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Multinucleation and Mesenchymal-to-Epithelial-Transition Alleviate Resistance to Combined Cabazitaxel and Antiandrogen Therapy in Advanced Prostate Cancer

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Abstract

Patients with metastatic castration resistant prostate cancer (mCRPC) frequently develop therapeutic resistance to taxane chemotherapy and antiandrogens. Cabazitaxel (CBZ) is a second-line taxane chemotherapeutic agent that provides additional survival benefits to patients with advanced disease. In this study we sought to identify the mechanism of action of combined CBZ and androgen receptor (AR) targeting, in pre-clinical models of advanced prostate cancer. We found tha CBZ induced mitotic spindle collapse and multi-nucleation by targeting the microtubule de-polymerizing kinesins and inhibiting AR. In androgen responsive tumors, treatment with the AR inhibitor, Enzalutamide, overcame resistance to CBZ. Combination treatment of human CRPC xenografts with CBZ and Enzalutamide reversed epithelial-mesenchymal transition (EMT) to mesenchymal-epithelial-transition (MET) and led to multi-nucleation, while retaining nuclear AR. In a transgenic mouse model of androgen-responsive prostate cancer, CBZ treatment induced MET, glandular re-differentiation and AR nuclear localization that was inhibited by androgen deprivation. Collectively, our pre-clinical studies demonstrate that prostate tumor resistance to Cabazitaxel can be overcome by antiandrogen-mediated EMT-MET cycling in androgen-sensitive tumors, but not in CRPC. Moreover, AR splice variants may preclude patients with advanced disease from responding to Cabazitaxel chemotherapy and antiandrogen combination therapy.

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This evidence enables a significant insight into therapeutic cross-resistance to taxane chemotherapy and androgen-deprivation therapy in advanced prostate cancer.

Introduction

Androgen deprivation therapy (ADT) has been used as standard treatment for patients with advanced prostate cancer, since the historic discovery by Charles Huggins 65 years ago (1). All patients however, ultimately develop castration resistant prostate cancer (CRPC) with recurrence to lethal disease. Progression to metastatic CRPC (mCRPC) is characterized by aberrant expression of the androgen receptor (AR), *de novo* intraprostatic androgen production, and cross talk between androgen signaling with other oncogenic pathways (2,3). Recent anti-androgen therapies such as Abiraterone Acetate and Enzalutamide although effectively target the androgen signaling axis (4-6), due to the addiction of CRPC cells to AR signaling and constitutively active AR splice variants, resistance develops and disease recurs (7-9). Taxanes (Docetaxel, Cabazitaxel) are the mainstay of chemotherapy for metastatic CRPC patients who developed resistance to anti-androgen therapy. First generation taxanes like Docetaxel (Taxotere[®]) target the cytoskeleton by stabilizing the interaction of β -tubulin subunits of microtubules preventing de-polymerization, inducing G2M arrest and apoptosis (10). Un-liganded AR is sequestered in the cytoplasm by the HSP90 super-complex and upon binding to the ligand, dihydrotestosterone (DHT), dimerizes and translocates to the nucleus (11,12). Nuclear AR binds androgen responsive elements of DNA and transcriptionally activates genes promoting prostate cell growth (11). Taxanes bind to β -tubulin subunits of microtubules stabilizing their interaction and preventing de-polymerization of the microtubule structure and leading to apoptosis (13,14). Work by our group and others established that Docetaxel chemotherapy inhibits AR trafficking and nuclear translocation, thus preventing its transcriptional activity (15-17). Taxanes also upregulate Forkhead box O1 (FOXO1), a transcriptional repressor of AR, resulting in inhibition of ligand-dependent and -independent transcription, and downregulation of AR and PSA expression (18,19).

The therapeutic impact of taxanes in metastatic CRPC and improving patient survival, has been attributed to microtubule stabilization and AR targeting ADT (18,20). Despite an initial efficacy and a survival advantage in patients with mCRPC, resistance to taxane chemotherapy invariably develops leading to disease progression (21). Mechanisms implicated in the development of Docetaxel resistance, include high affinity of the drug for the P-glycoprotein drug efflux pump, mutational alterations in tubulin and EMT (20-22). The FDA has recently approved several promising agents including Jevtana[®] (Cabazitaxel, CBZ), Xtandi[®] (Enzalutamide, MDV3100), and Provenge[®] (Sipuleucel-T) providing additional survival benefits to patients with advanced disease (23,24). Cabazitaxel is the second-line taxane chemotherapy, with significantly decreased affinity for the P-Glycoprotein pump for increased cellular retention (14). The non-steroidal, anti-androgen Enzalutamide (MDV3100) was rationally designed from the AR crystal structure (4,24,25). Functionally, MDV blocks androgen signaling by preventing binding of AR to DHT, blocking AR translocation into the nucleus, and inhibiting AR from binding to androgen

responsive elements on DNA (12,26). Recent clinical evidence suggests that the AR splice variant V7 confers therapeutic resistance to Enzalutamide in CRPC patients (8,9,27).

