

Tubulin-Targeting Chemotherapy Impairs Androgen Receptor Activity in Prostate Cancer

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Abstract

Recent insights into the regulation of the androgen receptor (AR) activity led to novel therapeutic targeting of AR function in prostate cancer patients. Docetaxel is an approved chemotherapy for treatment of castration-resistant prostate cancer; however, the mechanism underlying the action of this tubulin-targeting drug is not fully understood. This study investigates the contribution of microtubules and the cytoskeleton to androgen-mediated signaling and the consequences of their inhibition on AR activity in human prostate cancer. Tissue microarrays from docetaxel-treated and untreated prostate cancer patients were comparatively analyzed for prostate-specific antigen (PSA) and AR immunoreactivity. The AR transcriptional activity was determined in prostate cancer cells *in vitro*, based on PSA mRNA expression and the androgen response element reporter activity. The interaction of AR with tubulin was examined by immunoprecipitation and immunofluorescence. Treatment of prostate cancer patients with docetaxel led to a significant translocation of AR. In untreated specimens, 50% prostate tumor cells exhibited nuclear accumulation of AR, compared with docetaxel-treated tumors that had significantly depleted nuclear AR (38%), paralleled by an increase in cytosolic AR. AR nuclear localization correlated with PSA expression. *In vitro*, exposure of prostate cancer cells to paclitaxel (1 $\mu\text{mol/L}$) or nocodazole (5 $\mu\text{g/mL}$) inhibited androgen-dependent AR nuclear translocation by targeting AR association with tubulin. Introduction of a truncated AR indicated the requirement of the NH₂-terminal domain for AR-tubulin interaction. Our findings show that in addition to blocking cell division, docetaxel impairs AR signaling, evidence that enables new insights into the therapeutic efficacy of microtubule-targeting drugs in prostate cancer.

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Introduction

Prostate cancer is the most frequently diagnosed noncutaneous cancer and the third leading cause of cancer mortality in men. Prostate cancer growth is dependent on androgens and can be suppressed by androgen ablation (1). Nearly 90% of all patients with metastatic prostate cancer initially respond to castration-induced androgen withdrawal (2). Unfortunately, the therapeutic response is limited to a median duration of 18 to 24 months, with ultimate tumor recurrence to a castration-resistant prostate cancer (CRPC) and progression to advanced disease. Considerable efforts have been devoted toward understanding the mechanisms contributing to tumor therapeutic resistance and progression to metastasis.

This can be monitored indirectly through serum prostate-specific antigen (PSA), insofar as increasing PSA levels indicate that androgen receptor (AR) activity is functional in CRPC.

Prior work has shown that the tubulin/microtubule system, which is an integral component of the cytoskeleton, is a therapeutic target for prostate cancer treatment (3). Microtubules are highly dynamic structures that play a critical role in orchestrating the separation and segregation of chromosomes during mitosis (4). Once the motor protein kinesin-1 is recruited to the microtubules, it preferentially moves various cargoes, including vimentin filaments and transferrin, along detyrosinated microtubules (5, 6). Tubulin-binding agents are derived from natural sources and include a large number of compounds with diverse chemical structures, all sharing an ability to disrupt microtubule dynamics, induce mitotic arrest, and promote apoptosis. The best characterized of these agents are the *Vinca* alkaloids and taxanes, which at high doses cause microtubule destabilization and microtubule stabilization, respectively. Two independent multicenter phase III studies (Southwest Oncology Group 99-16 and TAX 327) compared taxane-based regimens with mitoxantrone/prednisone and showed a significant survival benefit in patients (7, 8). Docetaxel, a semisynthetic taxane, stabilizes the microtubule by promoting binding to β -actin. Once bound, microtubules cannot be disassembled, thereby disrupting mitosis, causing G₂M cell cycle arrest, and

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triggering apoptosis (9). Although docetaxel and prednisone chemotherapy has become a first-line standard treatment for metastatic CRPC, the efficacy of this therapy in combination with other chemotherapeutic agents is still under investigation (3).

Androgen deprivation therapy (ADT) is the first-line treatment for advanced metastatic prostate cancer. After the initial response, however, tumor relapse occurs in the majority of patients due to the emergence of an androgen-independent CRPC state (10). The dynamic relationship between prostate cancer growth and the androgen signaling axis features a unique complexity in its ability to drive tumor progression and simultaneously dictate therapeutic potential. Androgen-induced prostate epithelial cell proliferation engages indirect pathways involving paracrine mediators produced by stromal cells (11, 12). The long-term benefit of androgen deprivation in patients with metastatic disease has been the subject of debate (10, 13, 14). Recent breakthroughs in the development of novel AR antagonist strategies led to phase I clinical trials with the potential to improve the efficacy of AR targeting and consequently the therapeutic outcome in patients with CRPC (15). Paralleling these studies is the discovery that taxanes can target prostate tumors through alternative routes besides mitosis disruption. Docetaxel counteracts the prosurvival effects of *Bcl-2* gene expression (16, 17). *Bcl-2* is part of class of oncogenes that contributes to neoplastic progression by inhibition of apoptotic cell death. Phosphorylation of *Bcl-2* inhibits its activity (9).

The clinical knowledge of paclitaxel as the only effective treatment for CRPC calls for the need to understand the mechanisms of action of this drug to augment its therapeutic efficacy. Considering the requirement of AR signaling to drive prostate growth and survival, and because CRPC still retains AR activity, in this study we explored the impact of tubulin and microtubule-targeting drugs on AR signaling in prostate cancer. Our results show that microtubule-targeting agents play a prominent role in impairing AR nuclear transport and activity, thus promoting prostate tumor suppression. This evidence suggests a potential new mechanism underlying treatment failure (to paclitaxel) of prostate cancer patients within the microtubule repertoire in CRPC.

Materials and Methods

Patients and specimen processing

Between January 2001 and November 2004, 57 patients with high-risk localized prostate cancer (defined a cT2b or T3a, PSA ≥ 15 ng/mL, or Gleason grade $\geq 4+3$) were recruited for a phase II trial of neoadjuvant chemotherapy (using docetaxel and mitoxantrone). The design of the clinical trial has been previously described (18). The study was approved by the institutional review boards of the Oregon Health & Science University, Portland VA Medical Center, Kaiser Permanente Northwest Region, Legacy Health System, and the University of Washington, and all patients provided signed informed consent. From each patient, 10 standard prostate biopsies (bilateral at the apex, bilateral medial and lateral

at mid-gland, bilateral medial and lateral at the base of the gland) were obtained under ultrasound guidance and snap-frozen in liquid nitrogen.

Tissue microarray construction

A tissue microarray (TMA) was constructed from formalin-fixed representative tissues collected at prostatectomy from the first 50 patients enrolled on the neoadjuvant chemotherapy study. Tissue cores (0.6-mm diameter placed 0.2 mm apart) were removed from the paraffin-embedded prostate tissue blocks (donor blocks) and placed in a recipient paraffin block (30 \times 25 mm) by using a precision Tissue Arrayer (Beecher Instruments). Hematoxylin and eosin (H&E) slides of each donor block were examined microscopically and reviewed by a pathologist to determine the appropriate location to sample. From every study patient, three cores each of prostate cancer, normal prostate, and, where applicable, lymph nodes with metastatic cancer were placed in each block in a pseudorandomized fashion. Dispersed among the study cores were control tissues from non-study patients (liver, prostate, lymph node, salivary gland, kidney, testis), untreated cell lines (DU-145, PC-3, LNCaP), and the same cell lines treated with mitoxantrone and docetaxel (alone or in combination). After completion, the block was heated at 37°C for 30 minutes to ensure incorporation of the cores into the block. The block was then cut into 5- μ m-thick sections, and unstained slides were stored at 4°C until needed.

