cells can be sensitized by down-regulation of constitutively active AKT and nuclear factor- κ B (NF- κ B) or pretreatment with chemotherapeutic drugs and irradiation (13, 14). Similarly, DATS may enhance the therapeutic potential of TRAIL in prostate cancer.

Angiogenesis plays an important role in a multitude of biological processes including those of tumorigenesis and cancer progression. Hypoxia is the prime driving factor for tumor angiogenesis and the family of hypoxia-inducible factors (HIF) plays a pivotal role in this process. HIF-1, a heterodimer of HIF-1 α and HIF-1 β subunits, is a transcriptional activator central to the cellular response to low oxygen that includes metabolic adaptation, angiogenesis, metastasis, and inhibited apoptosis. Hypoxia is the key to increased expression of HIF-1 α resulting in increased expression of growth factors [e.g., vascular endothelial growth factor (VEGF) and epidermal growth factor]. Overexpression of HIF-1 α is correlated with increased tumor invasiveness and resistance to chemotherapy and has been associated with a poor prognosis in a variety of malignant tumors. Therefore, HIF expression could be a useful target for therapeutic intervention.

The purpose of our studies was to investigate the molecular mechanisms by which DATS enhanced the therapeutic potential of TRAIL *in vitro* and *in vivo* models of prostate cancer. Our results indicated that DATS inhibited PC-3 tumor growth, metastasis, and angiogenesis in an orthotopic model of nude mice through regulation of AKT and NF- κ B and its gene products and enhanced the apoptosis-inducing potential of TRAIL. Thus, DATS can be used alone or in combination with TRAIL for the treatment and/or prevention of prostate cancer.

Materials and Methods

Reagents

Antibodies against CD31, VEGF, Bcl-2, Bcl-X_L, Bax, Bak, TRAIL-R1/DR4, TRAIL-R2/DR5, Bid, PUMA, IKK γ , Noxa, Bim, HIF-1 α , p65-NF- κ B, and β -actin were purchased from Santa Cruz Biotechnology. Antibodies against phospho-AKT (S473), AKT, phospho-I κ B α (Ser³² and Ser³⁶), MMP-2, MMP-7, MT-1 MMP, and MMP-9 were purchased from Cell Signaling Technology. Enhanced chemiluminescence Western blot detection reagents were from Amersham Life

Sciences. TRAIL was purified as described elsewhere (15). DATS was purchased from LKT Laboratories. Anti-caspase-3, anti-caspase-8, anti-caspase-9, and anti-poly(ADP-ribose) polymerase antibodies were purchased from BD Biosciences/PharMingen. Kit for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling and JC-1 dye (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzamidazolocarboyanin iodide) were purchased from EMD Biosciences/Calbiochem. Antibodies against TRAIL-R1/DR4, TRAIL-R2/DR5, DcR1, and DcR2 for flow cytometry were purchased from R&D Systems.

Western Blot Analysis

Western blots were done as we described earlier (10, 11). In brief, cells were lysed in radioimmunoprecipitation assay buffer containing 1 \times protease inhibitor cocktail, and protein concentrations were determined using the Bradford assay (Bio-Rad). Proteins were separated by 12.5% SDS/PAGE and transferred to Immobilon membranes (Millipore) in a Tris (20 mmol/L), glycine (150 mmol/L), and methanol (20%) buffer at 55 V for 4 h at 4°C. After blotting in 5% nonfat dry milk in TBS, the membranes were incubated with primary antibodies at 1:1,000 dilution in TBS overnight at 4°C and then secondary antibodies conjugated with horseradish peroxidase at 1:5,000 dilution in TBS-Tween 20 for 1 h at room temperature. Protein bands were visualized on X-ray film using an enhanced chemiluminescence system.

Measurement of Death Receptors

Cells were detached with 0.5 mmol/L EDTA, and washed three times (spun at 500 \times g for 5 min) with an isotonic PBS wash buffer supplemented with 0.5% bovine serum albumin. Cells (1 \times 10⁵) were resuspended in 200 μ L PBS, stained with primary antibody (1 μ g/mL), and incubated for 30 min at 4°C. Unreacted antibody was removed by washing the cells twice with the same PBS buffer. Cells were stained with secondary antibody conjugated with phycoerythrin and incubated for 30 min at 4°C. Unbound phycoerythrin-conjugated antibody was washed twice with PBS. Cells were resuspended in 200 μ L PBS. Cell surface expression of DR4, DR5, DcR1 and DcR2 was measured by flow cytometry.

Measurement of Mitochondrial Membrane Potential

Mitochondrial energization was determined by retention of JC-1 dye (Molecular Probes) as we described earlier (16). Briefly, drug-treated cells (5 \times 10⁵) were loaded with JC-1 dye (1 μ g/mL) during the last 30 min of incubation at 37°C in a 5% CO₂ incubator. Cells were washed in PBS twice. Fluorescence was monitored in a fluorometer using 570 nm excitation/595 nm emission for the J-aggregate of JC-1 (17). Mitochondrial membrane potential was calculated as a ratio of the fluorescence of J-aggregate (aqueous-phase) and monomer (membrane-bound) forms of JC-1.

IKK Assay

Tumor lysates were incubated with 2 μ g/mL anti-NEMO/IKK γ antibody for 2 h at 4°C. Immunocomplex was precipitated using protein G-PLUS-Agarose beads overnight at 4°C. Beads were washed and then resuspended in 30 μ L kinase buffer [50 mmol/L Tris-HCl (pH 8), 100 mmol/L NaCl,

2 mmol/L MgCl₂, 1 mmol/L DTT, 1 mmol/L NaF, 1 mmol/L Na₃VO₄, 25 mmol/L β-glycerophosphate, 10 mmol/L NPP, and proteases inhibitor cocktail (complete, Roche) supplemented with ATP (1 mmol/L) in the presence of wild-type glutathione S-transferase-IκBα₁₋₅₅] and were incubated at 30°C for 30 min. Reactions were stopped by the addition of SDS loading buffer and were subjected to SDS-PAGE. Proteins were electrotransferred to polyvinylidene difluoride membranes and blotted with a phosphospecific anti-IκBα (Ser³²-Ser³⁶) antibody.

