Transcriptional Repression and Inhibition of Nuclear Translocation of Androgen Receptor by Diallyl Trisulfide in Human Prostate Cancer Cells

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Abstract

Purpose: The present study was undertaken to determine the effect of diallyl trisulfide (DATS), a promising cancer chemopreventive constituent of garlic, on androgen receptor (AR) protein expression and function using prostate cancer cells.

Experimental Design: The protein levels of AR and prostate-specific antigen (PSA) were determined by immunoblotting. The effect of DATS treatment on AR mRNA level and AR promoter activity was determined by quantitative reverse transcription-PCR and luciferase reporter assay, respectively. Expression of AR protein in poorly differentiated carcinoma and normal prostate of transgenic adenocarcinoma of mouse prostate (TRAMP) mice was determined by immunohistochemistry. Confocal microscopy was done to determine nuclear translocation of AR. Cell viability was determined by trypan blue dye exclusion assay.

Results: Exposure of prostate cancer cells (LNCaP, C4-2, and TRAMP-C1) to DATS resulted in a concentration-dependent decrease in protein level of AR, which was accompanied by suppression of intracellular and secreted levels of PSA. Structure-activity studies revealed critical roles for allyl groups and the oligosulfide chain length in DATS-mediated down-modulation of AR protein. Quantitative reverse transcription-PCR showed a dose-dependent decrease in AR mRNA level, which correlated with inhibition of AR promoter activity. DATS treatment inhibited synthetic androgen (R1881)-stimulated nuclear translocation of AR in LNCaP/C4-2 cells and proliferation of LNCaP cells. Oral gavage of 2 mg/day DATS (three times per week for 13 weeks) markedly suppressed AR protein level in poorly differentiated prostate cancer in TRAMP mice.

Conclusion: The present study shows, for the first time, that DATS treatment suppresses AR function in prostate cancer cells.

Prostate cancer continues to be one of the most frequently diagnosed malignancies and a leading cause of cancer-related deaths among men in the United States (1). Androgen ablation is the main treatment option for early-stage prostate cancer (2). After an initial response to androgen ablation therapy most prostate cancers progress to castration-resistant state that is highly aggressive, metastatic, and resistant to chemotherapy (2–4). Androgen receptor (AR) plays an important role in the development of prostate cancer as well as in its transition to castration resistance (4, 5). AR is a member of the nuclear receptor superfamily and a ligand-activated transcription factor (6, 7). Although the mechanism leading to hormone independence is not fully understood, AR overexpression is frequent in castration-resistant prostate cancers (8). Mutations in AR are also acquired during androgen ablation therapy and are found to occur more frequently with increasing tumor grade and stage (5, 9). Therefore, identification of agents that are nontoxic and could potentially block the AR signaling axis could be very valuable. Natural products have received increased attention for the discovery of novel cancer chemopreventive and therapeutic agents (10).

Epidemiologic studies continue to support the premise that dietary intake of Allium vegetables (e.g., garlic) may confer protection against various types of malignancies including prostate cancer (11–13). For example, a population-based, case-control study conducted in China indicated that men with high intake of total Allium vegetables (>10 g/d) were at a statistically significantly lower risk of developing prostate cancer than those with low Allium vegetable intake (<2.2 g/d; ref. 13). The association between Allium vegetable intake and the risk of prostate cancer was independent of body weight, consumption of other foods, and total caloric intake (13). The anticancer effect of Allium vegetables is attributed to organosulfur compounds such as diallyl sulfide, diallyl disulfide, and diallyl trisulfide (DATS), which are released during processing (crushing and chewing)
of these vegetables (14). Preclinical animal studies have shown that organosulfur compounds, including diallyl sulfide, diallyl disulfide, and/or DATS, are highly effective in affording protection against chemically induced cancers (15–17). Oral administration of DATS significantly retards growth of PC-3 human prostate cancer xenografts in nude mice (18). We also found that oral gavage of DATS inhibits prostate cancer progression and pulmonary metastasis multiplicity in transgenic adenocarcinoma of mouse prostate (TRAMP) mice (19). Studies using and DATS-mediated down-regulation of AR protein expression is also observed in vivo in a transgenic mouse model of prostate cancer. These preclinical results warrant clinical investigation to determine the efficacy of DATS against human prostate cancer.