Microscopic evaluation of frozen sections of tissue samples identified the presence of adequate number of cancer cells in both pretreatment and posttreatment samples for 31 subjects. Frozen sections (7 μ mol/L) were cut from tissue frozen in ornithine carbamyl transferase blocks, stained with Mayer's hematoxylin (Sigma), dehydrated in 100% (v/v) ethanol and xylene, and used for laser capture microdissection using an Arcturus PixCell Iie microscope (Arcturus, Inc.). To evaluate gene expression alterations after chemotherapy, malignant epithelium from pretreated biopsy and posttreated prostatectomy specimens were captured separately (3,000 cells per sample). The histology of captured cells was verified both by review of an H&E-stained frozen section from each sample and by review of the pre/post-laser capture microdissection images.

Cell lines and antibodies

The androgen-sensitive and transforming growth factor- β (TGF- β)-responsive human prostate cancer cells LNCaP T β R11 cells (19, 20), LNCaP, CWR22, and PC-3 prostate cancer cell lines were used in this study. To determine the effects of dihydrotestosterone (DHT) and TGF- β , cells were grown in DMEM or RPMI 1640 with 10% fetal bovine serum (without phenol red). The antibodies against E-cadherin, β -catenin, and poly(ADP-ribose) polymerase (PARP) were purchased from Cell Signaling Technology. The antibodies against the AR, tubulin, and N-cadherin proteins were purchased from Santa Cruz Biotechnology. The cofilin and actin antibodies were obtained from Sigma-Aldrich. The antibody against the human talin protein was purchased

from Upstate Biotechnology. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was obtained from Novus Biologicals.

Cell lines. The LNCaP, CWR22, and PC-3 cell lines were obtained from American Type Culture Collection and used within 6 to 12 months. The androgen-sensitive and TGF- β -responsive human prostate cancer LNCaP TRII cells were generated and characterized in our laboratory (19, 20).

Western blot analysis

Total cellular protein was extracted from the cell pellets by homogenization in radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 2 mmol/L EDTA, 1% NP40, 0.1% SDS]. Protein samples (20–60 μ g) were loaded on 4% to 12% SDS-polyacrylamide gels and subjected to electrophoretic analysis and subsequent blotting. Membranes were incubated with the primary antibody, overnight at 4°C, and the relevant secondary antibodies (1 hour at room temperature). Membranes were subsequently incubated with the enhanced chemiluminescence system (Amersham BioSciences) and autoradiographed using X-ray film (Amersham BioSciences). Densitometric analysis was performed using the Scion Image program. All bands were normalized to actin and shown as fold change compared with controls.

Immunofluorescence analysis

Cells were plated (1×10^5 per well) in chamber slides. After 24 hours, cells were exposed to RPMI 1640 supplemented with 10% charcoal-stripped serum in the presence of DHT (1 nmol/L), TGF- β (5 ng/mL), or a combination of DHT and TGF- β as indicated. Following treatment, cells were fixed in 2% (v/v) paraformaldehyde in PBS and permeabilized in 0.1% Triton X-100 in PBS. Cells were incubated with the primary antibody overnight at 4°C and secondary antibody for 1 hour at room temperature. Slides were mounted by Vectashield mounting medium (Vector Laboratories, Inc.) and were visualized and counted under a fluorescence microscope (Olympus IX70 Inverted Microscope, Olympus America, Inc.).

Immunohistochemical analysis

Prostate TMAs were subjected to immunohistochemical analysis by using the following antibodies: the mouse monoclonal antibody against PSA and the rabbit polyclonal antibody against E-cadherin from Cell Signaling Technology, Inc., and N-cadherin and AR (sc-7939 and sc815, respectively; Santa Cruz Biotechnology). After blocking nonspecific binding with goat serum (1.5% in TBS-T) for 30 minutes at room temperature, serial sections were exposed to the specific antibodies overnight at 4°C (negative controls were incubated with IgG). Sections were subsequently exposed to biotinylated goat anti-rabbit IgG (1 hour, room temperature) and horseradish peroxidase–streptavidin conjugate (Chemicon). Color development was accomplished using a standard immunoperoxidase method (DakoCytomation LSAB2 system-HPR) and counterstaining with hematoxylin. Images were captured using an Olympus BX51 microscope system (Olym-

pus America). Protein expression pattern, intensity, and localization were assessed in formalin-fixed, paraffin-embedded prostate cancer TMAs through light microscopy, performed by two independent observers (NK and CB) blinded to treatment modality. Three different cores were measured for each patient. The overall pattern of staining in human prostate tumor cells in the TMAs was determined as the average number of positive epithelial cells in three different fields for each tissue core.

Immunoprecipitation analysis

Cells were harvested in lysis buffer [10 mmol/L Tris (pH 7.5), 1 mmol/L EDTA, 400 mmol/L NaCl, 10% (v/v) glycerol, 5 mmol/L NaF, 0.5 mmol/L sodium orthovanadate, 1 mmol/L dithiothreitol] and Complete Mini Protease Inhibitor Cocktail (Roche Biochemicals). Cell extracts were homogenized, and protein content was quantitated using the Bio-Rad Protein Assay (Bio-Rad). Cell lysates (400 μ g) were precleared with protein A/G beads (Oncogene Research Products) and were subsequently incubated with the AR or the α -tubulin antibody (overnight at 4°C). Protein A/G beads were then added to the cell lysate/antibody mixture. Following incubation (1 hour at 4°C), the lysate/antibody/bead mixture was centrifuged at $14,000 \times g$ (30 seconds). Beads were subjected to elution with 100 mmol/L glycine (pH 3.0), and eluate fractions were centrifuged at $14,000 \times g$ in 1 mol/L phosphate buffer (pH 8.0). Samples were lysed in SDS-PAGE buffer and analyzed by Western blotting as described above.

RNA extraction and real-time reverse transcriptase-PCR

RNA samples extracted with Trizol reagent were treated with RNase-free DNase I and reverse transcript to cDNA (Bio-Rad) Taqman real-time reverse transcriptase-PCR analysis of the cDNA samples was conducted in an ABI 7700 Sequence Detection System (Applied Biosystems, Inc.) using specific primers for PSA (Applied Biosystems).

Transient transfections and luciferase activity assays

Cells were plated (10^5 per well) in six-well plates and treated as described above. After 48 hours, cells were transfected with the androgen response element (ARE) luciferase construct (1 mg/well; from Dr. Zoran Culig, Innsbruck Medical University, Innsbruck, Austria) in the presence of the control Renilla luciferase construct (Promega) using Tfx-50 transfection reagent (Promega). Following a 2-hour incubation with the DNA/Tfx50 mixture, serum-containing medium was added to the cells and incubation was continued for 22 hours. After treatment, cells were harvested and luciferase activity was determined according to the manufacturer's protocol (Promega, Dual Luciferase Assay). Data are representative of three independent experiments in duplicate.