Orthotopic Assays in Nude Mice

Athymic male nude mice (BALB/c *nu/nu*, 4-6 weeks old) were purchased from the National Cancer Institute. Nude mice were anesthetized with 5% halothane before inoculation with prostate cancer cells. Prostate gland was exposed

following a lower midline incision. Mice were inoculated with PC-3 cells (1 × 10⁶ per 100 μL medium) into the dorsolateral lobe of the prostatic capsule by means of a 21-gauge needle and a calibrated pushbutton syringe. Proper inoculation of cell suspension was indicated by blebbing under the prostatic capsule. The incision was closed by using a running suture of 5-0 silk. Tumor growth was measured by palpation. Tumor-bearing mice were randomized into four groups, and the following treatment protocols was implemented: group 1: vehicle control (0.1 mL normal saline containing 0.5% DMSO) administered by oral injection everyday 5 days/wk throughout the duration of experiment; group 2: TRAIL (15 mg/kg) administered i.v. on days 2, 8, 15, and 22; group 3: DATS (40 mg/kg in 0.1 mL normal saline containing 0.5% DMSO)

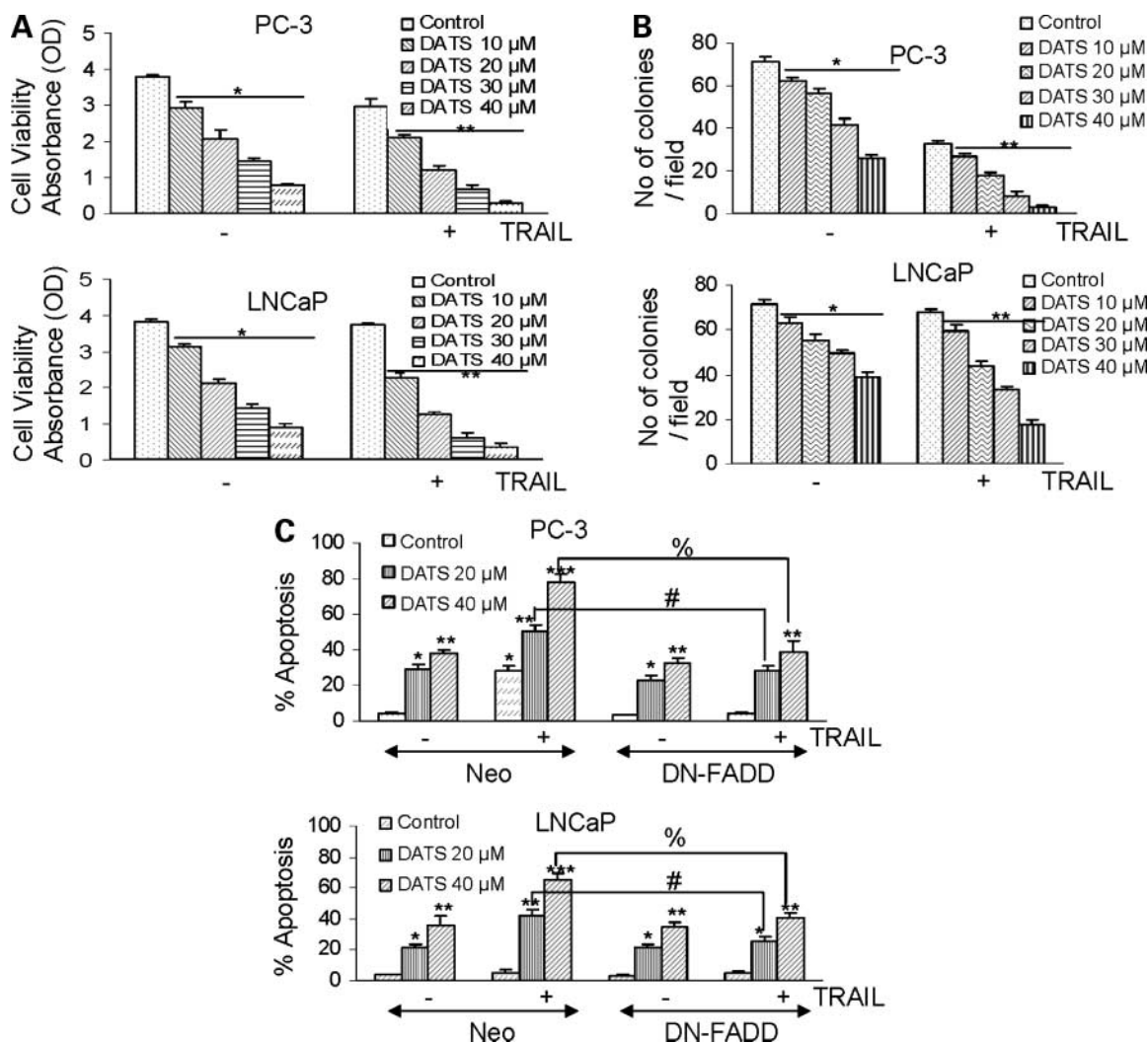
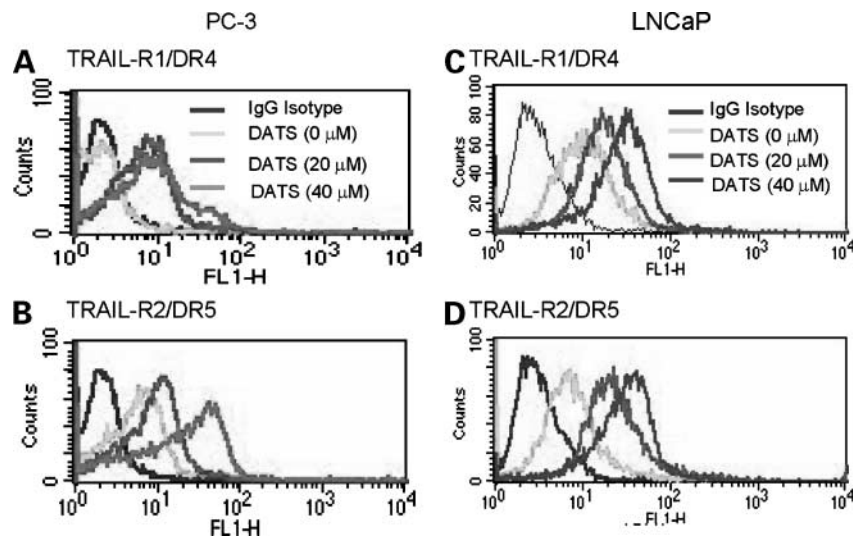