Translational Relevance
Androgen receptor (AR) plays an important role in prostate cancer development and in the transition to castration-resistant state. Accordingly, preclinical/clinical development of novel agents that are relatively safe but can inhibit AR function is highly desirable. The present study shows that pharmacologic concentration of diallyl trisulfide (DATS), a highly promising cancer chemopreventive constituent of garlic, decreases protein level of AR in LNCaP and C4-2 human prostate cancer cells by causing its transcriptional repression. In addition, DATS treatment inhibits synthetic androgen (R1881)-stimulated nuclear translocation of AR in both cell lines. The DATS-mediated down-regulation of AR protein expression is also observed in vivo in a transgenic mouse model of prostate cancer. The results of the present study together with our previous observations (18–25) merit clinical investigation of DATS for its efficacy against human prostate cancer.

Materials and Methods

Reagents. DATS (≥98% purity) was from LKT Laboratories. The diallyl sulfide, diallyl disulfide, dipropyl sulfide, dipropyl disulfide, allyl mercaptan, DMSO, bovine serum albumin (BSA), and 4′,6-diamidino-2-phenylindole (DAPI) were from Sigma. The antibiotic mixture, sodium pyruvate, and fetal bovine serum (FBS) were purchased from Invitrogen. Charcoal/dextran-treated FBS (cFBS) was obtained from HyClone, and RPMI 1640 medium with or without phenol red was purchased from Mediatech. Triton X-100 and glycine were purchased from Bio-Rad Laboratories. The antibody against AR was from Santa Cruz Biotechnology, the antibody against phospho-AR (Ser-210/213) was from Imgenex, and the antibody against prostate-specific antigen (PSA) was from DakoCytomation. The R1881 was from Perkin Elmer whereas the proteasomal inhibitor MG132 was from Calbiochem.

Cell culture. The LNCaP, C4-2, and TRAMP-C1 cells were maintained as we previously described (24, 28). To deplete the endogenous steroid hormones, cells were cultured in phenol red-free RPMI 1640 medium containing 10% cFBS before treatment. Stock solution of DATS was prepared in DMSO, and an equal volume of DMSO (final concentration <0.5%) was added to the controls.

Immunoblotting. Desired cells (0.7 × 10⁶) were seeded in 12-well plates in phenol red–free RPMI 1640 medium containing cFBS. After overnight incubation, the cells were treated with synthetic androgen R1881 (1 nmol/L) or the combination of R1881 (1 nmol/L) with DATS (20, 40, or 60 μmol/L) for 24 h. Cell viability was measured by trypan blue dye exclusion assay as described by us previously (23).

Measurement of PSA secretion. The LNCaP and C4-2 cells were plated and treated as described above. After 24-h incubation, the media were collected and the level of PSA (secreted) in the media was determined using an enzyme-linked immunosorbent assay kit from R&D Systems according to the manufacturer’s protocol.

Quantitative reverse transcription-PCR. Total RNA from control and DATS-treated cells was isolated using RNaseasy kit from Qiagen and was reverse-transcribed using an oligo(dT)_15 primer (Promega) and SuperScript III reverse transcriptase (Invitrogen) in 50 μL reaction mixture. PCR amplification was done as follows: initial denaturation at 94°C for 2 min, followed by 25 cycles of 94°C for 1 min, 57°C (AR) or 55°C (GAPDH) for 1 min, and 68°C for 1 min, followed by final extension at 68°C for 5 min. The AR primer (Invitrogen) had the following sequence: forward- 5′-ATGGTACAGGCTAAGATATG-3′ and reverse- 5′-GTGTCGTTATGACGTCC-3′. The GAPDH primer (Biomol) had the following sequence: forward- 5′-TCATGCGATACTCAT-3′ and reverse- 5′-TCCCGGGCTTCTA-3′. The PCR products were analyzed on 2% agarose gel containing ethidium bromide.

