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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 (CRPC), but 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 (TMAs) 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 (ARE) 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 to 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 (1μM) or Nocodazole (5μg/ml), inhibited androgen-dependent AR nuclear translocation, by targeting AR association with tubulin. Introduction of a truncated AR indicated the requirement of the N-terminal domain for AR-tubulin interaction. Our findings demonstrate 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.

Keywords

Androgens; microtubule-targeting drugs; androgen receptor translocation

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INTRODUCTION

Prostate cancer is the most frequently diagnosed non-cutaneous 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 18-24 months, with ultimate tumor recurrence to a castration-resistant-prostate cancer (CRPC) and progression to advanced disease Considerable efforts have been devoted towards 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 rising PSA levels indicate that 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 transferin, 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 (SWOG) 99-16 and TAX 327) compared taxane-based regimens with Mitoxantrone/Prednisone and demonstrated a significant survival benefit in patients (7-8). Docetaxel, a semi synthetic 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 triggering apoptosis (9). Although Docetaxel and Prednisone chemotherapy have become first-line standard treatment of 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 there is tumor relapse in the majority of patients due to emergence of 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 via 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, and the 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 in order to augment its therapeutic efficacy. Considering the requirement of AR signaling to drive prostate growth and survival and since 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 demonstrate 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 or PSA ≥ 15 ng/ml or Gleason grade ≥ 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, ten 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 × 25 mm) using a precision Tissue Arrayer (Beecher Instruments, Sun Prairie, WI). 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 pseudo-randomized fashion. Dispersed amongst 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 (singly or in combination). After completion, the block was heated at 37°C for 30 mins to ensure incorporation of the cores into the block. The block was then cut into 5μm 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 pre-treatment and post-treatment samples for 31 subjects. Frozen sections (7μM) were cut from tissue frozen in ornithine carbamyl transferase blocks, stained with Mayer’s hematoxylin (Sigma, St. Louis, MO), 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 post-treated 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 micro-dissection images.

Cell Lines and Antibodies

The androgen-sensitive and TGF-β responsive human prostate cancer cells LNCaP TβRII cells (19-20), LNCaP, CWR22 and PC-3 prostate cancer cell lines were used in this study.

To determine the effects of DHT and TGF-β, cells were grown in DMEM or RPMI1640 with 10% FBS (without phenol red). The antibodies against E-cadherin, β-catenin, and PARP were purchased from Cell Signaling Technology (Danvers, MA); The antibodies against the AR, tubulin, and N-cadherin proteins were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); The cofilin and actin antibodies were obtained from Sigma-Aldrich (St. Louis, MO); The antibody against the human talin protein was purchased from Upstate Biotechnology (Billerica, MA); The GAPDH antibody was obtained from Novus Biologicals (Littleton, CO).

Cell lines: The LNCaP, CWR22 and PC-3 cell lines were obtained from ATCC and used within 6-12 months. The androgen sensitive and TGF-beta 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 RIPA buffer (50 mM Tris HCl pH7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% SDS). Protein samples (20-60μg) were loaded on 4%-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 (1hr at room temperature). Membranes were subsequently incubated with the enhanced chemiluminescence system (Amersham BioSciences, Piscataway, NJ) and autoradiographed using X-ray film (Amersham BioSciences). Densitometric analysis was performed using the Scion Image program (NIH, USA, http://rsb.info.nih.gov/scoin-image/). All bands were normalized to actin and shown as fold-change compared to controls.

Immunofluorescence Analysis

Cells were plated (1×10^5 cells/well) in chamber slides and after 24 hrs, cells were exposed to medium RPMI 1640 supplemented with 10% charcoal-stripped-serum (CSS) in the presence of DHT (1nM), TGF-β (5ng/ml), or combination of DHT and TGF-β as indicated. Following treatment, cells were fixed in 2% (v/v) paraformaldehyde in phosphate-buffered saline (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 1hr at room temperature). Slides were mounted by Vectashield mounting medium (Vector laboratories Inc, Burlingame, CA) and were visualized and counted under fluorescence microscope (Olympus IX70 Inverted Microscope, Olympus America Inc. Center Valley, PA).