Figure 1. Interactive effects of DATS and TRAIL on cell viability, colony formation and apoptosis. **A**, PC-3 and LNCaP cells were treated with various doses of DATS (0-40 μmol/L) for 24 h followed by treatment with TRAIL for another 24 h. Cell viability was measured by XTT assay. **B**, PC-3 and LNCaP cells were seeded in soft agar and treated with various doses of DATS (0-40 μmol/L) for 24 h followed by treatment with TRAIL. After 3 wk of incubation, no of colonies were counted. **C**, PC-3 and LNCaP cells were treated with various doses of DATS (0-40 μmol/L) for 24 h followed by treatment with TRAIL for another 24 h. Apoptosis was measured by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay as per manufacturer's instructions. *, **, ***, $P < 0.05$, significantly different from respective control. #, %, significantly different between groups.

Figure 2. Effects of DATS on the expression of death receptors in prostate cancer cells. PC-3 (A and B) and LNCaP (C and D) cells were treated with or without DATS (20 or 40 $\mu\text{mol/L}$) for 24 h. Cell surface expression of TRAIL-R1/DR4 and TRAIL-R2/DR5 receptors was measured by flow cytometric analysis.



administered by oral injection everyday 5 days/wk throughout the duration of experiment; and group 4: DATS plus TRAIL, DATS (40 mg/kg in 0.1 mL normal saline containing 0.5% DMSO) administered by oral injection everyday 5 days/wk throughout the duration of experiment, and TRAIL (15 mg/kg) administered i.v. on days 2, 8, 15, and 22.

Immunohistochemistry

Immunohistochemistry was done as described earlier (18). In brief, tumor tissues were collected after 4 weeks of treatment, excised and fixed with 10% formalin, embedded in paraffin, and sectioned. Tissue sections were stained with various primary antibodies at room temperature for 4 h. Subsequently, slides were washed three times in PBS and incubated with secondary antibody at room temperature for 1 h. Finally, alkaline phosphatase or hydrogen peroxide polymer-AEC chromagen substrate kits were used as per manufacturer's instructions (Lab Vision). After washing with PBS, Vectashield (Vector Laboratories) mounting medium was applied and sections were cover-slipped and imaged. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assays were done as per manufacturer's instructions.

Statistical Analysis

The mean and SD were calculated for each experimental group. Differences between groups were analyzed by one- or two-way ANOVA followed by Bonferroni's multiple comparison tests using PRISM statistical analysis software (GraphPad Software). Significant differences among groups were calculated at $P < 0.05$.

Results

DATS Enhances the Apoptosis Inducing Potential of TRAIL in PC-3 Cells and Sensitizes TRAIL-Resistant LNCaP Cells

We have recently shown that chemopreventive agents such as curcumin and resveratrol can enhance the

therapeutic potential of TRAIL in prostate cancer cells (10, 11, 18, 19). Here, we have extended the hypothesis to examine whether DATS can enhance the therapeutic potential of TRAIL in PC-3 cells. We first examined the effects of DATS and TRAIL on cell viability and colony formation in PC-3 and LNCaP cells. DATS inhibited cell viability and colony formation in PC-3 and LNCaP cells in a dose-dependent manner (Fig. 1A and B). Whereas TRAIL alone was effective, the pretreatment of PC-3 cells with DATS followed by TRAIL further inhibited cell viability and colony formation.

We next examined whether DATS can sensitize TRAIL-resistant LNCaP cells. Whereas TRAIL was ineffective, DATS inhibited cell viability and colony formation in LNCaP cells (Fig. 1A and B). The pretreatment of LNCaP cells with DATS followed by TRAIL caused an inhibition of cell viability and colony growth. These data suggest that DATS can enhance the therapeutic potential of TRAIL in prostate cancer cells.

Because TRAIL induces apoptosis through activation of death receptor pathway, we next sought to examine the involvement of death receptor pathway in synergistic interaction between DATS and TRAIL. We have shown previously that dominant-negative FADD blocked TRAIL-induced apoptosis and also inhibited synergistic interaction between curcumin and TRAIL or resveratrol and TRAIL (10, 18). Treatment of PC-3 and LNCaP cells with DATS induced apoptosis in a dose-dependent manner. DATS enhanced apoptosis-inducing potential of TRAIL in PC-3 cells and sensitized TRAIL-resistant LNCaP cells to undergo apoptosis (Fig. 1C). Dominant-negative FADD blocked TRAIL-induced apoptosis in PC-3 cells. Although dominant-negative FADD had no effect on DATS-induced apoptosis, it inhibited the synergistic interaction between DATS and TRAIL in both cell lines. These data suggest the involvement of TRAIL death receptor pathway in the synergistic interaction between DATS and TRAIL.

DATS Induces Death Receptor TRAIL-R1/DR4 and TRAIL-R2/DR5 Expression

We have shown recently that histone deacetylase inhibitors, curcumin, resveratrol, chemotherapeutic drugs, and γ -irradiation induce expression of death receptors DR4 and/or DR5 in leukemia, multiple myeloma, and breast and prostate cancer cells, so that successive treatment with TRAIL results in apoptosis in an additive or synergistic manner (10–13, 18–23). Here, we have extended this concept to prostate cancer cells where we propose to examine whether DATS induces sensitivity by up-regulating DR4 and/or DR5 expression. Treatment of PC-3 cells with DATS resulted in an increased expression of TRAIL-R1/DR4 and TRAIL-R2/DR5 receptors (Fig. 2A and B) but had no significant effects on the expression of decoy receptors Dcr1 and Dcr2 (data not shown). Similarly, DATS up-regulated DR4 and DR5 expression in LNCaP cells (Fig. 2C and D). These data suggest that up-regulation of TRAIL-R1/DR4 and TRAIL-R2/DR5 by DATS may enhance the ability of TRAIL to induce apoptosis.