Statistical analysis

Student *t* test and one-way ANOVA was performed to determine the statistical significance between values

for the *in vitro* experiments. The data derived from the immunostaining analysis of human prostate tissue specimens were analyzed for statistical significance using the unpaired *t* test. All numerical data are presented as the mean values \pm SEM. Statistical significance was reached at a *P* value of <0.05 .

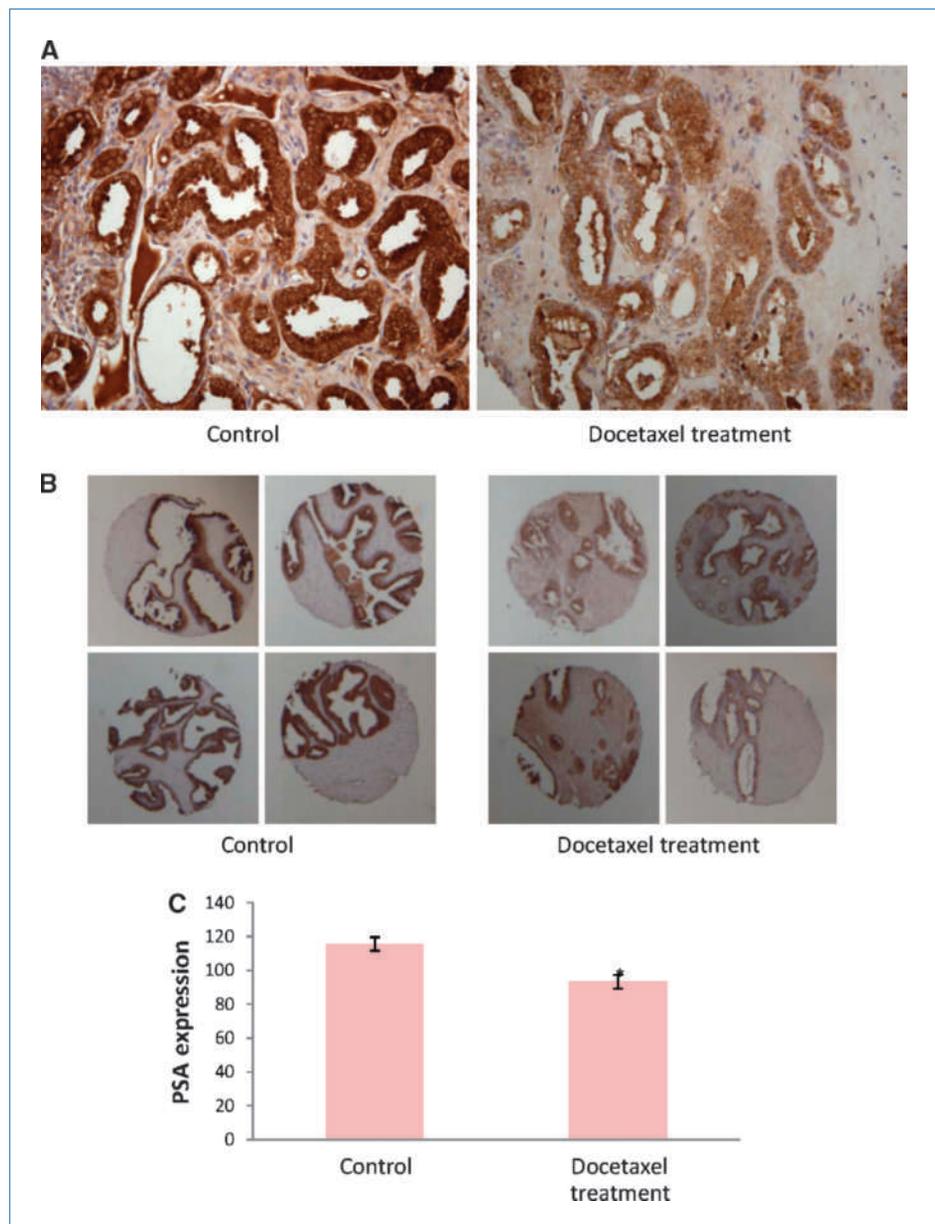
Results

Taxol chemotherapy inhibits PSA expression in prostate cancer

Taxol chemotherapy reduces the serum PSA levels in prostate cancer patients (21, 22). To investigate whether

the reduction in PSA is due to either tumor shrinkage or signaling axis impairment, PSA expression was profiled in prostate cancer epithelial cells by performing immunocytochemical analysis using the TMAs of human prostate specimens from docetaxel-treated prostate cancer patients. The results shown on Fig. 1A reveal the effect of docetaxel on tissue PSA expression in individual prostate tumor cells. There was a marked decrease in PSA immunoreactivity in the individual tissue arrays in response to microtubule-targeting drug treatment (Fig. 1B). Quantitative analysis of the data revealed a significant reduction (19%) in the intensity of PSA in prostate tumors from patients receiving docetaxel, compared with specimens from untreated patients (Fig. 1C).

Figure 1. Docetaxel suppresses PSA expression in human prostate tumors. A, PSA immunoreactivity pattern of prostate tissue array; left, untreated patients; right, docetaxel-treated patients. B, representative images of individual prostate tumor TMAs from untreated control and docetaxel-treated prostate cancer patients. Immunostaining for PSA was conducted as described in Materials and Methods. C, quantitative evaluation of PSA expression in tumor epithelial cells in prostate specimens from docetaxel-treated and untreated prostate cancer patients was determined as described in Materials and Methods. *, $P < 0.01$.



Taxol inhibits AR transcriptional activity

Paclitaxel and nocodazole were used to disrupt normal cellular function of the microtubule system. Similar to docetaxel, paclitaxel is a chemotherapy drug classified with the taxane group and used in the treatment of advanced prostate cancer (3). Nocodazole exerts its antitumor effect by interfering with the polymerization of microtubules. Subsequent experiments focused on determining the effect of microtubule-targeting drugs on AR activation *in vitro*. The mRNA levels of PSA were evaluated by quantitative PCR in response to DHT/microtubule-targeting drugs. Treatment of human prostate cancer LNCaP cells with DHT (1 nmol/L) for 24 hours led to a significant increase in the expression of PSA mRNA. Nocodazole completely abolished and paclitaxel partially inhibited this PSA induction (Fig. 2A). The changes in PSA protein levels were consistent with the mRNA changes in response to treatment (Fig. 2B). To further investigate the consequences of microtubule targeting on AR transcriptional activity, the ARE-luciferase vector was introduced to LNCaP cells in response to DHT in the presence of nocodazole or paclitaxel. Activation of ARE was detected within 24 hours of DHT treatment and was significantly inhibited by both nocodazole and paclitaxel (Fig. 2C).

Taxol inhibits ligand-independent AR transcriptional activity

Epidermal growth factor (EGF) induces ligand-independent AR activation in prostate cancer cells with hypophysical androgen level (23). To determine the effect of microtubule-targeting drugs on ligand-independent transcriptional activation of AR, EGF was used to induce the androgen-independent activation of AR. A significant increase in PSA mRNA expression was detected in response to EGF in combination with DHT, whereas nocodazole or paclitaxel ablated this PSA mRNA induction within 24 hours (Fig. 3A). To investigate whether the impaired AR transcriptional activity is specific to microtubule-targeting drugs, two different drugs, Velcade and Doxazosin, were examined (Fig. 3B). Exposure to either one of these agents did not affect the androgen-mediated PSA mRNA expression (Fig. 3C).