The process of epithelial mesenchymal transition (EMT) featuring characteristic phenotypic manifestations and driven by molecular programming, confers invasive, metastatic and stem cell-like properties in epithelial-derived tumors with acquired resistance to apoptosis (28-32). In this study we investigated the contribution of EMT to resistance to Cabazitaxel and antiandrogens in pre-clinical models of advanced prostate cancer. Our findings indicate for the first time that Cabazitaxel induces multi-nucleation by targeting kinesin expression, and reverses EMT to mesenchymal-epithelial transition (MET) *in vitro* and *in vivo*. Cabazitaxel also sustains nuclear AR conferring resistance that can be overcome by antiandrogens in androgen-responsive tumors. In CRPC models harboring a cohort of AR variants, combination treatment of Cabazitaxel with antiandrogen promoted epithelial re-differentiation by activating MET.

Materials and Methods

Cell Lines and Transfections

Human prostate cancer cell lines, the androgen independent cell lines PC3 and DU145, the CRPC cancer cell line 22Rv1, and the androgen sensitive human prostate cancer cell lines LNCaP and VCaP were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cell lines were obtained every year between 2010 and 2014 and have been authenticated and tested for mycoplasma in September 2011, June 2012, and November 2013, by Q11 short tandem repeat (STR; method of Masters et al. 2012: Authentication of human cell lines: standardization of STRprofiling; DDC Medical). PC3 AR variant transfectants PC3v7, PCv12 and PC3v567es were generated in this laboratory using plasmids provided by Drs. S. Plymate (University of Washington) and J. Luo (Johns Hopkins Brady Urologic Institute). TGF- β responsive LNCaPT β R11 were generated and characterized in this lab (33,34). All cell lines, but VCaP cells, were maintained in RPMI 1640 (Invitrogen, Grand Island, NY) and 10% fetal bovine serum (FBS), 100units/ml penicillin and 100 μ g/ml streptomycin in a 5% CO₂ incubator (37°C). The VCaP cells were cultured in DMEM (ATCC, Manassas, VA). Cells were seeded in 10% charcoal-stripped serum (CSS) and stimulated by dihydrotestosterone (DHT) (Sigma-Aldrich, St. Louis, MO) or R1881 (1nM).

Drugs

Cabazitaxel (Jevtana©) was generously provided by Sanofi Aventis. For the *in vivo* administration, Cabazitaxel was prepared by mixing ethanol, polysorbate 80, and 5% (w/v) glucose in sterile water (1:1:18). Solutions were administered intravenously as a slow bolus. CBZ stock (500 μ M) was prepared in 100% ethanol and stored at -20°C. MDV3100 was purchased from Selleck Chemicals (Houston, TX). For *in vivo* administration in mice, MDV3100 was prepared in dimethyl-sulphoxide (DMSO), diluted with sterile PBS (75% PBS: 25% CBZ DMSO Solution) and injected intraperitoneally.

Antibodies

The antibody against the AR (N-20) protein was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against Tubulin, N-cadherin, MCAK (KIF2C), HSET, CD31, and phospho Histone H3 were obtained from AbCam Cell Signaling (Cambridge, UK); antibodies against cleaved Caspase-3, GAPDH, E-Cadherin and Cytokeratin-18 proteins were obtained from Cell Signaling Technology (Danvers, MA). The ZEB1 antibody was obtained from Bethyl Laboratories (Montgomery, TX).

Western Blot Analysis

Total cellular protein was extracted from cell lysates by homogenization with RIPA buffer (Cell Signaling Technology, Danvers, MA); subcellular fractionation was performed using NE-PER nuclear-cytoplasmic fraction kit (Thermo Scientific). Protein samples were loaded into 4%-15% SDS-polyacrylamide gels (Bio-Rad, Hercules, CA) and subjected to electrophoretic analysis and blotting. Membranes were subsequently incubated with the Amersham ECL Plus Western Blotting Detection System (Amersham, GE Healthcare, Buckinghamshire, UK) and auto-radiographed using X-ray film (Denville Scientific, South Plainfield, NJ). Protein expression bands were normalized to GAPDH expression.

Cell Viability Assay

The effect of the various treatments on prostate cancer cell viability was evaluated using the Thiazolyl Blue Tetrazolium bromide (MTT) assay. Cells were seeded into 24-well plates and after grown to 60-75% confluence, were treated with vehicle control (DMSO, Sigma-Aldrich, St. Louis, MO), Cabazitaxel, MDV3100, and combination in RPMI 1640 with 10% CSS (Charcoal Stripped Serum) for 24hrs (or DMEM for VCaP). At termination of exposure, cells were aspirated and rinsed with PBS then treated with 250µl/well MTT (1mg/ml) for 30mins at 37°C. Absorbance was measured at 570nm using µQuant Spectrophotometer (Biotech Instruments Inc., Winooski, VT).

Migration Assays

Cells were seeded in 6-well plates and at 65% to 70% density the cell monolayers were wounded. After 24hrs the number of migrating cells towards center of the wound is counted in three different fields.

Quantitative RT-PCR Analysis

In vitro samples: RNA was extracted with the Trizol© reagent (Life Technologies, Grand Island, NY) and RNA samples (1µg) were subjected to reverse transcription using the Reverse Transcription System (Promega, Madison, WI). TaqMan real time reverse transcriptase-PCR (Life Technologies, Grand Island, NY) analysis of the cDNA samples was conducted in an ABI7700 Sequence Detection System (Applied Biosystems, Inc, Branchburg, NJ), using the following specific primers: for Prostate Specific Antigen (KLK3; Hs02576345_m1), AR (Hs00171172_m1), KIF2C (Hs00901710_m1), FOXO1 (Hs01054576_m1), KIFC1 (Hs00954801_m1), E-Cadherin (CDH1; Hs01023894_m1), N-Cadherin (CDH2; Hs00983056_m1), Twist (TWIST1; Hs01675818_s1), Vimentin (VIM; Hs00185584_m1) and 18S rRNA (4319413E) (Applied Biosystems, Life Technologies,

Grand Island, NY). Data represent average values from three independent experiments; Numerical data were normalized to 18s rRNA and expressed relative to controls.