Regulation of Bcl-2 Family Members, Inhibitors of Apoptotic Proteins, and Caspases by DATS and/or TRAIL

Deregulation of apoptotic pathways plays a central role in cancer pathogenesis. Members of the Bcl-2 protein family control the integrity and response of mitochondria to apoptotic signals (24, 25). Most members of this family are targeted to mitochondria, which serve as a pivotal component of the apoptotic cell death machinery (24, 25). We have shown that overexpression of Bcl-2 or Bcl-X_L causes cancer cells to resist chemotherapy (26–28). Therefore, we sought to examine the regulation of Bcl-2 family members by DATS (Fig. 3A). DATS inhibited expression of antiapoptotic Bcl-2, Bcl-X_L, and Mcl-1 and induced expression of proapoptotic Bax, Bak, Bim, NOXA, and PUMA in PC-3 cells. Induction of proapoptotic members of Bcl-2 family by DATS suggests that these proteins may cause disruption of mitochondrial homeostasis.

Overexpression of inhibitors of apoptotic proteins (IAP) due to their genetic amplification have been reported in

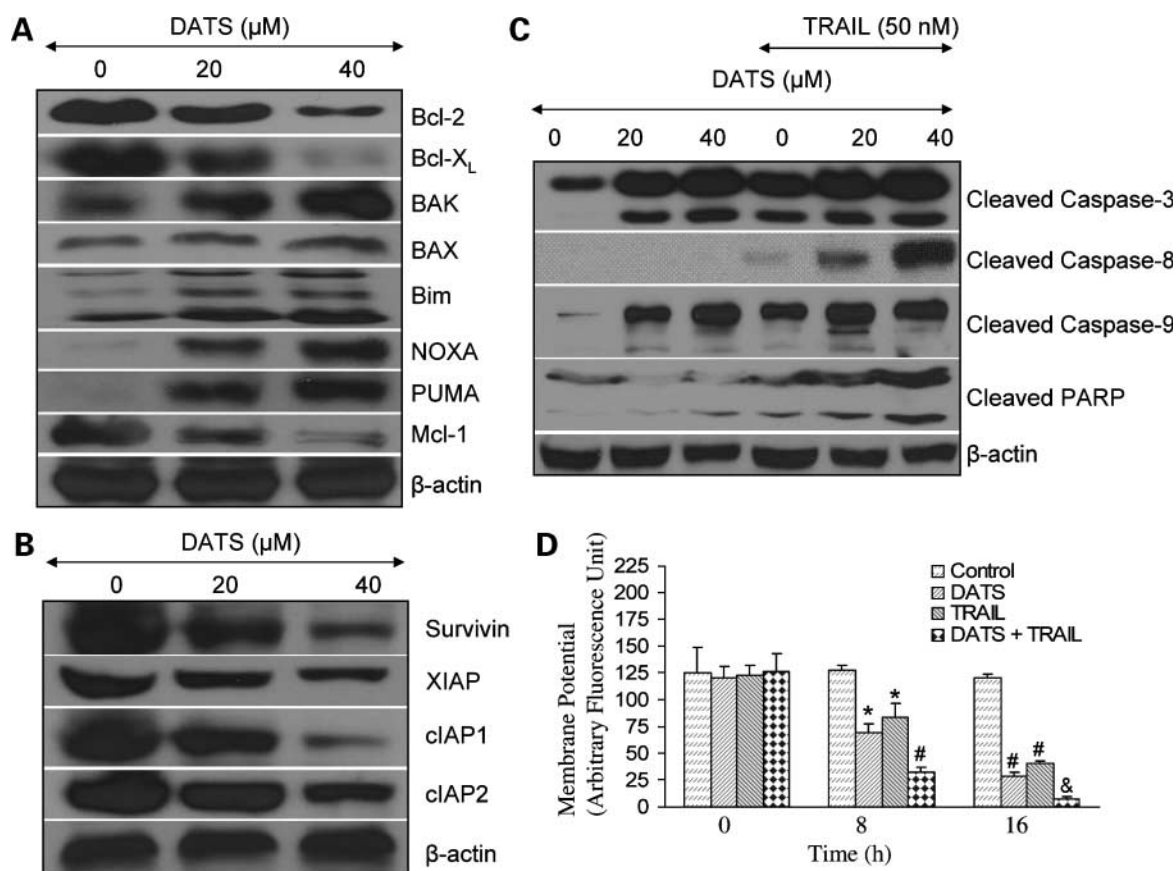


Figure 3. Effects of DATS and/or TRAIL on Bcl-2 family members, IAPs, and caspase activation. Prostate cancer PC-3 cells were treated with DATS (0–40 μ mol/L) for 48 h. Expression of Bcl-2 family proteins (A) and IAPs (B) was measured by the Western blotting. Anti- β -actin antibody was used as a loading control. C, interactive effects of DATS and TRAIL on caspase-3, caspase-8, and caspase-9 activation. PC-3 cells were pretreated with DATS (20 or 40 μ mol/L) for 24 h followed by treatment with TRAIL (50 nmol/L) for another 24 h. Cleavage/activation of caspase-3, caspase-8, and caspase-9 was measured by the Western blotting. Anti- β -actin antibody was used as a loading control. D, interactive effects of DATS and TRAIL on mitochondrial membrane potential. PC-3 cells were treated with DATS (20 μ mol/L) for 0 to 24 h. Cells were stained with JC-1 dye, and mitochondrial membrane potential was measured by a fluorometer as per manufacturer's instructions.