Immunohistochemical Analysis

Prostate TMAs were subjected to immunohistochemical analysis using the following antibodies were used: the mouse monoclonal antibody against PSA and the rabbit polyclonal antibody against E-cadherin from Cell Signaling Technology Inc. (Danvers, MA); N-cadherin, and AR (# sc-7939, and sc815 respectively, Santa Cruz Biotechnology, Santa Cruz, CA). After blocking nonspecific binding with goat serum (1.5% in TBS-T) for 30min at room temperature, serial sections were exposed to the specific antibodies overnight at 4°C (Negative controls consisted of incubation with IgG). Sections were subsequently exposed to biotinylated goat anti-rabbit IgG (1hr, room temperature) and horseradish peroxidase-streptavidin conjugate (Chemicon, Billerica, MA). Color development was accomplished using a standard immunoperoxidase method (Dako cytomation LSAB2 system-HPR, Carpinteria, CA) and counterstaining with hematoxylin. Images were captured using an Olympus BX51 microscope system (Olympus America, Lake Success, NY). Protein expression pattern, intensity and localization were assessed in formalin-fixed, paraffin-embedded prostate cancer TMAs via light microscopy, was 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 [10mM Tris pH 7.5, 1mM EDTA, 400mM NaCl, 10% (v/v) glycerol, 5 mM NaF, 0.5mM sodium orthovanadate, 1 mM dithiothreitol (DTT)], and Complete Mini Protease Inhibitor Cocktail (Roche Biochemicals, Indianapolis, IN). Cell extracts were homogenized and protein content was quantitated using the BioRad Protein Assay (BioRad, Hercules, CA). Cell lysates (400μg) were pre-cleared with protein A/G beads (Oncogene Research Products, #IP05, Boston, MA) and were subsequently incubated with the AR or the a-tubulin antibody (overnight at 4°C). Protein A/G beads were then added to the cell lysate/antibody mixture. Following incubation (1hr at 4°C), the lysate/antibody/bead mixture was centrifuged at 14,000g (30sec). Beads were subjected to elution with 100mM glycine pH 3.0 and eluate-fractions were centrifuged at 14,000g in 1M phosphate buffer pH8.0. Samples were lysed in SDS-PAGE buffer and analyzed by Western blotting as described above.

RNA extraction and Real-Time RT-PCR

RNA samples extracted with Trizol Reagent were treated with RNase-free DNase I and reverse transcript to cDNA (BioRad) Taqman realtime RT-PCR analysis of the cDNA samples was conducted in an ABI 7700 Sequence Detection System (Applied Biosystems Inc, Foster City, CA) using specific primers for PSA (Applied Biosystems Inc, CA).

Transient Transfections and Luciferase Activity Assays

Cells were plated (10^5 cells/well) in 6-well plates and treated as described above. After 48 hrs, cells were transfected with the ARE luciferase construct (1 mg/well) (from Dr. Zoran Culig, Innsbruck Medical University, Austria) in the presence of the control Renilla luciferase construct (Promega, Madison, WI) using Tfx-50 transfection reagent (Promega, # E1811). Following a 2hr-incubation with the DNA/Tfx50 mixture, serum-containing medium was added to the cells and incubation was continued for 22 hrs. After treatment, cells were harvested and luciferase activity was determined according to the manufacturer's protocol (Promega, Dual Luciferase Assay, # E1920). Data are representative of three independent experiments in duplicate.

Statistical Analysis

Student *t*-test and one-way analysis of variance (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 ± SEM (standard error of the mean). Statistical significance was reached at a p value of less than 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 shrinking or impairing the signaling axis, 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 Figure 1 reveal the effect of Docetaxel on tissue PSA expression in individual prostate tumor cells (Panel A). There was a marked decrease in PSA immunoreactivity in the indivdual tissue arrays in response to microtubule-targeting drug treatment (Fig.1 panel B). Quantitative analysis of the data revealed a significant reduction (19%) in the intensity of PSA in prostate tumors from patients receiving Docetaxel, compared to specimens from non-treated patients (Fig. 1, Panel C).

Taxol Inhibits AR Transcriptional Activity

Paclitaxel and Nocodazole were used to disrupt normal cellular function of microtubule system. Similar to Docetaxel, Paclitaxel is chemotherapy drug classified with the taxane group and used in the treatment of advanced prostate cancer (3). Nocodazole exerts its anti-tumor 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 (1nM) for 24hrs led to a significant increase in the expression of PSA mRNA. Nocodazole completely abolished and Paclitaxel partially inhibited this PSA induction (Fig. 2, Panel A). The changes in PSA protein levels were consistent with the mRNA changes in response to treatment (Fig. 2, Panel B). 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 24hrs of DHT treatment and was significantly inhibited by both Nocodazole and Paclitaxel (Fig. 2, Panel C).