Immunofluorescent Confocal Microscopy

Cells were plated (1×10^5) on cover glass in 6-well plates. After 24–48hrs, cells were exposed to medium (RPMI 1640 with 10% CSS) in the presence of DHT (1nM), CBZ (35–100nM), MDV3100 (1 μ M) or in combination of the two agents. Following treatment, cells were fixed in 100% methanol and permeabilized with 0.1% Triton X-100 in sterile phosphate buffer saline (PBS). Fixed cells were incubated overnight with primary antibody specific for AR (N-20), and Tubulin (AbCam Cell Signaling, Cambridge, UK), (at 4°C) with gentle rocking and the appropriate Alexa-Fluor (Life Technologies, Grand Island, NY) fluorescent secondary (1.5hrs, room temperature). Slides were mounted using Vectashield mounting medium with DAPI and were visualized using a FV1000 Confocal Microscope (Markey Cancer Center Core, University of Kentucky).

Flow Cytometric Analysis

The human prostate cancer cells harboring the AR variants PC3v567es and 22Rv1 cells were exposed to various treatments (MDV, CBZ, or combination for 24–96hrs), and subjected to washing with PBS in 0.1% bovine serum albumin. Cells were subsequently fixed with 100% ethanol (–20°C). For cell cycle analysis, cells were incubated with propidium iodide solution with RNase A (10 μ g/mL) overnight, at 4°C. Samples were analyzed using Becton-Dickinson FACSCalibur (Flow cytometry core at the University of Kentucky).

In Vivo Tumor Targeting Studies

All animal experiments were performed in accordance with the guidelines approved by the Animal Care and Use Committee of the University of Kentucky and according to the NIH recommendations and reporting standards. Male nude mice (5–6 weeks old) mice were subcutaneously injected with 22Rv1 cells (2×10^6 cells) and after tumors were palpable, mice were divided into four groups, 5 mice/group: (a) Control group receiving 1% medium /0.1% Tween-20 daily via oral gavage; (b) CBZ treatment group, mice received CBZ (Day 1 and 4: 5 mg/kg, Day 8 and 14: 2.5 mg/kg) via tail vein injection for two weeks (c) Enzalutamide group, mice receiving 30 mg/kg MDV3100 via intraperitoneal injection for 2-weeks; (d) CBZ and MDV3100 combination group, mice receiving both Cabazitaxel and Enzalutamide for 2wks. Tumors were measured twice a week and the volume was calculated (length $\times$ width $\times$ 0.5236). Prostate tumor xenografts were harvested at 4days after last treatment and histopathologically analyzed. Formalin-fixed paraffin embedded sections were subjected to immuno-staining for expression and localization of AR, mitotic kinesins, EMT, vascularity (CD31), and apoptosis (TUNEL, EMD Millipore, Billerica, MA). TUNEL analysis of apoptotic cells *in situ* was performed as previously described (35).

Transgenic Mouse Model of Prostate Cancer Progression

Mice were maintained under environmentally controlled conditions and subject to a 12-h light/dark cycle with food and water ad libitum. Mice TRAMP+/DNTGF β R11+ (35) (16–18

weeks) were matched with littermates and were treated with either vehicle control (VHC) or highest non-toxic dose (HNTD) of CBZ (Day 0 and 3: 10mg/kg; Day 7 and 11: 5mg/kg) dosed intraperitoneally and harvested on Day 14. TRAMP+/DNTGFβRII+ male mice were castrated and treated with CBZ (Day 3 and 7: 10mg/kg; Day 11 and 15: 5mg/kg) and harvested on Day 18.

Immuno-histochemical Analysis

Tissue specimens from human CRPC (22Rv1) xenografts and transgenic mouse prostate tumors were formalin fixed and paraffin-embedded; serial sections (5μ), were subjected to immuno-histochemical analysis using antibodies against E-cadherin, N-cadherin, Androgen Receptor (N-20), MCAK, HSET, pH3, ZEB1, Cytokeratin-18, and CD31. After blocking nonspecific binding, sections were incubated with primary antibody (overnight, 4°C) and were subsequently exposed to biotinylated goat anti-rabbit IgG (2hrs, room temperature) and horseradish peroxidase-streptavidin (EMD Millipore, Billerica, MA). Signal/Color detection was achieved with SigmaFast 3, 3'-Diaminobenzidine tablets (Sigma-Aldrich, St. Louis, MO) and counterstained with haematoxylin. TUNEL analysis of apoptotic cells was performed as previously described (35). Images were captured via light microscopy (40× and 100×) using an Olympus BX51 microscope (Olympus America, Center Valley, PA). The intensity and level of immunoreactivity was recorded by two independent observers.

Statistical Analysis

Student's t test, one-way, or two-way ANOVA were performed using Graph Pad Prism 6 software to determine the statistical significance of difference between means / treatments. All numerical data are presented as mean ± standard error of the mean (SEM). Statistical significance was set at P value < 0.05.