Microtubule-targeting chemotherapy inhibits AR nuclear translocation

To further investigate the effect of Taxol drugs on AR function in prostate cancer cells, AR expression was evaluated in docetaxel-treated prostate cancer patients. There was no significant change in AR levels in prostate epithelial cells between the two groups (Fig. 4A). However, marked changes in the cellular localization of AR were observed in response to docetaxel treatment. For the prostate specimens derived from patients not receiving chemotherapy, 50% prostate cancer epithelial cells exhibited nuclear accumulation of AR, whereas only 10% of the cell population had cytoplasmic localization of AR (Fig. 4B and C). For docetaxel-treated patients, there was a marked reduction in nuclear translocation of AR (to 38%), with a parallel increase (to 29%) predominantly in the cytoplasm (Fig. 4B and C). The AR localization also correlated with PSA expression level in prostate epithelial cells. Cells with nuclear AR localization exhibited a higher PSA expression (Fig. 4D). The impact of microtubule targeting on AR localization in prostate cancer cells *in vitro* was assessed by immunofluorescence staining to evaluate the AR nuclear translocation in response to Taxol treatment. As shown on Fig. 5 (A and B), DHT treatment (4 hours) induces a massive AR nuclear translocation in LNCaP cells. Pretreatment of paclitaxel and nocodazole for 24 hours

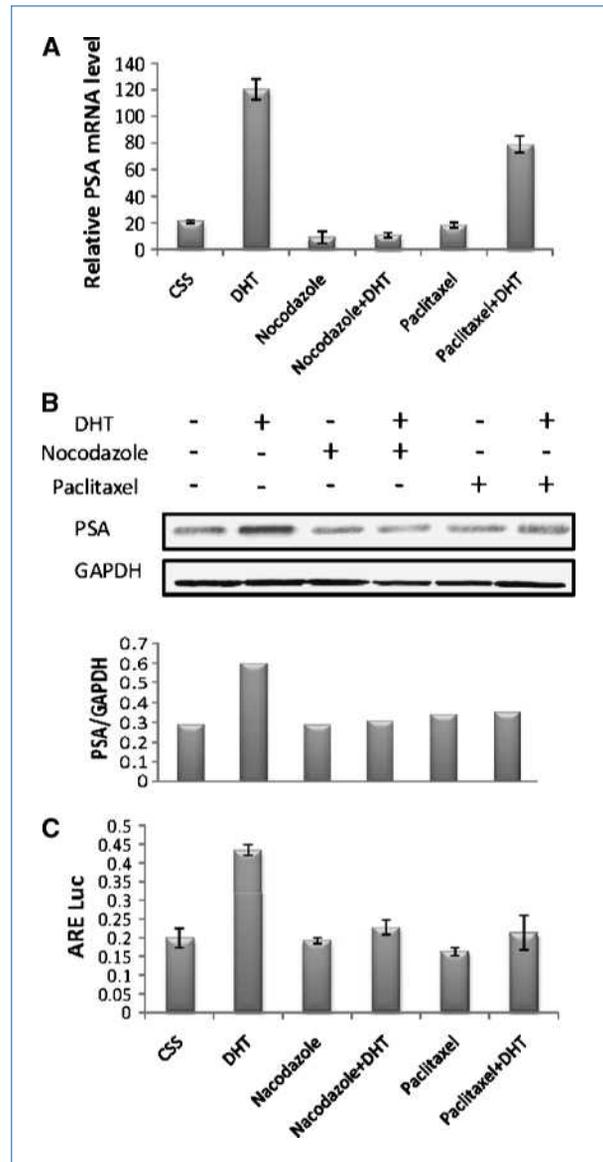


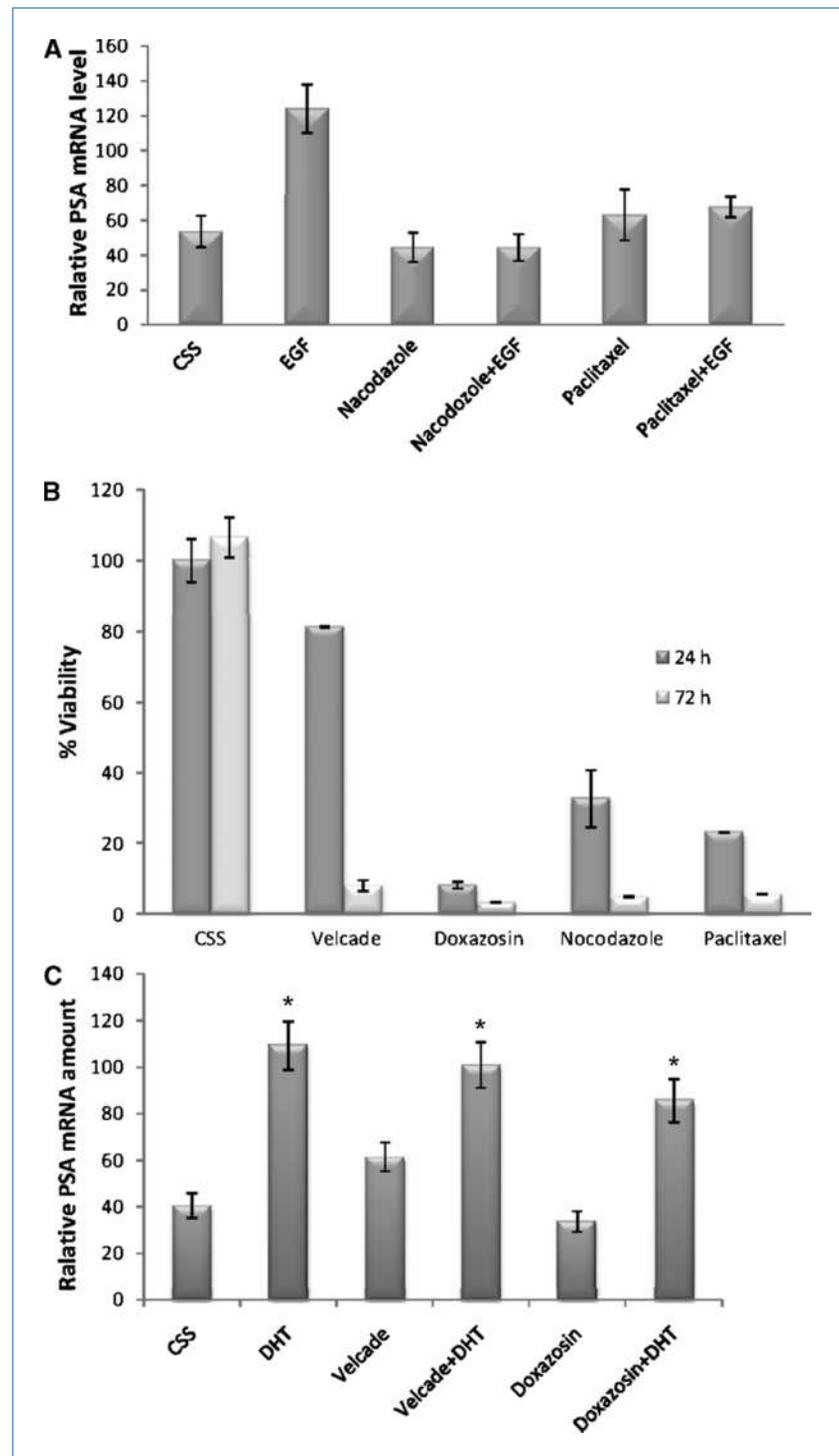
Figure 2. Microtubule-targeting drugs inhibit ligand-dependent AR transcriptional activity. LNCaP cells were treated with DHT (1 nmol/L) in the presence or absence of nocodazole (5 μ g/mL) or paclitaxel (1 μ mol/L). A, PSA mRNA expression was determined by real-time PCR. B, PSA protein levels were assessed by Western blotting, and relative expression was quantitated (bottom). C, AR transcriptional activity in response to microtubule-targeting drugs was determined using the ARE luciferase reporter vector in LNCaP cells.