Luciferase reporter assay. The LNCaP and C4-2 cells were transiently cotransfected with 1 μg/mL of pRLUC plasmid and 0.01 μg/mL of pRL-CMV plasmid using FuGENE 6. Twenty-four hours after transfection, the cells were exposed to specified concentrations of DATS for 24 h, washed with PBS, and collected in reporter lysis buffer. The samples were centrifuged, and a 20 μL supernatant fraction was used for measurement of dual-luciferase reporter activity using a luminometer. The luciferase activity was normalized against protein concentration and expressed as a ratio of firefly luciferase to Renilla luciferase units.
Confocal microscopy. Confocal microscopy was done to determine the effect of DATS treatment on R1881-stimulated nuclear translocation of AR according to the method described by Deep et al. (30) with some modifications. Briefly, the LNCaP or C4-2 cells grown in phenol red–free RPMI 1640 medium containing cFBS were plated on coverslips and allowed to attach by overnight incubation. The cells were treated with DATS (40 μmol/L) or DMSO for 3 h and then incubated with synthetic androgen R1881 (1 nmol/L) for an additional 3 h. The cells were then fixed in 2% paraformaldehyde at 4°C overnight. Fixed cells were permeabilized with 0.5% Triton X-100 for 15 min at room temperature, washed with PBS, and incubated with PBS containing 0.5% (w/v) BSA and 0.15% (w/v) glycine (BSA buffer) for 1 h at room temperature. Subsequently, the cells were treated with anti-AR antibody (1:100 in BSA buffer) for 1 h at room temperature. The cells were then washed with BSA buffer and incubated with 1 μg/mL Alexa Fluor 568-conjugated secondary antibody for 1 h at room temperature followed by counterstaining with 100 ng/mL DAPI for 2 min. Slides were mounted and examined using an Olympus FV1000 confocal microscope at 100× objective lens magnification.

Immunohistochemical analysis of AR protein expression in dorsolateral prostate of TRAMP mice. Prostate/tumor tissues from control and DATS-treated TRAMP mice were obtained from a study we previously described (19). Briefly, the mice in the control group received 0.1 mL PBS by oral gavage 3 times per wk for 13 wk (19). A portion of the prostate tissue was placed in 10% neutral buffered formalin, paraffin-embedded, and sectioned at 4- to 5-μm thickness. Deparaffinized and rehydrated sections were quenched with 3% hydrogen peroxide and blocked with normal serum. The sections were then incubated with anti-AR 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 hematoxylin (Sigma) and examined under a Leica microscope. At least three non-overlapping images of each dorsolateral prostate section were captured using a camera mounted on to the microscope. The images were analyzed using Image ProPlus 5.0 software (Media Cybernetics).

### Results

DATS treatment decreased AR protein level in prostate cancer cells. We determined the effect of DATS treatment on AR
protein level using LNCaP (androgen responsive) and C4-2 (an androgen-independent variant of LNCaP cells) human prostate cancer cells. The AR in LNCaP cells was mutated with alanine to threonine substitution at position 877 (31). The C4-2 cell line was generated through coculture of parental LNCaP cells with human bone fibroblasts in vivo in castrated male athymic mice, and displayed elevated PSA expression and increased anchorage-independent growth in soft agar (32, 33). As can be seen in Fig. 1A, exposure of LNCaP and C4-2 cells to DATS resulted in a concentration-dependent decrease in AR protein level. The DATS-mediated down-regulation of AR protein level was also observed in a cell line (TRAMP-C1) derived from prostate tumor of a TRAMP mouse (Fig. 1A). Next, we raised the question of whether the DATS-mediated suppression of AR protein level was reversible. To test this possibility, the C4-2 cells treated for 24 hours with either DMSO (control) or 40 μmol/L DATS were

Fig. 2. Secreted levels of PSA in the medium of (A) LNCaP and (B) C4-2 cells following 24-h treatment with DMSO or DATS. Columns, mean (n = 3); bars, SE; *, significantly different (P < 0.01) compared with control by one-way ANOVA followed by Dunnett’s test. C, immunoblotting for PSA using lysates from LNCaP and C4-2 cells treated with DMSO or DATS. Numbers on top of the bands, change in protein level compared with DMSO-treated control. Each experiment was done at least twice and the results were consistent.