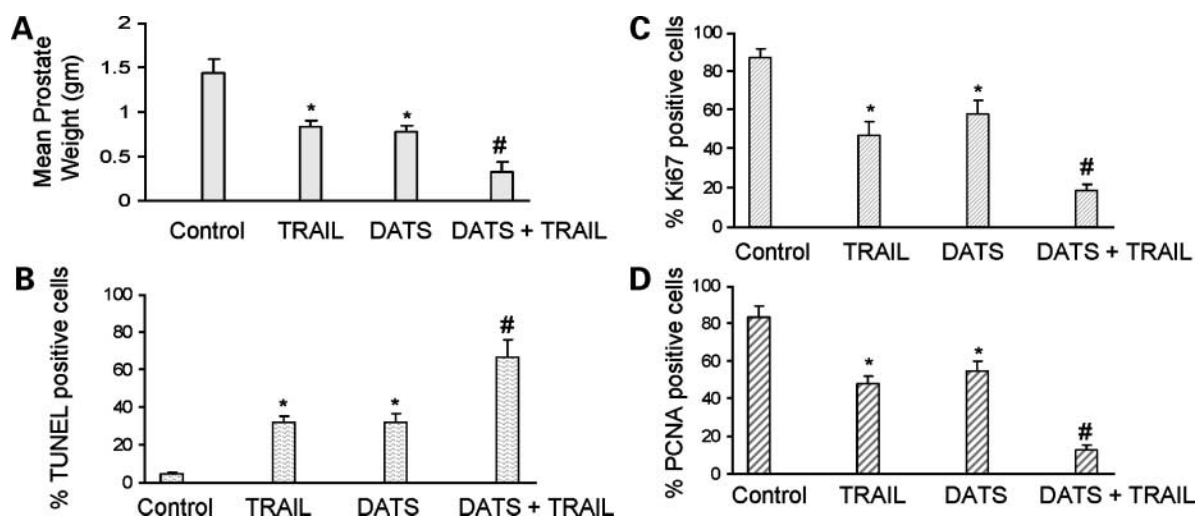


Figure 4. DATS enhances the antitumor activity of TRAIL in orthotopically implanted prostate tumor. PC-3 cells (1×10^6 per 100 μ L medium) were orthotopically implanted in prostate gland of BALB/c nude mice. Treatment of mice began on the day of tumor cell implantation as follows. Group 1: vehicle control (0.1 mL normal saline) administered by oral injection everyday 5 d/wk throughout the duration of experiment; group 2: TRAIL (15 mg/kg) administered i.v. on days 2, 8, 15, and 22; group 3: DATS (40 mg/kg) administered by oral injection everyday 5 d/wk throughout the duration of experiment; group 4: DATS and TRAIL, DATS (40 mg/kg) administered by oral injection everyday 5 d/wk throughout the duration of experiment, and TRAIL (15 mg/kg) administered i.v. on days 2, 8, 15, and 22. After 4 wk, mice were euthanized, and prostate tumor weight was recorded. *, #, $P < 0.05$, significantly different from respective control. **B**, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay was done to examine the induction of apoptosis as per manufacturer's instructions (BD Biosciences). **C** and **D**, tumor tissues were fixed and immunohistochemistry was done using antibody against Ki-67 or proliferating cell nuclear antigen (PCNA).

certain cancers including prostate cancer, which confers resistance to chemotherapy and radiotherapy (29, 30). We therefore examine the regulation of IAPs in PC-3 cells (Fig. 3B). DATS inhibited the expression of survivin, XIAP, cIAP1, and cIAP2 in a dose-dependent manner. These data suggest that the down-regulation of IAPs may also contribute to the proapoptotic effects of DATS.

Because DATS enhances the apoptosis-inducing potential of TRAIL, we examined the role of caspases in DATS-induced apoptosis (Fig. 3C). DATS induced caspase-3 and caspase-9 activities in PC-3 cells. Similarly, TRAIL induced caspase-3, caspase-8, and caspase-9 activities. Pretreatment of PC-3 cells with DATS followed by TRAIL further enhanced caspase-3, caspase-8, and caspase-9 activities. These data suggest that the DATS induces apoptosis through caspase-3 activation and further enhances TRAIL-induced caspase-8 activity.

Because Bcl-2 family members regulate mitochondrial homeostasis (24, 25), we next examined the effects of DATS and/or TRAIL on mitochondrial membrane potential (Fig. 3D). Treatment of PC-3 cells with DATS and TRAIL alone resulted in a drop in mitochondrial membrane potential. The combination of DATS and TRAIL was more effective in dropping mitochondrial membrane potential than single agent alone. These data suggest that DATS and TRAIL induce apoptosis by engaging mitochondria.

DATS Enhances the Antitumor Activity of TRAIL in Prostate Cancer PC-3 Cells Orthotopically Implanted in Nude Mice

Because DATS enhances the apoptosis-inducing potential of TRAIL *in vitro*, we sought to validate this hypothesis

in vivo orthotopic nude mice model of prostate cancer. PC-3 cells were implanted in prostate gland of BALB/c nude mice and treated with TRAIL, DATS, and DATS plus TRAIL (Fig. 4). Treatment of mice with TRAIL and DATS alone caused significant inhibition in tumor growth compared with control group. The combination of DATS and TRAIL further inhibited tumor growth compared with single agent alone. These data suggest that DATS can enhance the antitumor activity of TRAIL *in vivo*.

We next sought to examine whether antitumor activity of DATS and TRAIL was exerted due to inhibition in tumor cell proliferation and induction in apoptosis (Fig. 4B-D). Treatment of mice with TRAIL and DATS alone caused significant inhibition in Ki-67 and proliferating cell nuclear antigen staining (markers of tumor cell proliferation) and an increase in terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling-positive tumor cells (a marker of apoptosis) compared with control group. The combination of DATS and TRAIL further inhibited tumor-cell proliferation and increased tumor-cell apoptosis compared with single agent alone. These data suggest that DATS can enhance the antiproliferative and proapoptotic activities of TRAIL *in vivo*.

Regulation of Death Receptors (DR4 and DR5), Caspase-8 Activity, and Bcl-2 Family Members by TRAIL and/or DATS in Prostate Tumors

Because DATS enhanced the antitumor activity of TRAIL, we sought to examine the expression of DR4 and DR5 receptors and caspase-8 activity in prostate tumor tissue. The expression of death receptors (DR4 and DR5) and caspase-8 activity in tumor tissues was measured by

immunohistochemistry (Fig. 5A-C and B). The data showed that TRAIL and DATS alone induced DR4 and DR5 expression compared with control group (Fig. 5A). The combination of DATS and TRAIL was more effective in inducing DR4 and DR5 expression than single agent alone. These data suggest that DATS can enhance the apoptosis-inducing potential of TRAIL by up-regulating DR4 and DR5 expression.