Results

Significance of AR Status in Prostate Cancer Cell Response to Cabazitaxel

The pre-clinical efficacy of Cabazitaxel chemotherapy against prostate cancer was originally demonstrated using the androgen-independent human cancer cell line, DU145 (lacking AR) as a model (14). To establish the cellular response of androgen sensitive and CRPC cancer cells to CBZ treatment, a panel of human prostate cancer cell lines with varying AR expression status was used. A dose response analysis of prostate cancer cell viability to increasing concentrations of CBZ (10-500 nM) for 96hrs, demonstrated that DU145 cells were highly sensitive to CBZ treatment consistent with earlier reports (14) (Fig. 1A). A time course analysis of the temporal response to increasing treatment periods to CBZ (100nM) was also conducted (Fig. 1B). The androgen-independent PC3 cells exhibited a similar sensitivity to CBZ as the DU145 cells (Fig. 1A and B). In contrast, PC3 cells with forced overexpression of the AR splice variant v567es (PC3v567es) were resistant to CBZ even at very high doses of the drug, compared to parental PC3 cells (Fig. 1A). The CRPC cell line 22Rv1 harboring a mix of AR variants as well as the full length AR, exhibited relative resistance to low doses of CBZ and short treatment periods (24-48hrs), but after longer treatment (over 72hrs), there was significant loss of cell viability (Fig.1A and B). The

androgen responsive cells VCaP (full length AR) were resistant to CBZ (500nM) compared to LNCaP and LNCaPT β RII cells (Fig.1A and B).

To examine the effect of AR targeting inhibition by the antiandrogen (MDV3100, MDV) to sensitize prostate cancer cells to CBZ, human prostate cancer cell lines were treated with CBZ alone (100nM) or in combination with MDV (1-10 μ M). The PC3v567es cells did not exhibit further cell death as a result of antiandrogen treatment compared to CBZ alone (except at supra-physiological concentrations) (Fig. 1C). For the androgen-sensitive and CBZ resistant VCaP cells (Fig. 1A), exposure to increasing concentrations of MDV in combination with CBZ resulted in a significant loss of viability ($P<0.05$) (Fig. 1C). The CRPC 22Rv1 cells (harboring a mixture of full length AR and AR splice variants) exhibit loss of viability in response to CBZ alone; however combination of the taxane with MDV at high concentrations (10 μ M) led to an increase in cell viability compared to single CBZ treatment (Fig. 1C), and compared to untreated control cells. Cell cycle analysis revealed that for the PC3v567es cells CBZ treatment promotes G2 and S phase arrest (Fig. 1D and E). For CRPC 22Rv1 cells, exposure to CBZ alone or in combination with Enzalutamide (96hrs) resulted in a significant G2 arrest (Fig.1F and G). Treatment of PC3v567es and 22Rv1 cells with CBZ led to increased prostate cell population in the S and G2 phase, regardless of androgens.

Recent work from this laboratory demonstrated that combination therapy of taxanes (Docetaxel) and N-terminal targeting of AR with novel anti-androgens enhanced the therapeutic efficacy of taxane against CRPC tumor growth (36). Thus, we comparatively analyzed the dose response of prostate cancer cells to Docetaxel (microtubule targeting), MDV (AR targeting), given as single agents or in combination (Supplementary Fig. S1A, B, C). The PC3, PCv567es, 22Rv1 and LNCaP cells exhibited resistance to MDV but all the cell lines showed partial sensitivity to Docetaxel (Supplementary Fig.S1, panels A and B, respectively). Combination of Docetaxel and MDV led to a significant loss of cell viability for all of the prostate cancer cell lines (Supplementary Fig. S1, panel C). Overexpression of AR variants in PC3 cells resulted in stable clones PC3567es, PC3v7 and PCv12 that all exhibited a significant increase in their migration potential compared to parental control cells (Supplementary Fig. S1, panel D).

Effect of Cabazitaxel on AR Expression, Localization and Activity

Docetaxel chemotherapy has been shown to impair prostate cancer growth by preventing the physical translocation of cytoplasmic AR into the nucleus ultimately inhibiting the activity of AR-regulated target genes (PSA) (15-17). LNCaP and VCaP cells grown in CSS media were subjected to confocal microscopy analysis that indicated a diffused distribution of AR between cytoplasm and nucleus in LNCaP control cells (Fig. 2A). Treatment with DHT (Fig. 2A) resulted in AR translocation to the nucleus. Treatment with the antiandrogen MDV (1 μ M; 24 hrs) increased cytoplasmic AR with no apparent effect on microtubule structural network (4) (Fig. 2A). CBZ reduced overall AR immunoreactivity, while it sustained nuclear localization of AR regardless of androgens or antiandrogens. Treatment of VCaP cells for 24hrs with MDV, followed by pulsing with DHT (2hrs) was analyzed by confocal microscopy (Supplementary Fig.S2A). AR localization was primarily confined to the

nucleus, indicating that MDV was unable to completely block the androgen-mediated AR nuclear translocation (Supplementary Fig. S2A). Treatment of VCaP with CBZ (96hrs) leads to AR nuclear localization independently of androgens (Fig. 2A; Supplementary Fig. S2). There was a significant impact by CBZ treatment on the microtubule structure with tubule bundling on the periphery of the cell and complete loss of fibrous microtubule network appearance (Fig. 2A; green). The effects on the microtubule appearance were consistently detected in response to CBZ, associated with remarkable multi-nucleation in both cell lines (Zoom images) (Fig. 2A; Supplementary Fig. S2A). The PC3v567es cells expressing AR variant v567es (resistant to CBZ) (Fig. 1A and B) were treated with CBZ and subjected to confocal microscopy. CBZ exerts the bundling effect on microtubule structures (Supplementary Fig. S3), reduces AR levels and fails to sustain nuclear localization of AR v567es. Extensive multi-nucleation is observed for PC3v567es cells in response to CBZ.