Taxol Inhibits Ligand-independent AR Transcriptional Activity

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, while Nocodazole or Paclitaxel ablated this PSA mRNA induction within 24hrs (Fig. 3, Panel A). To investigate whether the impaired AR transcriptional activity is specific to microtubule targeting drugs, two different drugs, Velcade and Doxazosin were examined (Fig. 3, Panel B). Exposure to either one of these agents did not affect the androgen-mediated PSA mRNA expression (Fig. 3, Panel C).

Microtubule Targeting Chemotherapy Inhibits AR Nuclear Translocation

In order 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. 4, Panel A). 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, while only 10% of the cell population had cytoplasmic localization of AR (Fig. 4, Panels B 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. 4, Panels B 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. 4, Panel D). 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 Figure 5 (Panels A and B), DHT treatment (4hrs) induces a massive AR nuclear translocation in LNCaP cells. Pre-treatment of Paclitaxel and Nocodazole for 24hrs abrogated this AR nuclear translocation (Fig. 5, Panels A 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. 5, Panel C).

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. Since we recently reported that androgens and the AR regulate EMT and 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 Figure 2).

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. Interaction of endogenous AR and α-tubulin was detected in both LNCaP and CWR22 cells (Fig.5, Panels D and E). The co-localization of AR and tubulin was detected by immunofluorescence staining as yellow color (Supplementary Figure 1, Panel A). This co-localization was reduced by DHT treatment (Fig. 5, Panels D and E; Supplementary Fig. 1, Panel A). To further determine the interaction site of AR with tubulin, PC-3 prostate cancer cells were transfected with different truncated forms of AR (Fig. 5, Panel F). Loss of either the Ligand-binding domain (LBD)-hinge domain or the DNA-binding domain (DBD) cannot inhibit the interaction of AR and tubulin, implicating the N-terminal domain as being responsible for the AR and tubulin association and potential interaction (Fig. 5, Panel G).

Androgens Downregulate Tubulin in Prostate Cancer Cells

To determine whether androgen signaling can impact the microtubules, tubulin expression was evaluated by Western blot analysis and immunofluorescence. Treatment of prostate cancer cells with DHT, significantly inhibited tubulin expression (Fig. 6, Panel A). There was a marked reduction in tubulin levels, an effect that was enhanced by TGF-β (Supplementary Figure 1, Panel B). Immunofluorescence staining revealed that the microtubule spindles were undetectable after androgen treatment (Fig. 6, Panel B).

DISCUSSION

The present study documented 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 -independent activation of AR and its nuclear translocation towards downstream androgen signaling (28). In conjunction with the demonstrated association and potential interaction of AR and tubulin, our findings raise the possibility that preferential 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 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 non-targeting microtubules, such an effect appears 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 accord 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 regimes lead to a significant survival benefit 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, arrests cells in G2M phase, ultimately inducing apoptotic cell death. Another action of Docetaxel is its antagonistic activity against the prosurvival 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 towards 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, since 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 tumor suppression action by ADT; Addition of an AR-binding moiety to a therapeutic agent like 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 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 N-teminal 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 6, Panel C). This feedback loop may explain the reduced association and co-localization between AR and tubulin, due to tubulin downregulation. Androgen signaling is important in cell differentiation and regulates cell cycle including G2M arrest (33-34), consistent with its function in inhibiting microtubule structures (Supplementary Figure 3). 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 anatagonizes 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 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, towards 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 Figure 4) and supports a combination strategy of ADT with tubulin-targeting chemotherapy towards an improved therapeutic response in

CRPC. The mechanisms via which this multifunctional tubulin-targeting compound hinders the AR ligand-binding pocket is currently being investigated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AR	androgen receptor
DHT	dihydrotestosterone
ARE	androgen response element
PSA	prostate-specific antigen
TGF-*β*	transforming growth factor- *β*
TGF*β*RII	TGF-*β* receptor II
BPH	benign prostatic hyperplasia
ADT	androgen deprivation therapy
CRPC	castration resistant prostate cancer
LBD	Ligand-binding domain-hinge domain
DBD	DNA-binding domain