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abrogated this AR nuclear translocation (Fig. 5A and B). Western blot analysis of the cellular compartments after subcellular fractionation also revealed that DHT-induced nuclear translocation of AR was blocked in response to either paclitaxel or nocodazole (Fig. 5C).

The process of epithelial-mesenchymal-transition (EMT) during which cells lose their polarity and cell-junction proteins and acquire mesenchymal cell markers is linked to tumor progression and metastasis. Because we recently reported that androgens and the AR regulate EMT and

Figure 3. Microtubule targeting inhibits ligand-independent AR transcriptional activity. A, LNCaP cells were treated with a combination of DHT (0.1 nmol/L) and EGF (5 nmol/L) with or without nocodazole (5 μ g/mL) or paclitaxel (1 μ mol/L). AR transcriptional activity was evaluated on the basis of PSA expression by using real-time PCR. B, LNCaP cells were treated with the following chemotherapeutic agents for 24 to 72 h: tumor necrosis factor-related apoptosis-inducing ligand, Velcade, Doxazosin, nocodazole (5 μ g/mL), or paclitaxel (1 μ mol/L), and cell death was determined using the MTT assay. C, LNCaP cells were treated with DHT (1 nmol/L) in the presence or absence of Velcade or Doxazosin. PSA mRNA expression was evaluated by real-time PCR. CSS, charcoal-stripped serum. *, $P < 0.05$.



cytoskeleton organization involved in the invasive behavior of prostate tumor epithelial cells (24), we subsequently examined the consequences of Taxol chemotherapy on EMT. Expression of E-cadherin, β -catenin (epithelial cell markers), and N-cadherin (mesenchymal cell marker) was immunohistochemically profiled in the prostate TMAs from treated and untreated patients. We found that docetaxel treatment had no significant impact on EMT (Supplementary Fig. S2).

Tubulin interacts with the AR

Microtubule is the main cytoskeleton protein component responsible for intracellular protein transportation and facilitates many cellular events. The potential interaction between AR and microtubules was subsequently investigated. The interaction of endogenous AR and α -tubulin was detected in both LNCaP and CWR22 cells (Fig. 5D and E).

The colocalization of AR and tubulin was detected by yellow immunofluorescence staining (Supplementary Fig. S1A). This colocalization was reduced by DHT treatment (Fig. 5D and E; Supplementary Fig. S1A). To further determine the interaction site of AR with tubulin, PC-3 prostate cancer cells were transfected with different truncated forms of AR (Fig. 5F). Loss of either the ligand-binding domain-hinge domain or the DNA-binding domain cannot inhibit the interaction of AR and tubulin, implicating the NH₂-terminal domain as being responsible for the AR and tubulin association and potential interaction (Fig. 5G).

Androgens downregulate tubulin in prostate cancer cells

To determine whether androgen signaling can affect the microtubules, tubulin expression was evaluated by Western

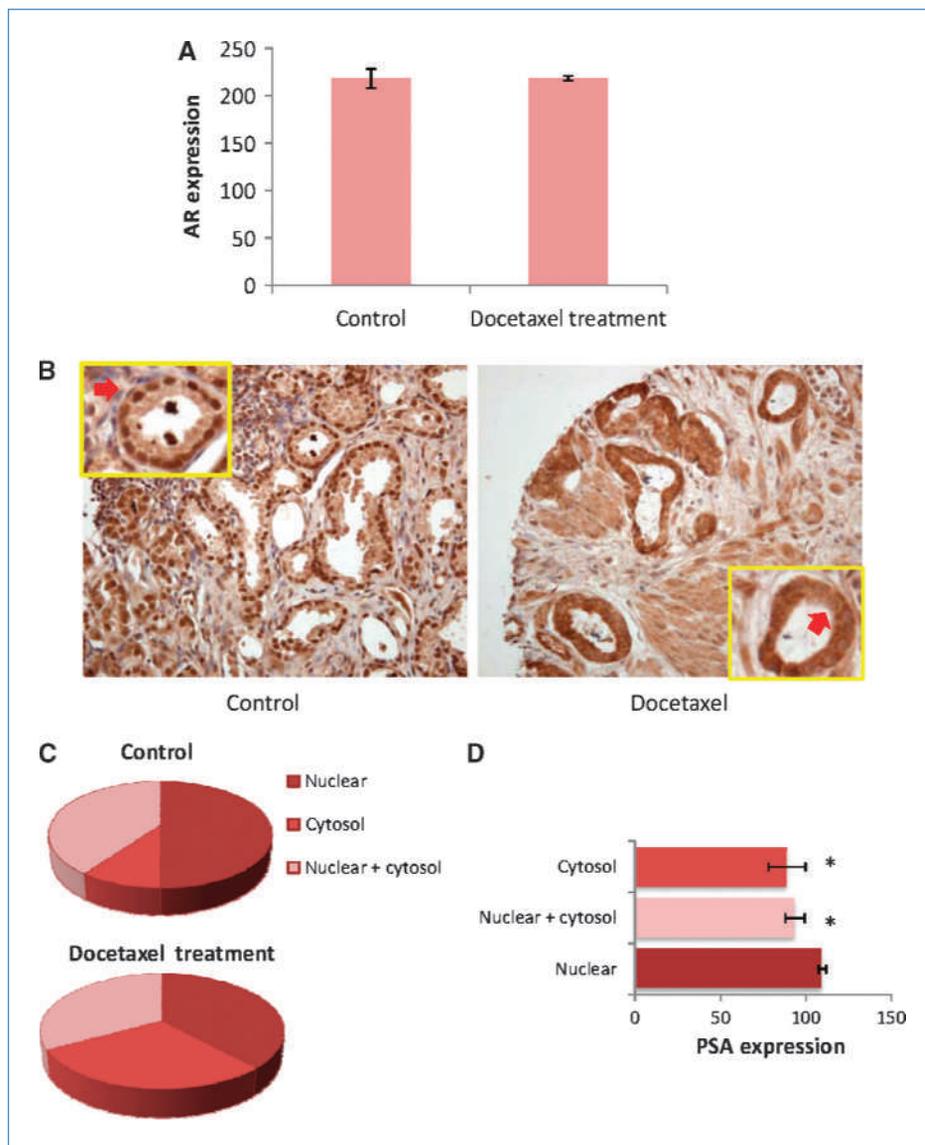


Figure 4. Docetaxel suppresses AR nuclear translocation in prostate cancer. A, AR protein expression levels in prostate cancer epithelial cells of docitaxel-treated and untreated patients was evaluated by immunohistochemical staining. There was no significant change in AR levels in prostate epithelial cells between the two groups. B, representative images of the subcellular AR localization in human prostate tissue. The presence of AR was assessed in formalin-fixed, paraffin-embedded prostate cancer tissue microarrays through light microscopic examination in which observers were blinded to treatment modality. The overall pattern of staining (specifically the presence or absence of AR localization in the nucleus, cytoplasm, or both) was determined for each tissue core. Left, tissue from untreated patients; right, tissue from docetaxel-treated patients. C, percentage of nuclear and cytoplasmic AR in docetaxel-treated and untreated tumors. For the prostate specimens from untreated patients, 50% prostate cancer epithelial cells exhibited nuclear AR. In docetaxel-treated patients, a reduction in nuclear translocation of AR was paralleled by an increase in cytosolic AR. D, AR localization correlated with PSA levels in prostate epithelial cells.