Fig. 3. A, chemical structures of organosulfur compounds. B, immunoblotting for AR and PSA using lysates from LNCaP cells treated for 24 h with DMSO or 40 μmol/L diallyl sulfide (DAS), diallyl disulfide (DADS), DATS, dipropyl sulfide (DPS), dipropyl disulfide (DPDS), or allyl mercaptan (AM). Numbers on top of the bands, change in protein level compared with DMSO-treated control. Immunoblotting for each protein was done twice and the results were consistent.
incubated in drug-free medium for an additional 24 hours prior to immunoblotting. As shown in Fig. 1B, the DATS-mediated suppression of AR protein level was maintained for at least up to 24 hours after drug removal.

**DATS treatment inhibited R1881-stimulated growth of LNCaP cells.** We have shown previously that DATS treatment inhibits proliferation of cultured human prostate cancer cells including the LNCaP and C4-2 cells (23, 24). Because proliferation of LNCaP cells depends on functional AR signaling, it was of interest to determine whether androgen-stimulated growth of LNCaP cells was inhibited by DATS exposure. Treatment of LNCaP cells with the synthetic androgen R1881 (1 nmol/L) resulted in about a 2.6-fold increase in cell viability as judged by trypan blue dye exclusion assay (Fig. 1C). The R1881-stimulated growth of LNCaP cells was inhibited significantly in a concentration-dependent manner by DATS exposure (Fig. 1C).

**DATS treatment decreased the levels of secreted and intracellular PSA protein in prostate cancer cells.** Next, we determined the effect of DATS treatment on level of PSA protein, which is an AR-regulated gene product belonging to the kallikrein family of serine proteases and represents a sensitive biomarker for screening of prostate cancer (2, 34, 35). Twenty-four-hour exposure of LNCaP (Fig. 2A) and C4-2 cells (Fig. 2B) with DATS resulted in a concentration-dependent decrease in secreted levels of PSA protein in both cell lines. For example, the secreted levels of PSA were decreased by 23% and 52% by a 24-hour treatment of LNCaP cells with 20 and 40 μmol/L DATS, respectively (Fig. 2A). We also determined the effect of DATS treatment on intracellular levels of PSA protein by immunoblotting. As shown in Fig. 2C, treatment with DATS resulted in a concentration-dependent decrease in intracellular levels of PSA in both LNCaP and C4-3 cells.

**Allyl groups and the oligosulfide chain length were critical for DATS-mediated decline in AR protein level.** We conducted a structure-activity relationship study using naturally occurring organosulfur compound analogues (Fig. 3A) to determine the possible impact of allyl groups and the oligosulfide chain length on DATS-mediated decline in AR and PSA protein levels. Down-regulation of AR and PSA protein levels was relatively more pronounced in the DATS-treated LNCaP cells than in cells exposed to the monosulfide diallyl sulfide or the disulfide diallyl disulfide (Fig. 3B). Furthermore, the level of AR protein was only minimally affected in the presence of dipropyl sulfide or dipropyl disulfide. An intermediate response was observed in cells treated with allyl mercaptan, a putative metabolite of DATS (Fig. 3B). These results indicated that the presence of allyl groups and the oligosulfide chain length were critical determinants of DATS-mediated down-regulation of AR and PSA protein levels.