To determine the effect of CBZ treatment on AR expression, LNCaP and VCaP prostate cancer cells were treated for 24, 48, or 72hrs with CBZ alone or pulsed with DHT for 3hrs prior to cell lysis and Western blot analysis. CBZ treatment for 72hrs markedly reduced AR protein levels in the androgen sensitive prostate cancer cell lines, LNCaP and VCaP (Fig. 2B and Supplementary Fig. S2A), respectively. Expression of β -tubulin was not affected. CBZ treatment (48hrs) led to caspase-3 cleavage indicating apoptosis induction (Fig. 2B). To confirm the effect of CBZ on AR localization, we performed subcellular fractionation analysis in LNCaP cells after exposure to CBZ (24, 48, or 72hrs), alone or pulsed with R1881 (1nM for 2hrs). Androgens predictably increased nuclear AR expression compared to controls (Fig. 2C). CBZ treatment for 24-48hrs decreased AR levels in LNCaP cells, while there was nuclear retention of AR (Fig. 2C); by 72hrs AR levels were diminished in both cytosolic and nuclear fractions (Fig. 2C). To establish that the effect of CBZ on AR expression was a consequence of transcriptional inhibition, the AR mRNA levels were evaluated. LNCaP cells were treated with CBZ (24, 48 or 72hrs) alone or pulsed with DHT for 2hrs prior to mRNA extraction. CBZ treatment (24hrs) led to downregulation of AR mRNA (Fig. 2D). Moreover CBZ significantly inhibited expression of the AR-regulated gene PSA and AR interactor FOXO1, (Fig. 2E and F), respectively indicating targeting of AR transcriptional activity by CBZ. A similar effect of Cabazitaxel on AR regulated gene expression was also found for the VCaP cells (Supplementary Fig. S2C, D and E).

Cabazitaxel Causes Multi-nucleation in Prostate Cancer Cells by Targeting Kinesins

As chemotherapeutic agents, taxanes can effectively target the microtubules and the mitotic spindle apparatus thus blocking cellular division by inducing G2M arrest and apoptosis (37). Confocal microscopy analysis revealed that three human prostate cancer cell lines with different AR status, DU145 cells (AR negative), PC3v7 AR variant, LNCaP (mutant AR) and VCaP (full length AR) cells, exhibit increased incidence of multi-nucleation in response to CBZ treatment (Fig. 3A). In the LNCaP cells, there was a disruption of the mitotic spindle and mono-astral spindle formation. Overexpression of pro-mitotic kinesins can facilitate taxane resistance due to their microtubule depolymerizing action. Targeting of kinesins by CBZ, was profiled by analyzing the expression of a subset of mitotic kinesins in human prostate cancer cell lines (Fig. 3B). MCAK kinesin plays an important role in facilitating spindle pole capture and also acts as a microtubule de-polymerizing factor. HSET

functionally mediates cytokinesis. LNCaP cells express relatively low kinesin levels, while VCaP cells exhibit high expression of both proteins (Fig. 3B). DU145 and PC3v567es have high expression of MCAK but completely lack HSET protein expression (Fig. 3B). In response to CBZ (24, 48 or 72hrs) alone or under androgenic pulse, there was a transient increase in expression of MCAK and HSET kinesins within the first 24hrs in the VCaP cells; by 72hrs of treatment kinesin levels were significantly down regulated (Fig. 3C and Supplemental Fig. S4A). In addition there was a significant decrease in MCAK (KIF2C) mRNA expression in LNCaP and VCaP cells (Fig. 3D and Supplementary Fig S4), and in HSET (KIFC1) mRNA levels for VCaP cells (Supplementary Fig. S4, panels B and C) in response to CBZ. To determine the potential link between action of CBZ on mitotic spindle formation and resistance, we subsequently examined pericentrin (marker of centrosomes) expression in the Cabazitaxel-resistant prostate cancer PCv567es and VCaP cells and the CBZ sensitive CRPC 22Rv1 cells. As shown on Figure 3E, in response to CBZ, PC3v567es and VCaP cells exhibited centrosome clustering and amplification accompanied with severe multi-nucleation; these effects were not influenced by the status of androgen axis (presence of androgens or antiandrogen, MDV) (Fig. 3F). In the CRPC 22Rv1 cells, CBZ treatment led to multi-nucleation but not centrosomal amplification (Supplemental Fig. S5A).