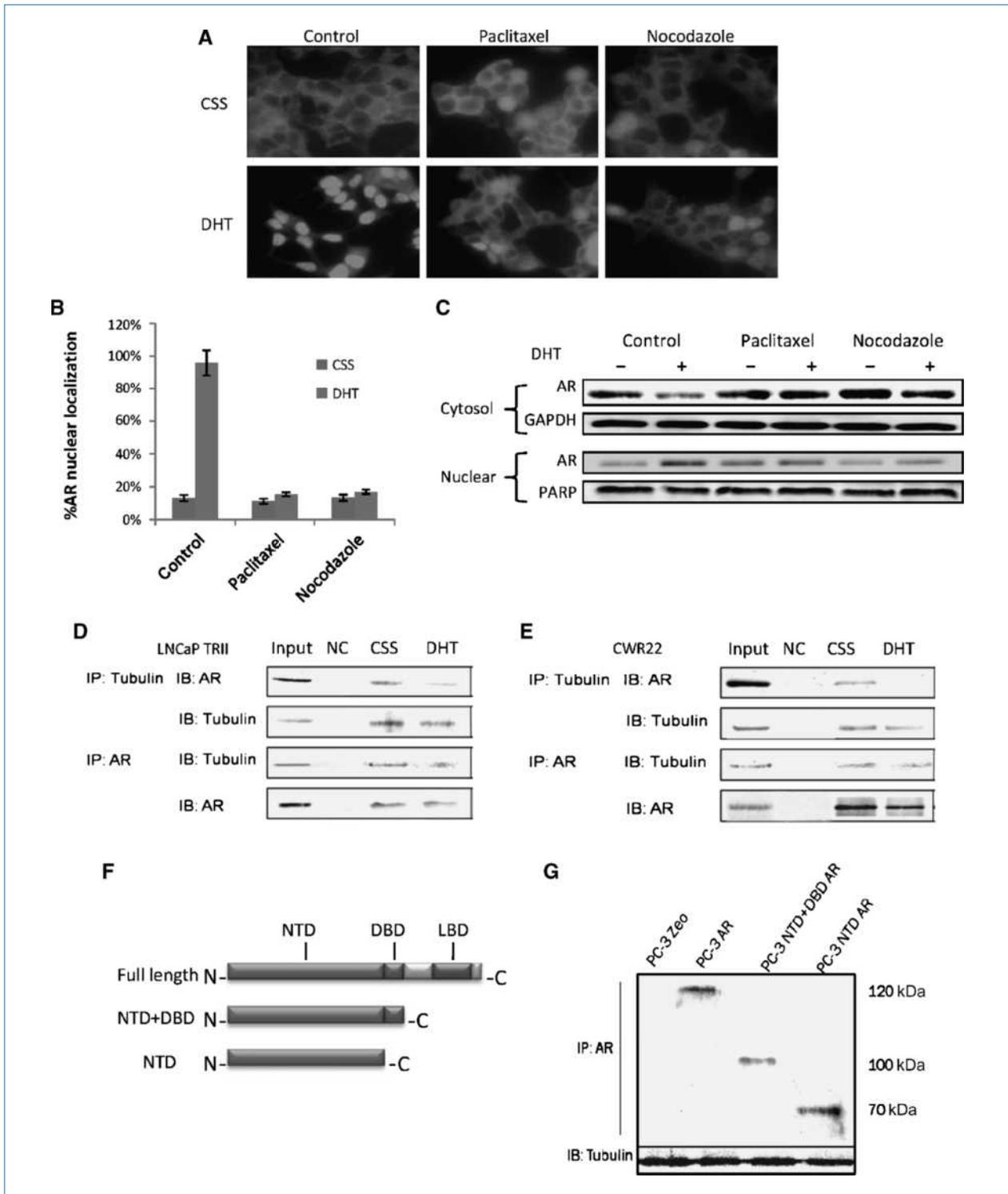


Figure 5. Tubulin interacts with AR. A and B, androgens induce AR nuclear translocation in LNCaP cells, and pretreatment of paclitaxel and nocodazole for 24 h abrogated this AR nuclear translocation. Subcellular localization of AR was detected by fluorescent staining (red; 40 \times magnification). Western blot analysis of the cellular compartments after subcellular fractionation also revealed that DHT-induced nuclear translocation of AR was blocked in response to either paclitaxel or nocodazole treatment (C). GAPDH and PARP were used as loading controls. D and E, LNCaP TRII and CWR22 cells were treated with DHT (1 nmol/L) in the presence or absence of TGF- β (5 ng/mL), respectively. Immunoprecipitation was performed by using the antibodies against either tubulin or AR to show the AR-tubulin association. F, truncated forms of AR transfected in PC-3 cells. Immunoprecipitation analysis of AR and tubulin interaction indicates that loss of ligand-binding and DNA-binding domain and hinge domain did not inhibit the AR-tubulin association (G).

blot analysis and immunofluorescence. Treatment of prostate cancer cells with DHT significantly inhibited tubulin expression (Fig. 6A). There was a marked reduction in tubulin levels, an effect that was enhanced by TGF- β (Supplementary Fig. S1B). Immunofluorescence staining revealed that the microtubule spindles were undetectable after androgen treatment (Fig. 6B).

Discussion

The present study documents that microtubule-stabilizing chemotherapeutic agents interfere with AR nuclear localization and activity in human prostate tumors. The microtubule network has been implicated in facilitating the nuclear import of cancer regulator proteins, including pThrP, P53, and Rb (25–27). There is a solid body of evidence supporting the requirement for androgen-dependent and androgen-independent activation of AR and its nuclear translocation toward downstream androgen signaling (28). In conjunction with the shown association and potential interaction of AR and tubulin, our findings raise the possibility that preferen-

tial binding of microtubules to AR may recruit AR to determine its transcriptional activity. Significantly enough, microtubule-targeting chemotherapy was found to suppress both ligand-dependent and ligand-independent AR signaling in prostate cancer cells. Considering that nuclear protein importation is not directly dependent on microtubules (27), and because AR function was not impaired by chemotherapeutic agents nontargeting microtubules, such an effect seems to be specific and indeed may represent a new mode of action for microtubule-targeting drugs to regulate AR intracellular distribution in prostate tumors. Thus, the AR cytoplasmic “zip code” determined by its localization becomes critical in targeting CRPC. In accordance with our findings, it was recently reported that paclitaxel treatment increases the association of FOXO1 (an AR-suppressive nuclear transcription factor) with nuclear AR in prostate cancer cells (29).