**DATS treatment caused transcriptional repression of AR in human prostate cancer cells.** The ubiquitin-proteasomal pathway is implicated in degradation of AR (36). Next, we designed experiments to test whether DATS-mediated decline in AR protein level was caused by the proteasomal pathway. The DATS-mediated down-modulation of AR protein was maintained in the presence of proteasomal inhibitor MG132 (Fig. 4A). Figure 4B depicts the effect of DATS treatment on AR message as determined by quantitative reverse transcription-PCR. Twenty-four hours’ exposure of LNCaP and C4-2 cells to DATS resulted in a marked and concentration-dependent decrease in AR mRNA levels (Fig. 4B). We determined the effect of DATS...
treatment on AR promoter activity using luciferase reporter assay, and the results are shown in Fig. 4C. The pARLUC plasmid used in the present study is a modification of the pAR1.1LUC plasmid containing the proximal 1047 bp rat AR promoter fragment inserted upstream of the luciferase gene in the pGL2-enhancer vector (37). Exposure of LNCaP cells to DATS for 24 hours resulted in about 38% to 43% decrease in AR promoter activity (P < 0.05 by one-way ANOVA followed by Dunnett’s test). The DATS-mediated inhibition of AR promoter activity was also observed in the C4-2 cell line (results not shown).

DATS treatment inhibited R1881-stimulated nuclear translocation of AR protein in prostate cancer cells. Because AR transactivation is regulated by its phosphorylation (5, 6), we determined the effect of DATS treatment on AR phosphorylation at Ser-210/213 using LNCaP and C4-2 cells. As can be seen in Fig. 4D, DATS treatment caused a concentration-dependent decrease in phosphorylation of AR in LNCaP and C4-2 cells. Nuclear localization of AR is critical for its transcriptional activity (5–7). Because DATS treatment inhibited transcriptional activity of AR as evidenced by suppression of PSA levels, we raised the question of whether DATS treatment inhibited nuclear translocation of AR. To explore this possibility, we determined the effect of DATS treatment on R1881-stimulated nuclear localization of AR. As can be seen in Fig. 5, the AR staining was both cytoplasmic and nuclear in DMSO-treated control cells as evidenced by red fluorescence. Exposure of LNCaP (Fig. 5) and C4-2 (results not shown) cells to R1881 resulted in nuclear translocation of AR as evidenced by merging of red and blue fluorescence. DATS treatment inhibited nuclear translocation of AR stimulated by R1881 in LNCaP (Fig. 5) and C4-2 (results not shown) cells.

DATS administration decreased AR protein level in poorly differentiated prostate cancer in TRAMP mice. We have shown
previously that oral gavage of DATS, 3 times a week for 13 weeks, significantly inhibits the incidence as well as the burden of poorly differentiated cancer in the dorsolateral prostate of TRAMP mice (19). These studies revealed that the incidence of the poorly differentiated carcinoma in the dorsolateral prostate of mice treated with 1 and 2 mg/d DATS was lower by 34% ($P = 0.0147$) and 41% ($P = 0.035$), respectively, in comparison with control mice (19). Moreover, the area occupied by the poorly differentiated carcinoma in the dorsolateral prostate of DATS-treated mice was statistically significantly lower compared with that in vehicle-treated control mice. We used the dorsolateral prostate tissue sections from the same study (control and 2 mg DATS-treated mice; ref. 19) to determine the effect of DATS administration on AR protein expression. As shown in Fig. 6A, the poorly differentiated cancer in the dorsolateral prostate of 2 mg DATS-treated mice exhibited an approximate ~71% decrease in AR protein level compared with control. The DATS-mediated decline in AR protein level was relatively less pronounced in the normal prostate (Fig. 6B). Consistent with the results in Fig. 6A, immunoblotting revealed suppression of AR protein level in the prostate tumor of DATS-treated mice compared with control (Fig. 6C).

**Discussion**

AR plays an important role in the development of prostate cancer as well as in the transition to castration-resistant state, which is highly aggressive and resistant to chemotherapy (4, 38). Thus, the preclinical/clinical development of novel agents that are safe but can suppress AR signaling is highly desirable. The present study shows that DATS treatment suppresses protein level of AR in both LNCaP cells and their androgen-independent variant. The DATS-mediated AR protein down-regulation is maintained for at least up to 24 hours even after removal of the drug. In addition, DATS treatment exhibits significant antiproliferative effect against androgen-stimulated growth of LNCaP cells.