***In Vivo* Novel Action of Cabazitaxel in Models of Advanced Prostate Cancer via Induction of Mesenchymal-Epithelial Transition (MET) and Glandular Differentiation**

To define the physiological significance of our *in vitro* findings we investigated the anti-tumor action of Cabazitaxel in an *in vivo* model we previously established (TRAMP mice crossed with mice expressing a conditional dominant negative TGF β RII) (35). This TRAMP/DNTGF β RII model is characterized by aggressive tumor progression to metastasis driven by EMT. The consequences of Cabazitaxel treatment on the phenotypic landscape and growth dynamics were evaluated in prostate tumors from (N=10) 16-18-wks male mice (Castrate versus non-castrate groups) receiving treatment for 14 days with pharmacological dose of CBZ (Supplemental Fig. S6; panels A and B). CBZ treatment led to a significant decrease in the body weight as well as the prostate weight ($P<0.005$) (Supplemental Fig. S6C and D respectively; $P<0.005$). At 18-20wks, TRAMP+DNTGFBRII+ mice progressed to poorly differentiated prostate cancer (35); Histopathological evaluation (H&E staining) revealed that CBZ alone or in combination with androgen depletion restored the glandular structures and luminal secretions of the prostate epithelium compared to controls (Fig. 4A). Immunoreactivity of cytokeratin-18, a luminal cell and glandular differentiation marker, was markedly increased in tumors from CBZ-treated mice (non-castrate and castrate) compared to VHC (Fig. 4E).

Evaluation of prostate tumor cell proliferative capacity based on Ki-67 and phospho-Histone 3 (pH3) immunoreactivity revealed an increased proliferative activity in response to CBZ alone, while castration-induced-androgen deprivation resulted in a significant decrease in the proliferative index (Fig. 4A and D). To correlate the effect by CBZ on the mitotic spindle with resulting multi-nucleation (observed *in vitro*; Fig. 3), expression of the nuclear protein phospho-H3 histone, was examined in prostate tumors. As shown on Figure 4 (panel A, lower section), CBZ, resulted in distinct multi-nucleation among prostate tumor glands from CBZ-treated mice, compared to VHC controls (Fig.4B, arrows). There was an increased

number of TUNEL positive prostate tumor cells after castration-induced androgen withdrawal indicating apoptosis induction ($P<0.005$) (Fig. 4A and C). Treatment of either intact or androgen-depleted mice, with CBZ (2wks) did not induce significant apoptosis (Fig.4A and C).

The potential contribution of EMT-MET cycling to the glandular formation and reversion to differentiated prostate epithelium by CBZ was subsequently interrogated. Figure 5, panel A indicates representative images of immunoreactivity analysis of the EMT landscape. Certain populations of tumor epithelial cells exhibited strong E-cadherin immunoreactivity paralleled by decreased N-cadherin expression in response to CBZ, supporting an effect on reversing EMT (Fig. 5A). We previously showed that prostate tumors from TRAMP +DNTGFBRII mice exhibit accelerated progression to metastatic disease via changes in the tumor microenvironment driven by increased inflammation and EMT (35). We found that CBZ alone or in combination with androgen-depletion reversed EMT to MET as reflected by elevated E-Cadherin and decreased N-Cadherin immunoreactivity (Fig. 5A). Intense nuclear immunoreactivity for AR was detected in prostate tumors from VHC mice; upon castration-induced ADT there was a marked reduction in nuclear AR associated with a diffused localization to the cytoplasm (Fig. 5B and C). Treatment of intact mice with CBZ significantly increased nuclear AR, compared to androgen-depletion mediated cytoplasmic translocation (Fig. 5B and C). Prostate tumor epithelial cells in castrate mice treated with CBZ (for 2wks) exhibited a reduced AR expression with a significant reduction of nuclear AR compared to controls or CBZ-alone treated mice (Fig.5B and C).

The kinesin immunoreactivity profile in prostate tumors from the transgenic mouse model revealed decreased MCAK expression in response to CBZ (given as a single agent), compared to VHC controls, castration-androgen depletion or combination of CBZ with castration (Fig. 5B). Impairing the androgen axis (castration) alone or in combination with CBZ led to increased kinesin expression (Figure 5, panel B). We subsequently examined the effect of CBZ on the gene expression profile of EMT regulators in androgen-responsive prostate cancer cell lines, VCaP and LNCaP RT-PCR analysis of mRNA expression for E-cadherin, Vimentin and Twist1 (Fig. 5, panels D, E and F respectively) in VCaP cells in response to CBZ, demonstrated a significant downregulation in all three genes within 24hrs of treatment, that was not affected by DHT. A similar profile of mRNA downregulation for EMT effectors in response to CBZ was observed in LNCaP cells (Fig.5, panels G, H and I).

The *in vivo* anti-tumor effect of CBZ alone or in combination with anti-androgen (MDV) against the human CRPC 22Rv1 xenografts is shown on Figure 6 (Dosing regimen described in Supplementary Fig. S7). CBZ alone significantly decreased tumor mass in tumor-bearing mice (when compared initiation vs termination of treatment per individual mouse), although this failed to reach statistical significance (Fig.6A and B). CBZ alone or in combination with MDV3100 led to a significant reduction in body weight (Supplementary Fig. S7, panel C). Tumor specimens from control (VHC) and treated mice were subjected to immunohistochemical analysis for the tumor growth kinetics, apoptosis and cell proliferation, vascularity, AR and kinesin expression. Treatment with CBZ alone or in combination with MDV induces significant apoptosis (Fig. 6, panels C and D), while the antiandrogen alone failed to induce apoptosis in the CRPC 22Rv1 tumors. Tumor

vascularity was inhibited in response to CBZ alone, an effect that was reversed by the combination treatment (Fig.6, panels C and E). There was a significant increase in Ki-67 immunoreactivity in response to CBZ treatment, but combination with the antiandrogen suppressed prostate tumor proliferation (Fig.6, panels C and F). Treatment of CRPC 22Rv1 cells with CBZ downregulates MCAK protein in a temporal correlation with loss of E-cadherin driven by CBZ (Fig.6G). A pattern of transient changes in AR levels in CRPC 22Rv1 cells was observed in response to CBZ; full length AR levels were reduced within 24hrs, followed by a significant increase at 48hrs, compared to controls (Fig. 6G).