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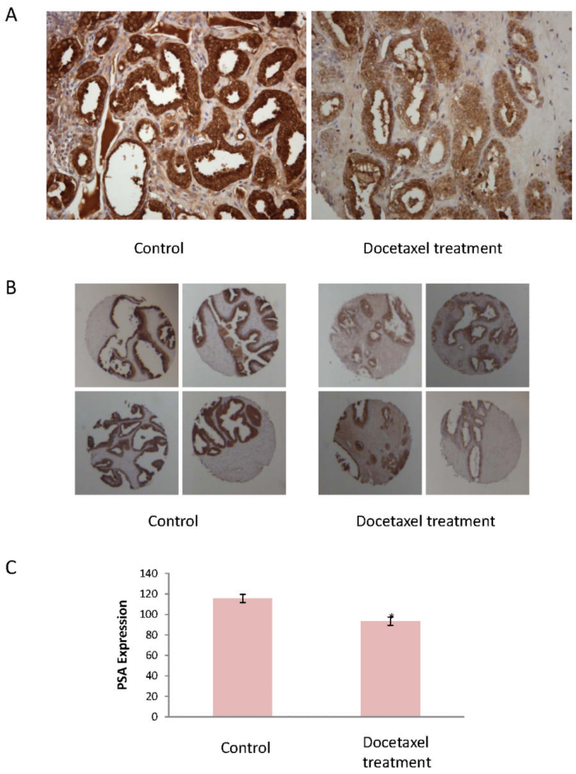

Figure 1. Doclitaxel Suppresses PSA Expression in Human Prostate Tumors

Panel A reveals the PSA immunoreactivity pattern of prostate tissue array: from the left panel, untreated patients; right, Docetaxel-treated patients. Panel B shows 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”. Panel C; Quantitative evaluation of PSA expression in tumor epithelial cells in prostate specimens from Docetaxel-treated and untreated prostate cancer patients was determine as described in “Materials and Methods”. * $P < 0.01$.

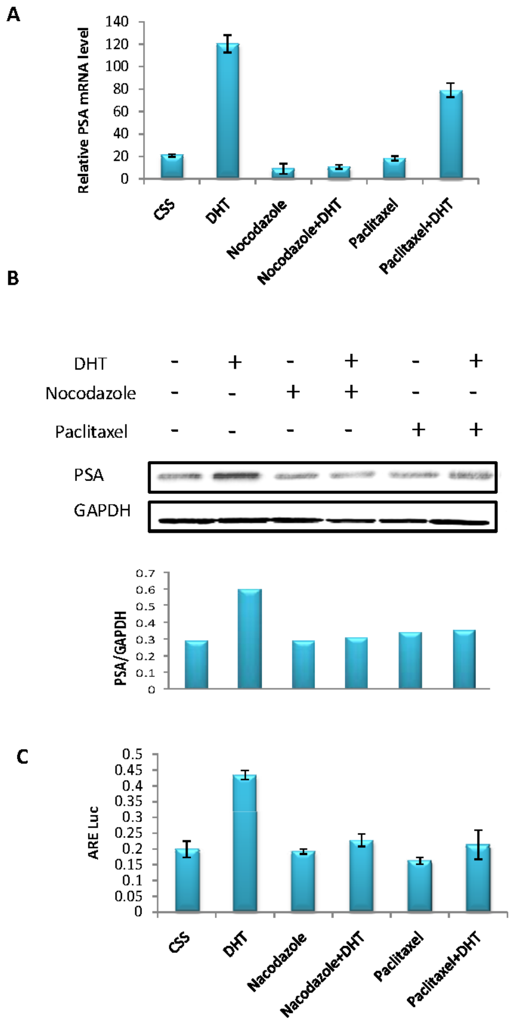

Figure 2. Microtubule Targeting Drugs Inhibit Ligand-dependent AR Transcriptional Activity

LNCaP cells were treated with DHT (1nM) in the presence or absence of Nocodazole (5ug/ml) or Paclitaxel (1μM). Panel A; PSA mRNA expression was determined by realtime PCR. Panel B; PSA protein levels were assessed by Western blotting and relative expression was quantitated (lower panel). Panel C; The AR transcriptional activity in response to microtubule-targeting drugs was determined using the ARE luciferase reporter vector in LNCaP cells.