Because caspase-8 is activated on activation of death receptor pathway, we sought to measure the activation of caspase-8 in tumor tissues derived from DATS- and/or TRAIL-treated mice (Fig. 5C). Whereas DATS was ineffective in inducing caspase-8 activity, treatment of mice with TRAIL caused activation of caspase-8. The combination of DATS plus TRAIL induced higher caspase-8 activity than TRAIL alone. These data suggest that caspase-8 activation is required for synergistic interaction between DATS and TRAIL.

Bcl-2 family members play a measure role in drug-induced apoptosis (31). Therefore, we sought to examine the effects of DATS and TRAIL on the expression of Bcl-2 family members. The data showed that TRAIL and DATS alone inhibited the expression of Bcl-2 and Bcl-X_L and induced the expression of Bax and Bak compared with control group (Fig. 5D). The combination of DATS and TRAIL was additive

in inhibiting Bcl-2 and Bcl-X_L expression and inducing Bax and Bak expression than single agent alone. These data suggest that DATS and TRAIL can regulate mitochondrial pathway of apoptosis through Bcl-2 family members.

Effects of DATS and TRAIL on Angiogenesis and Metastasis

Because angiogenesis plays a major role in tumor growth (32), we sought to measure the effects of DATS and/or TRAIL on angiogenesis by measuring microvessel density (staining tumor tissues with anti-CD31 and anti-factor VIII antibodies) and the expression of VEGF and interleukin-6 (IL-6; marker of angiogenesis; Fig. 6A-C). Treatment of mice with DATS and TRAIL alone resulted in significant reduction in microvessel density and the expression of VEGF and IL-6 compared with control mice. The combination of DATS and TRAIL was significantly more effective in inhibiting microvessel density and the expression of VEGF and IL-6 compared with single agent alone. We further confirmed the involvement of NF- κ B and phosphatidylinositol 3-kinase/AKT pathways by measuring IKK kinase activity and phosphorylation/activation of AKT in tumor tissues derived from TRAIL- and/or DATS-treated mice (Fig. 6C). The data showed that TRAIL and DATS alone inhibited IKK activity and phosphorylation of AKT. Furthermore, the combination of TRAIL plus DATS was

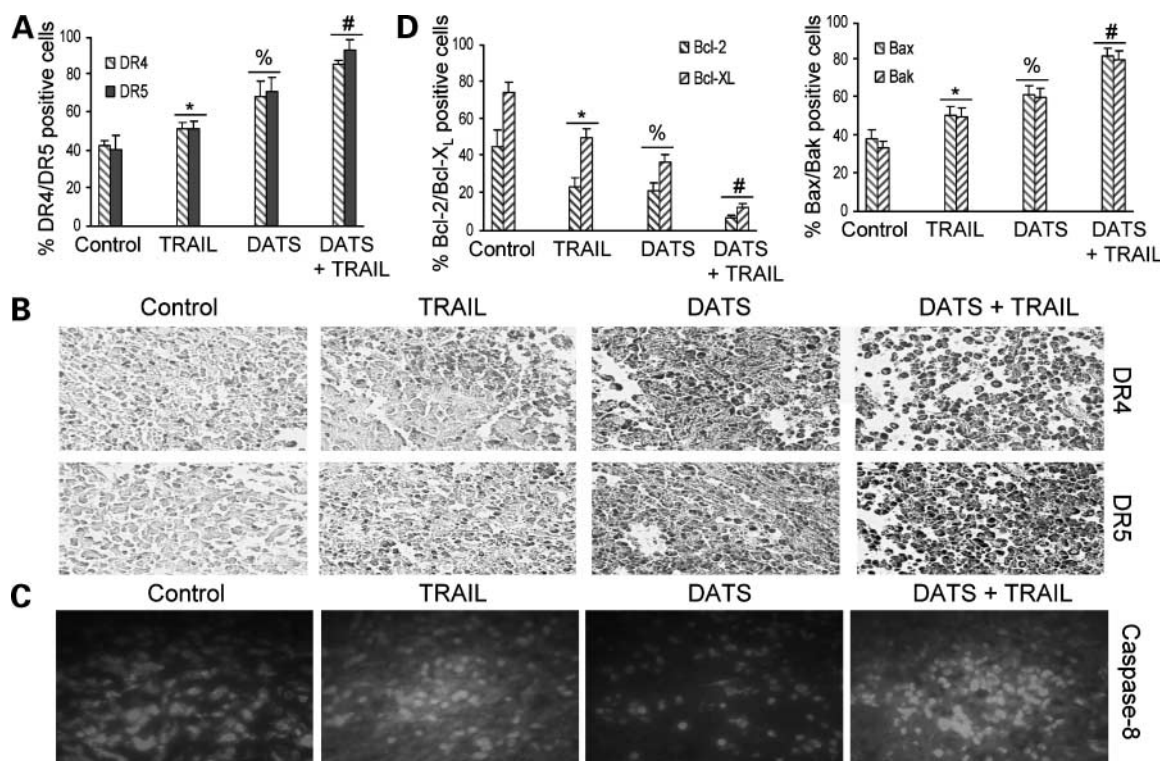


Figure 5. Regulation of death receptors, Bcl-2 family members, and caspase-8 by DATS and TRAIL. Orthotopically implanted nude mice were treated as we described in Fig. 4. Tumor tissues were fixed and immunohistochemistry was done using antibody against DR4 or DR5 (A and B), active caspase-8 (C), Bcl-2, Bcl-X_L, Bax, or Bak (D). *, %; #, $P < 0.05$, significantly different from respective control.