These findings are important in enhancing the clinical benefit generated by taxane-based regimens in prostate cancer patients with advanced disease. Clinical data reported by two independent teams established that docetaxel-based chemotherapy regimens lead to a significant survival benefit

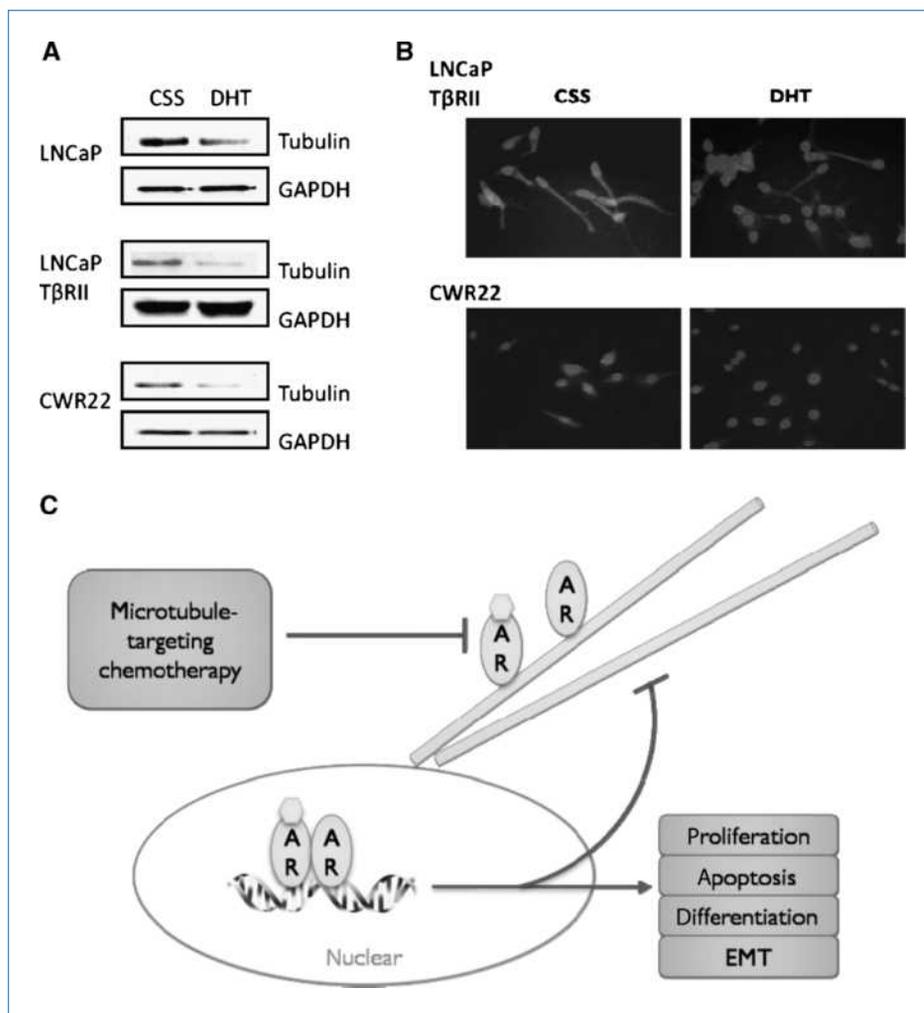


Figure 6. Androgens inhibit tubulin expression in prostate cancer cells. A, prostate cancer cells LNCaP, LNCaP T β RII, and CWR22 were treated with DHT (1 nmol/L, 72 h), and tubulin expression was evaluated by Western blotting using GAPDH as an internal normalizing control. B, LNCaP T β RII and CWR22 cells were treated with DHT (1 nmol/L) with or without TGF- β (5 ng/mL) for 72 h. Tubulin expression was detected by immunofluorescence (red); nuclei were stained by 4',6'-diamidino-2-phenylindole (blue). C, emerging mechanistic scenario: microtubules facilitate AR nuclear translocation and enhance downstream AR transcriptional activity in prostate cancer cells. Microtubule-targeting chemotherapy blocks this pathway and suppresses AR signaling through a negative feedback mechanism. AR signaling inhibits tubulin expression, thus impairing the cytoskeleton structure and organization.

in men with CRPC (7, 8). Microtubule stabilization through binding of docetaxel to β -tubulin is the most widely accepted mechanism of action. Once bound with taxanes, microtubules cannot disassemble; thus, the static polymerization disrupts the normal mitotic process and arrests cells in the G₂M phase, ultimately inducing apoptotic cell death. Another action of docetaxel is its antagonistic activity against the pro-survival effects of *bcl-2*. Treatment of prostate cancer cells overexpressing *bcl-2* with Taxol induces *bcl-2* phosphorylation. Bcl-2 phosphorylation inhibits its binding to bax and consequently apoptosis of prostate cancer cells in response to Taxol (30). The present findings suggest another mechanism for taxane-based regimens toward impairing nuclear localization and activity of AR.

A popularized underlying mechanistic basis for the therapeutic failure to ADT is the emergence of androgen-independent activation of AR responsible for driving and maintaining uncontrolled prostate tumor growth because ADT cannot impair the ligand-independent pathway (31). In our study, microtubule-targeting chemotherapy drugs could inhibit both the androgen-dependent and androgen-independent activation of AR by blocking AR nuclear translocation. Thus, it is tempting to speculate on an additional level of tumor suppression action by ADT. Addition of an AR-binding moiety to a therapeutic agent such as Taxol could selectively target AR-expressing prostate cancer cells. A potential combination of ADT and taxanes may augment efficacy by targeting both androgen-dependent and androgen-independent prostate tumor growth.

Modification of tubulin (detyrosination/tyrosination) can affect the microtubule stability (32). The present data indicate that binding of α -tubulin to AR engages the NH₂-terminal domain of AR as the required anchoring site. One could argue that the evidence suggesting that androgens suppress α -tubulin and impair the microtubules in prostate cancer implicates a potential negative feedback regulation in microtubule AR (Fig. 6C). This feedback loop may explain the reduced association and colocalization between AR and tubulin due to tubulin downregulation. Androgen signaling is important in cell differentiation and regulates the cell cycle, including G₂M arrest (33, 34), consistent with its function in inhibiting microtubule structures (Supplementary Fig. S3).

Our findings are in accordance with recent elegant molecular dynamics-based studies indicating that a conjugate of colchicine and an AR antagonist (cyanonilutamide) with tubulin-inhibiting activity increases cytoplasmic AR levels and antagonizes AR activity in prostate cancer cells (35). Moreover, indirect support for a microtubulin-targeting action influencing steroid receptor activity is gained from evidence on the ability of estrogens to regulate β -tubulin synthesis and decrease microtubule density, ultimately blocking prostate cancer cells at the G₂M phase (36, 37).