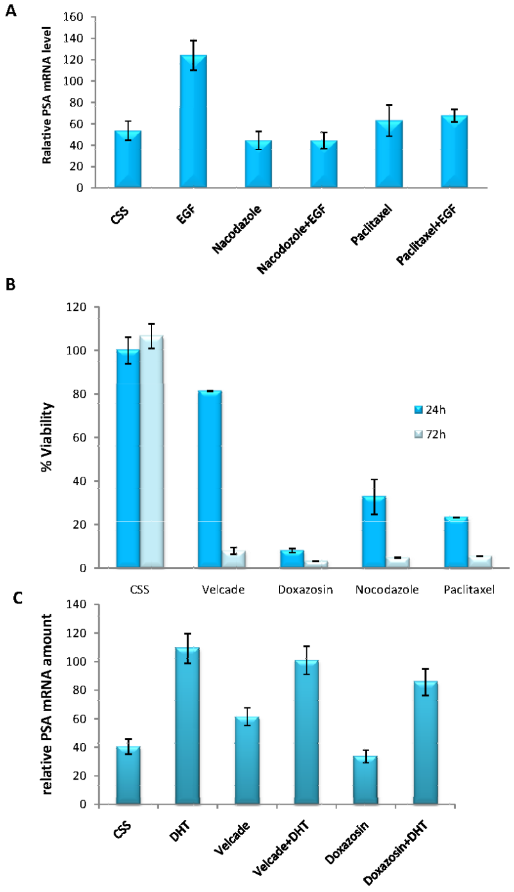

Figure 3. Microtubule Targeting Inhibits Ligand-independent AR Transciptional Activity
Panel A; LNCaP cells were treated with a combination of DHT (0.1nM) and EGF (5nM) with or without Nocodazole (5μg/ml) or Paclitaxel (1μM). AR transcriptional activity was evaluated on the basis of PSA expression using realtime PCR. Panel B; LNCaP cells were treated with the following chemotherapeutic agents for 24-72hrs: TRAIL, Velcade, Doxasosin, Nocodazole (5μg/ml) or Paclitaxel (1μM) and cell death was determined using the MTT assay. Panel C; LNCaP cells were treated with DHT (1nM), in the presence or absence of Velcade or Doxasosin as shown. PSA mRNA expression was evaluated by Real-time PCR. * P<0.05

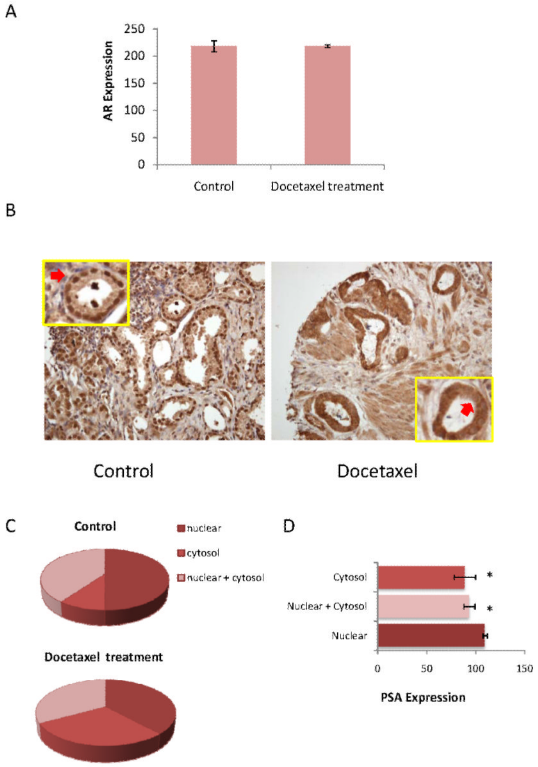

Figure 4. Docetaxel Suppresses AR Nuclear Translocation in Prostate Cancer

Panel A; AR protein expression levels in prostate cancer epithelial cells of Doclitaxel 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. Panel B reveals a representative image of the subcellular AR localization in human prostate tissue. AR presence was assessed in formalin-fixed, paraffin-embedded prostate cancer tissue microarrays via light microscopic examination, while 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 panel, tissue from untreated patients; right, tissue from Docetaxel-treated patients. Panel C indicates the 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 Panel D; AR localization correlated with PSA levels in prostate epithelial cells.