The impact of Cabazitaxel on EMT landscape in the CRPC xenografts was profiled with three marker proteins, E-cadherin, N-cadherin and ZEB-1. As shown on Figure 7 (panel A), CBZ treatment resulted in increased E-cadherin, while it decreased N-cadherin and ZEB1 immunoreactivity indicating abrogation of EMT programming in response to the taxane. These *in vivo* phenotypic findings in the CRPC xenograft model are consistent with the effect of the drug in the transgenic model of EMT-driven prostate tumor progression (Fig.5). Also shown on Figure 7 is the effect of CBZ on reducing kinesin levels, both MCAK and HSET, in CRPC tumors (Fig. 7B). Antiandrogen treatment had no significant effect on HSET levels, while it reduced MCAK expression. CBZ treatment reduced AR expression but it maintained a strong AR nuclear localization in CRPC 22Rv1 tumors compared to MDV3100 or the combination (Fig. 7C).

Discussion

Microtubule-stabilizing chemotherapeutic agents such as Docetaxel and Paclitaxel have been shown to inhibit AR nuclear localization and activity in human prostate cancer, an action that correlates with the therapeutic response in patients (16,17). The present results demonstrate that in *in vitro* and *in vivo* models of androgen sensitive and CRPC advanced prostate tumors, Cabazitaxel chemotherapy maintains AR nuclear localization, while it inhibits AR expression. These observations are in contrast to the effect of Docetaxel on preventing nuclear translocation of the AR from the cytosol, we and others have reported (16,17). Moreover, our data demonstrate that the sensitivity of human prostate cancer cells to Cabazitaxel was not associated with the AR variant status. Thus PC3 AR v567es (androgen independent) and VCaP (androgen responsive) cells exhibited comparable degree of resistance to Cabazitaxel treatment, in accord with recent evidence that the antitumor effect of Cabazitaxel proceeds via an AR-independent mechanism (38). The mechanisms driving therapeutic cross-resistance to taxanes and antiandrogens in CRPC involve microtubule stabilization and inhibition of AR activity and nuclear localization by interfering with tubulin-AR association (16-18). Compelling evidence identified new signaling effectors conferring mechanistic resistance to taxane chemotherapy in CRPC, including overexpression of ERG genes (39) and activation of the GATA2-IGF2 signaling axis (40).

During cell migration in interphase, centrosome-mediated nucleation of a microtubule array enables directionality and centrosome amplification promotes transient spindle multipolarity during mitosis and correlates with tumor aggressiveness (41-43). The phenomenon of severe multinucleation that predominated the Cabazitaxel-treated prostate cancer cells regardless of

AR status, as well as the ability of the drug to induce centrosome amplification promoting spindle multipolarity in the resistant cancer cell lines, provides a shift in our understanding of therapeutic resistance to taxane chemotherapy. This study identified the mitotic centromere-associated kinesin (MCAK) is a direct target of Cabazitaxel in both androgen-sensitive and CRPC tumors. The kinesin spindle protein (KSP) is a molecular motor that crawls along the microtubules to assist cell division (also known Eg5) and different mitotic kinesins serve specific functions during cell division. In accordance with our findings, MCAK was implicated as a potential mitosis phase target due to its overexpression in CRPC detected in gene expression datasets (44). The effect of Cabazitaxel on nuclear AR retention in pre-clinical models of androgen-sensitive prostate tumors and CRPC is in contrast to Docetaxel action (1st line taxane) on promoting cytosolic AR (16, 17). Disruption of the mitotic spindle via direct targeting of mitotic kinesins inducing multi-nucleation of prostate cancer cells, might drive the nuclear retention of AR and inability of microtubules to navigate its cytoplasmic distribution/propagation. Since we showed that this effect of Cabazitaxel on AR can be overcome by treatment with Enzalutamide in androgen-sensitive prostate cancer, the above argument drives a molecular rationale for the therapeutic sequencing of antitumor action of Cabazitaxel and the antiandrogen in CRPC tailored to the AR variant status (Fig. 6H).

Cabazitaxel can effectively stabilize microtubule structures in androgen-sensitive and CRPC cells in accordance with the known action of this taxane chemotherapeutic agent (14,45). With the knowledge that Cabazitaxel was designed to bind with β -tubulin subunits and stabilize their interaction preventing depolymerization of the structure, once assembled, the microtubules cannot disassemble leading to mitotic blockade. In contrast to Docetaxel that induced microtubule stabilization leading to classic G2M arrest and apoptosis as well as AR cytoplasmic localization, we report for the first time that Cabazitaxel treatment induces severe multi-nucleation and centrosome clustering that led to the development of mono-astral spindle formations in the androgen-sensitive prostate cancer cells. Thus in addition to microtubule stabilization, Cabazitaxel targets expression of pre-mitotic kinesins which facilitate this process (46). The inhibitory effect of Cabazitaxel on protein and mRNA expression of MCAK and HSET kinesins provides an initial mechanistic insight into the ability of a microtubule chemotherapy to effectively target a mitotic kinesin and consequently interfering with AR transport across the microtubules, leading to tumor suppression (illustrated on Figure 6, panel H). Although early-phase clinical trials in CRPC patients using the first generation Eg5 inhibitor, Ispinesip, have met limited success (45), the concept that kinesins may define therapeutic targeting of Cabazitaxel in advanced prostate cancer, gains support from evidence documenting a correlation between overexpression of kinesin spindle protein MCAK with CRPC progression and functional involvement of motor protein Eg5 in prostate cancer cell growth (44).