In summary, our study documents the contribution of the tubulin/microtubule repertoire to AR signaling in human prostate cancer by sequestering AR in the cytoplasm toward apoptotic signaling promotion and tumor growth inhibition. Impairing this AR activity enables a previously unrecognized, targeting forum for Taxol-based chemotherapy during prostate cancer progression (Supplementary Fig. S4) and supports a combination strategy of ADT with tubulin-targeting chemotherapy toward an improved therapeutic response in CRPC. The mechanisms through which this multifunctional tubulin-targeting compound hinders the AR ligand-binding pocket is currently being investigated.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

- Wang X, Yin L, Rao P, et al. Targeted treatment of prostate cancer. *J Cell Biochem* 2007;102:571–9.
- Petrylak DP. The current role of chemotherapy in metastatic hormone-refractory prostate cancer. *Urology* 2005;65:3–7, discussion 8.
- Mancuso A, Oudard S, Sternberg CN. Effective chemotherapy for hormone-refractory prostate cancer (HRPC): present status and perspectives with taxane-based treatments. *Crit Rev Oncol Hematol* 2007;61:176–85.
- Giannakakou P, Sackett D, Fojo T. Tubulin/microtubules: still a promising target for new chemotherapeutic agents. *J Natl Cancer Inst* 2000;92:182–3.
- Kreitzer G, Liao G, Gundersen GG. Detyrosination of tubulin regulates the interaction of intermediate filaments with microtubules *in vivo* via a kinesin-dependent mechanism. *Mol Biol Cell* 1999;10:1105–18.
- Liao G, Gundersen GG. Kinesin is a candidate for cross-bridging microtubules and intermediate filaments. Selective binding of kinesin to detyrosinated tubulin and vimentin. *J Biol Chem* 1998;273:9797–803.
- Petrylak DP, Tangen CM, Hussain MH, et al. Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer. *N Engl J Med* 2004;351:1513–20.
- Tannock IF, de Wit R, Berry WR, et al. Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. *N Engl J Med* 2004;351:1502–12.
- Kraus LA, Samuel SK, Schmid SM, Dykes DJ, Waud WR, Bissery MC. The mechanism of action of docetaxel (Taxotere) in xenograft models is not limited to bcl-2 phosphorylation. *Invest New Drugs* 2003;21:259–68.
- Shahinian VB, Kuo YF, Freeman JL, Goodwin JS. Risk of fracture

- after androgen deprivation for prostate cancer. *N Engl J Med* 2005; 352:154–64.
11. Cunha GR, Donjacour AA. Mesenchymal-epithelial interactions in the growth and development of the prostate. *Cancer Treat Res* 1989;46: 159–75.
 12. Byrne RL, Leung H, Neal DE. Peptide growth factors in the prostate as mediators of stromal epithelial interaction. *Br J Urol* 1996;77: 627–33.
 13. Makhsida N, Shah J, Yan G, Fisch H, Shabsigh R. Hypogonadism and metabolic syndrome: implications for testosterone therapy. *J Urol* 2005;174:827–34.
 14. Lu-Yao GL, Albertsen PC, Moore DF, et al. Survival following primary androgen deprivation therapy among men with localized prostate cancer. *JAMA* 2008;300:173–81.
 15. Tran C, Ouk S, Clegg NJ, et al. Development of a second-generation antiandrogen for treatment of advanced prostate cancer. *Science* 2009;324:787–90.
 16. Debes JD, Tindall DJ. Mechanisms of androgen-refractory prostate cancer. *N Engl J Med* 2004;351:1488–90.
 17. Oliver CL, Miranda MB, Shangary S, Land S, Wang S, Johnson DE. (-)-Gossypol acts directly on the mitochondria to overcome Bcl-2- and Bcl-X(L)-mediated apoptosis resistance. *Mol Cancer Ther* 2005;4:23–31.
 18. Beer TM, Garzotto M, Lowe BA, et al. Phase I study of weekly mitoxantrone and docetaxel before prostatectomy in patients with high-risk localized prostate cancer. *Clin Cancer Res* 2004;10:1306–11.
 19. Guo Y, Kyprianou N. Restoration of transforming growth factor β signaling pathway in human prostate cancer cells suppresses tumorigenicity via induction of caspase-1-mediated apoptosis. *Cancer Res* 1999;59:1366–71.
 20. Guo Y, Kyprianou N. Overexpression of transforming growth factor (TGF) β 1 type II receptor restores TGF- β 1 sensitivity and signaling in human prostate cancer cells. *Cell Growth Differ* 1998;9:185–93.
 21. Beer TM, Pierce WC, Lowe BA, Henner WD. Phase II study of weekly docetaxel in symptomatic androgen-independent prostate cancer. *Ann Oncol* 2001;12:1273–9.
 22. Berry W, Dakhil S, Gregurich MA, Asmar L. Phase II trial of single-agent weekly docetaxel in hormone-refractory, symptomatic, metastatic carcinoma of the prostate. *Semin Oncol* 2001;28:8–15.
 23. Oosterhoff JK, Grootegoed JA, Blok LJ. Expression profiling of androgen-dependent and -independent LNCaP cells: EGF versus androgen signalling. *Endocr Relat Cancer* 2005;12:135–48.
 24. Zhu M, Kyprianou N. Role of androgens and the androgen receptor in epithelial-mesenchymal transition and invasion of prostate cancer cells. *FASEB J* 2010;24:769–77.
 25. Giannakakou P, Sackett DL, Ward Y, Webster KR, Blagosklonny MV, Fojo T. p53 is associated with cellular microtubules and is transported to the nucleus by dynein. *Nat Cell Biol* 2000;2:709–17.
 26. Jiao W, Datta J, Lin HM, Dundr M, Rane SG. Nucleocytoplasmic shuttling of the retinoblastoma tumor suppressor protein via Cdk phosphorylation-dependent nuclear export. *J Biol Chem* 2006;281: 38098–108.
 27. Roth DM, Moseley GW, Glover D, Pouton CW, Jans DA. A microtubule-facilitated nuclear import pathway for cancer regulatory proteins. *Traffic* 2007;8:673–86.
 28. Feldman BJ, Feldman D. The development of androgen-independent prostate cancer. *Nat Rev Cancer* 2001;1:34–45.
 29. Gan L, Chen S, Wang Y, et al. Inhibition of the androgen receptor as a novel mechanism of Taxol chemotherapy in prostate cancer. *Cancer Res* 2009;69:8386–94.
 30. Haldar S, Chintapalli J, Croce CM. Taxol induces bcl-2 phosphorylation and death of prostate cancer cells. *Cancer Res* 1996;56: 1253–5.
 31. Burnstein KL. Regulation of androgen receptor levels: implications for prostate cancer progression and therapy. *J Cell Biochem* 2005; 95:657–69.
 32. Hammond JW, Cai D, Verhey KJ. Tubulin modifications and their cellular functions. *Curr Opin Cell Biol* 2008;20:71–6.
 33. Heisler LE, Evangelou A, Lew AM, Trachtenberg J, Elsholtz HP, Brown TJ. Androgen-dependent cell cycle arrest and apoptotic death in PC-3 prostatic cell cultures expressing a full-length human androgen receptor. *Mol Cell Endocrinol* 1997;126:59–73.
 34. Litvinov IV, Vander Griend DJ, Antony L, et al. Androgen receptor as a licensing factor for DNA replication in androgen-sensitive prostate cancer cells. *Proc Natl Acad Sci U S A* 2006;103:15085–90.
 35. Sharifi N, Hamel E, Lill MA, et al. A bifunctional colchicinoid that binds to the androgen receptor. *Mol Cancer Ther* 2007;6:2328–36.
 36. Bonham MJ, Galkin A, Montgomery B, Stahl WL, Agus D, Nelson PS. Effects of the herbal extract PC-SPES on microtubule dynamics and paclitaxel-mediated prostate tumor growth inhibition. *J Natl Cancer Inst* 2002;94:1641–7.
 37. Montgomery RB, Bonham M, Nelson PS, et al. Estrogen effects on tubulin expression and taxane mediated cytotoxicity in prostate cancer cells. *Prostate* 2005;65:141–50.

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Tubulin-Targeting Chemotherapy Impairs Androgen Receptor Activity in Prostate Cancer

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