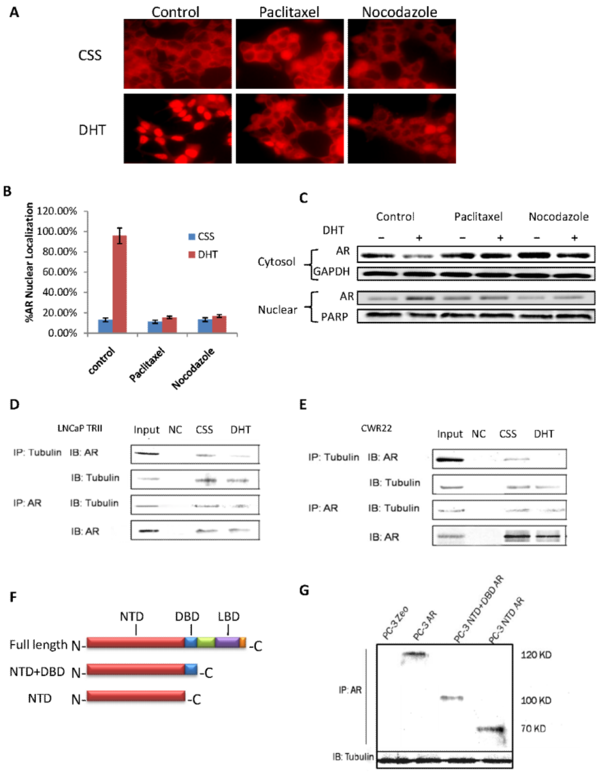

Figure 5. Tubulin Interacts with AR

Panels A and B; Androgens induce AR nuclear translocation in LNCaP cells and pre-treatment of Paclitaxel and Nocodazole for 24hrs abrogated this AR nuclear translocation. Subcellular localization of AR was detected by fluoresencent staining (red) (40x 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 (Panel C). GAPDH and PARP were used as loading controls. Panels D and E; LNCaP TβRII and CWR22 cells, respectively were treated with DHT (1nM), in the presence or absence of TGF-β (5ng/ml). Immunoprecipitation was performed by using the antibodies against either tubulin or AR to show the AR-tubulin association). Panel 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 (Panel G).

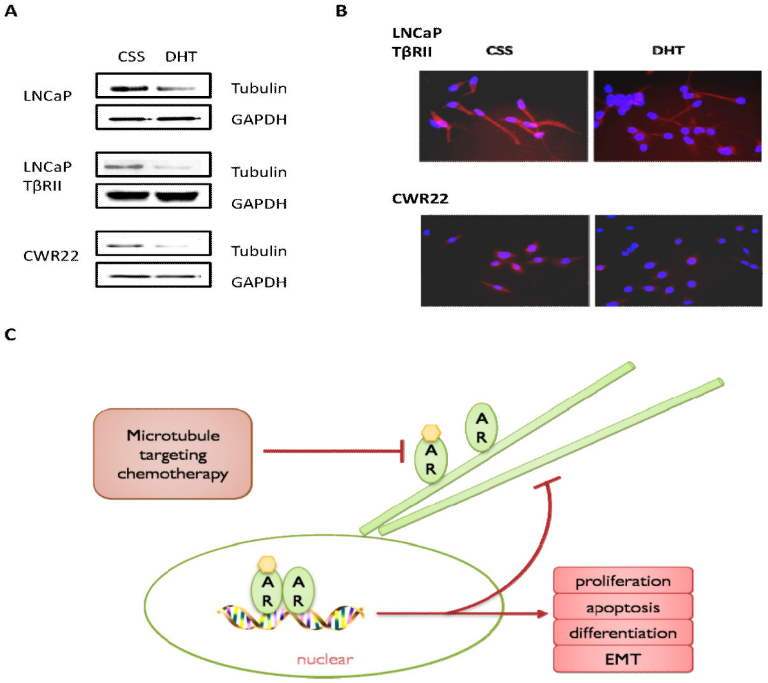

Figure 6. Androgens Inhibit Tubulin Expression in Prostate Cancer Cells

Panel A; Prostate cancer cells LNCaP, LNCaP TβRII and CWR22, were treated with DHT (1nM; for 72hrs) and tubulin expression was evaluated by Western blotting using GAPDH as internal normalizing control. Panel B; LNCaP TβRII and CWR22 cells were treated with DHT (1nM) with or without TGF-β (5ng/ml) for 72hrs. Tubulin expression was detected by immunofluorescence (red); nuclei were stained by DAPI (blue). Panel C illustrates the 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, via a negative feedback mechanism; AR signaling inhibits tubulin expression thus impairing the cytoskeleton structure and organization.