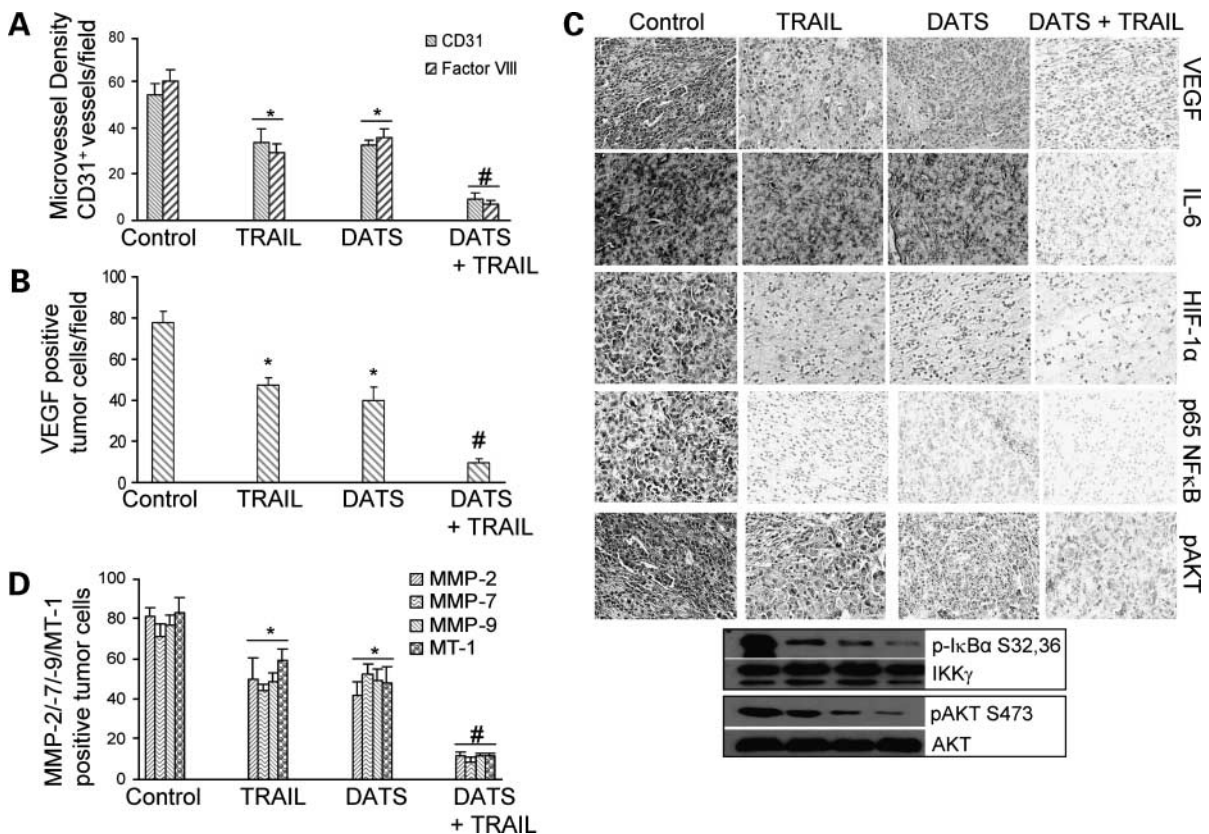


Figure 6. Effects of DATS and TRAIL on angiogenesis and metastasis. Tumor tissue sections were stained with anti-CD31, anti-factor VIII, anti-VEGF, or anti-IL-6 antibodies. **A**, microvessel density was calculated by counting CD31-positive and factor VIII-positive tumor tissues. **B** and **C**, expression of VEGF, IL-6, HIF-1 α , phospho-p65 NF- κ B, and phospho-AKT in tumor tissues. Mean \pm SE. *, #, $P < 0.05$, significantly different from control. After immunoprecipitation of the IKK complex with an anti-IKK γ antibody, an *in vitro* IKK kinase assay was carried out by incubation of the immunoprecipitated proteins with a purified glutathione *S*-transferase-I κ B α_{1-55} fusion protein as substrate. A Western blotting was then done using an antibody specific for Ser³²-Ser³⁶ phosphorylated I κ B α and IKK γ (*top*). Expression of phospho-AKT and total AKT by Western blotting (*bottom*). **D**, tumor tissue sections were stained with anti-MMP-2, MMP-7, MMP-9, or MT-1 MMP antibodies, counted, and quantified. Mean \pm SE. *, #, $P < 0.05$, significantly different from control.

more effective in inhibiting IKK and AKT activities than single agent alone. These data show that DATS and/or TRAIL can inhibit tumor angiogenesis by regulating NF- κ B and AKT pathways.

MMPs are a family of zinc-dependent endopeptidases (33, 34). They are capable of digesting the different components of the extracellular matrix and basement membrane (33, 34). Treatment of mice with DATS and TRAIL alone down-regulated the expression of MMP-2, MMP-7, MMP-9, and MT-1 MMP in tumor tissues compared with untreated control group (Fig. 6D). The combination of DATS plus TRAIL had significantly more effects than single agent alone. Our data show that DATS and TRAIL can inhibit tumor metastasis through regulation of MMPs.

Discussion

In the present study, we showed that DATS inhibited cell proliferation and induced apoptosis through activation of caspase-3 and caspase-9 in prostate cancer cells. DATS

induced expression of proapoptotic proteins (Bax, Bak, PUMA, Noxa, and Bim) and death receptors (TRAIL-R1/DR4 and TRAIL-R2/DR5) and inhibited expression of antiapoptotic proteins (Bcl-2 and Bcl-X_L) and IAPs (XIAP, cIAP1, cIAP2, and survivin). DATS enhanced the apoptosis-inducing potential of TRAIL in PC-3 cells and sensitized TRAIL-resistant LNCaP cells by engaging cell-intrinsic and cell-extrinsic pathways of apoptosis. The death receptor pathway was required for the synergistic interaction between DATS and TRAIL on apoptosis because dominant-negative FADD inhibited this synergistic interaction. *In vivo*, oral administration of DATS inhibited the growth of orthotopically implanted PC-3 tumors, metastasis, and angiogenesis. Most importantly, the combination of DATS plus TRAIL had greater effect on tumor growth inhibition, metastasis, and angiogenesis than either agent alone.