We also conclude that the DATS-mediated suppression of AR protein level leads to inhibition of transcriptional activity of AR as evidenced by a significant decrease in secreted as well as cultured media of LNCaP and its androgen-independent variant.
as intracellular levels of PSA in both LNCaP and C4-2 cells. Structure-activity analysis reveals that even a subtle change in the structure of DATS (e.g., the number of sulfur atoms) markedly affects its anti-AR effect. The DATS-mediated decline in AR protein level is not blunted in the presence of MG132 but correlates with reduction in AR message as judged by reverse transcription-PCR and inhibition of AR promoter activity as revealed by the luciferase reporter assay.

Ligand-free AR predominantly resides in the cytoplasm complexed with chaperone proteins including Hsp90 but in a conformational state receptive to ligand binding (6, 39). Ligand-activated regulation of gene expression by AR is achieved by its nuclear translocation, dimerization, and binding to androgen response elements in the DNA of target genes (6, 39). AR transactivation is also regulated by its phosphorylation (5, 6). The present study indicates that DATS treatment suppresses phosphorylation of AR in a concentration-dependent manner. Moreover, the nuclear translocation of AR stimulated by R1881 is also inhibited in the presence of DATS in both LNCaP and C4-2 cell lines. These results are consistent with repression of transcriptional activity of AR in DATS-treated prostate cancer cells.

The in vivo validation of the cellular findings is critical for the further clinical development of potential cancer chemopreventive and therapeutic agents. We found that oral administration of DATS suppresses AR protein expression in poorly differentiated cancer in the dorsolateral prostate of TRAMP mice, which correlates with a significant decrease in the incidence and burden of poorly differentiated carcinoma (19). Interestingly, the DATS-mediated decrease in AR protein expression is relatively less pronounced in the normal prostate. These results suggest that DATS administration is unlikely to interfere with AR function in the normal prostate. However, the mechanism behind selectivity of DATS for suppression of AR protein level in cancer versus normal prostate remains to be elucidated.

Plasma achievable concentration is a critical issue in the clinical development of DATS as an effective AR antagonist. Even though pharmacokinetic parameters for DATS in humans are yet to be determined, this information is available in rats. The concentration of DATS in rat blood following treatment with 10 mg of the compound was shown to be around 31 μmol/L (40). The DATS-mediated suppression of AR protein level/function is observed at 20 μmol/L concentration (present study). It is important to point out that 200 mg of synthetic DATS (also known as allitridum) in combination with 100 μg selenium has been safely administered to humans for 1 month without any harmful side effects (41). Thus, we remain optimistic that a pharmacologic dose of DATS necessary for suppression of AR function may be achievable in humans. However, a carefully designed pharmacokinetic study is needed to substantiate this speculation.

Previous studies from our laboratory have revealed that DATS treatment suppresses viability of human prostate cancer cells lacking functional AR (e.g., PC-3 and DU145 cells; refs. 18, 23). The antiproliferative effect of DATS against PC-3 and DU145 cells is accompanied by the activation of an irreversible checkpoint kinase 1–dependent prometaphase arrest and reactive oxygen species and Bax/Bak-mediated apoptosis induction (20, 22, 23). It is of interest to note that the checkpoint kinase 1–mediated prometaphase arrest as well as the reactive oxygen species and Bax/Bak-mediated apoptosis resulting from DATS exposure are not unique to AR-independent human prostate cancer cells because similar cellular responses are evident in the LNCaP cell line (24, 42). Based on these results, we conclude that both mechanisms involving the suppression of AR function and AR-independent effects are likely to contribute to the growth suppressive effects of DATS against prostate cancer cells.

In conclusion, the present study shows that (a) DATS treatment decreases protein level of AR in cultured prostate cancer cells by causing its transcriptional repression; (b) allyl groups and the number of sulfur atoms are critical for DATS-mediated suppression of AR protein level; (c) DATS treatment inhibits synthetic androgen-stimulated nuclear translocation of AR in prostate cancer cells; and (d) the DATS-mediated decrease in AR protein level is observed in vivo. These results together with our previous observations (18–24) warrant investigation of DATS against prostate cancer in a clinical setting.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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