Bcl-2 members are crucial regulators of apoptotic cell death (31). Several mechanisms exist allowing cells to escape programmed cell death among them is the

overexpression of the antiapoptotic proteins. Cancer cells are often found to overexpress many of these members such as Bcl-2, Bcl-X_L, Mcl-1, Bcl-w, and A1/Bfl1 and are usually resistant to a wide range of anticancer drugs and treatments. *In vitro*, DATS down-regulated the expressions of Bcl-2, Bcl-X_L, and Mcl-1 and up-regulated the expressions of Bax, Bak, PUMA, Noxa, and Bim protein levels in PC-3 cells. Furthermore, tumor tissues derived from DATS- or TRAIL-treated mice showed significantly less expression of Bcl-2 and Bcl-X_L and induced expression of Bax and Bak compared with control group. The combination of DATS and TRAIL was more effective in regulating Bcl-2 family members than single agent alone. Our studies show that DATS can engage cell-intrinsic pathway of apoptosis and can further enhance the proapoptotic effects of TRAIL by regulating the expression of Bcl-2 family of proteins.

Angiogenesis is a physiologic process involving the growth of new blood vessels from preexisting vessels and is required for tumor growth and metastasis (32). AKT plays an important role in regulating normal vascularization and pathologic angiogenesis (35, 36). Recent studies have shown that AKT activation is necessary and sufficient to regulate VEGF and HIF-1 expression in human cancer cells (37–39). VEGF, IL-6, and HIF-1 α are potent inducers of angiogenesis. AKT activation induces VEGF and HIF-1 α expression through its two downstream molecules HDM2 and p70S6K1. On the other hand, AKT transmits the upstream signals from growth factors, cytokines, heavy metals, and oncogenes for regulating VEGF and HIF-1 expression in human cancer cells (40–42). Our studies show that DATS can inhibit AKT activation and expression of VEGF, IL-6, and HIF-1 α , which are associated with inhibition of angiogenesis. These observations support the hypothesis that DATS may inhibit prostate tumor angiogenesis through the suppression of VEGF-mediated autocrine and paracrine signaling pathways between tumor cells and vascular endothelial cells. Similarly, we have shown that other chemopreventive agents such as EGCG and curcumin can inhibit angiogenesis (11, 43). Thus, inhibition of AKT and its downstream targets offers a new approach for targeting angiogenesis, which could be important for the development of new cancer therapeutics in the future.

Metastases formation is a major factor in disease progression and accounts for the majority of cancer deaths (33, 34). Recent observations indicate that the metastatic phenotype may already be present during the angiogenic switch of tumors. Intratumoral hypoxia correlates with poor prognosis and enhanced metastases formation. MMPs are up-regulated in many cancers and play significant role in tumor progression and metastasis (44). MMPs degrade extracellular matrix, thereby promoting tumor cell invasion and dissemination. To grow efficiently *in vivo*, tumor cells induce angiogenesis in both primary solid tumors and metastatic foci. Our results showed that DATS and TRAIL alone significantly inhibited the expression of MMP-2, MMP-7, MMP-9, and MT-1 MMP in PC-3 implanted

tumors, and the combination treatment was more effective in inhibiting the expression of these MMPs. These data suggest that DATS and/or TRAIL can inhibit metastasis by regulating MMPs.

Death receptors TRAIL-R1/DR4 and TRAIL-R2/DR5 are selectively expressed in cancer cells and thus offer an advantage for targeted therapy and prevention (7, 13, 45). TRAIL induces apoptosis in cancer cells that express DR4 and DR5 (7, 45). We have shown that the up-regulation of death receptors DR4 and/or DR5 by chemotherapeutic drugs, ionizing radiation, and chemopreventive agents can enhance the therapeutic potential of TRAIL (10–13). Specifically, TRAIL-resistant LNCaP cells can be sensitized by chemotherapeutic drugs, ionizing radiation, and chemopreventive agents through up-regulation of death receptors DR4 and/or DR5 (10–12, 19, 20). From these data, it is clear that up-regulation of DR4 and DR5 may enhance the apoptosis-inducing potential of TRAIL. In the present study, DATS also up-regulated DR4 and DR5 expression, which could be one of the mechanisms of sensitizing cells to TRAIL. Therefore, from a cancer management point of view, it will be beneficial to combine chemopreventive agents with chemotherapeutic drugs.

The NF- κ B is constitutively active in various human malignancies, including several solid tumors, leukemias, and lymphomas (47). NF- κ B contributes to development and progression of malignancy by regulating the expression of genes involved in cell growth, differentiation, apoptosis, angiogenesis, and metastasis (47). Prostate cancer cells have been reported to have constitutive NF- κ B activity due to increased activity of the I κ B kinase complex (48). In prostate cancer cells, NF- κ B may promote cell growth and proliferation by regulating expression of genes such as c-myc, cyclin D1, and IL-6 and inhibit apoptosis through activation of expression of antiapoptotic genes, such as Bcl-2 and Bcl-X_L (47, 49). NF- κ B-mediated expression of genes, involved in angiogenesis, invasion, and metastasis, may further contribute to the progression of prostate cancer. Constitutive NF- κ B activity has also been shown in primary prostate cancer tissue samples and suggested to have prognostic importance for a subset of primary tumors. In the present study, DATS inhibited the activation of NF- κ B and its gene products such as VEGF, IL-6, HIF-1 α , Bcl-2, Bcl-X_L, MMP-2, MMP-7, MMP-9, and IL-6 in PC-3 orthotopic tumors. These findings suggest that NF- κ B may play a role in human prostate cancer development and/or progression and DATS can inhibit these processes through regulation of NF- κ B-regulated gene products.

In summary, our *in vivo* experiments have shown that DATS enhances the apoptosis-inducing potential of TRAIL through multiple mechanisms. *In vitro*, it induces death receptors and proapoptotic members of Bcl-2 family and inhibits antiapoptotic Bcl-2 proteins and markers of cell proliferation. *In vivo*, DATS induces apoptosis and inhibits tumor cell proliferation, metastasis, and angiogenesis. Furthermore, immunohistochemical data on tumor tissues show that DATS inhibits the activation of AKT and NF- κ B and its gene products, which play significant roles in cell

proliferation, apoptosis, metastasis, and angiogenesis. Our studies provide strong preclinical evidence that DATS either alone or in combination with TRAIL can be used to prevent and/or treat prostate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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