Utilizing a transgenic mouse model of androgen-responsive prostate cancer that is driven by EMT to advanced metastatic disease, we analyzed the consequences of Cabazitaxel on EMT in prostate tumor progression *in vivo*. Prostate tumors from mice treated with Cabazitaxel alone or in combination with ADT, exhibited a phenotypically re-differentiated glandular prostate epithelium with intact luminal secretions. It could be postulated that the plasticity

afforded to a fully differentiated epithelium by Cabazitaxel allows individual cells to de-differentiate into mesenchymal-like derivatives in reversible phenotypic transformative process. Thus several rounds of EMT and MET allow for the formation of well-differentiated glandular epithelial structures in response to Cabazitaxel. In a twist of growth kinetics, Cabazitaxel increased the proliferative activity of prostate tumor cells. Considering that a dynamic EMT-MET cycling has been functionally implicated in the formation of epithelial tissues (47), our data support the ability of Cabazitaxel to induce epithelial differentiation of aggressive prostate tumors via reversal of EMT to MET. Thus, we speculate that upon elimination of the primary population of prostate cancer cells targeted by Cabazitaxel, a subset of prostate tumor epithelial cells undergo MET and this reversion to glandular differentiated epithelial cells, primes prostate tumors to the anti-androgen action towards overcoming resistance, highlighting the therapeutic value in the cycling of EMT to MET (Fig.6H). Moreover our work provides a molecular basis for the emerging role of AR variants in predicting therapeutic resistance of advanced CRPC to Enzalutamide (9) and taxanes in experimental models of CRPC (48). Overexpression of AR splice variants may preclude patients from undergoing Cabazitaxel and antiandrogen combination therapy due to therapeutic resistance driven by centrosome clustering. An insight into this cross-resistance is enabled by our observations that combination of CBZ and Enzalutamide leads to a significant growth stimulatory response in the CRPC 22Rv1 cells (harboring AR variants), while in the androgen-sensitive VCaP cells, Enzalutamide overcame CBZ resistance. Interestingly, it was recently reported that Enzalutamide treatment of CRPC 22Rv1 cells had no effect on AR V7 variant expression, while it upregulated V7 in VCaP cells (49). Attractive as the biomarker value of AR profiling might emerge in predicting cross-resistance to taxane chemotherapy and antiandrogens in CRPC, one may also consider this new action of Cabazitaxel in prostate tumors that bypasses AR and points to an optimized sequencing of these therapeutics, tailored to the EMT landscape.

In summary, this is the first evidence to indicate that Cabazitaxel chemotherapy reverses EMT to MET towards phenotypically differentiated prostate glandular/luminal architecture. Cellular re-differentiation via MET conversions may account for the similarities in the phenotypic landscape between primary tumors and bone distant metastatic lesions that can dictate their therapeutic resistance. Ongoing studies investigate (a) the mechanisms driving this remarkable effect of Cabazitaxel on tumor glandular differentiation by engaging the microtubule/cytoskeleton interaction and (b) the consequences of Cabazitaxel combination with antiandrogens in clinical prostate tumor specimens from patients with metastatic disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ADT	androgen deprivation therapy
AR	Androgen Receptor
AR-V	androgen receptor splice variant
CRPC	castration-resistant prostate cancer
DHT	dihydrotestosterone
EMT	Epithelial mesenchymal transition
FDA	Food and Drug Administration
FOXO1	Forkhead box 01
LH	luteinizing hormone
LHRH	luteinizing hormone releasing hormone
PSA	prostate specific antigen
TGF-β	Transforming growth factor- β
DMSO	dimethyl sulfoxide
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick-end-labeling

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Figure 1. Human Prostate Cancer Cell Response to Cabazitaxel and AR Targeting
Panel A, Dose response analysis of human prostate cancer cells, DU-145, PC-3, PC3v567es, 22Rv1, LNCaP, LNCaPT β R11 and VCaP to Cabazitaxel (10-500nM) for 96hrs. Cell viability was evaluated by the MTT assay. Panel B, Time course of cell viability response to CBZ (100nM); Panel C, Effect of CBZ alone or in combination with the antiandrogen (MDV, 1-10 μ M). Panels D and E, CBZ induces G2 and S phase arrest in PC3v567es cells. Panels F and G, Cell cycle analysis of CRPC 22Rv1 cells in response to treatment. Data from three independent experiments $\pm$ SEM; * indicates $P < 0.05$ as determined by one-way ANOVA.

RT-PCR analysis shows CBZ-induced downregulation of AR, PSA, and FOXO1 gene expression. Data represent mean of three independent experiments in duplicate $\pm$ SEM; * indicates $P < 0.05$ as determined by a two-way ANOVA.

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Figure 3. Cabazitaxel Results in Multi-nucleation and Centrosome Clustering in Prostate Cancer Cells by Targeting Kinesins

Panel A, From left to right: DU145 treated with CBZ (35nM; 96hrs) exhibit multipolar spindle and microtubule bundling; LNCaP cells treated with CBZ (35nM; 48hrs) exhibit mono-astral spindle formation; LNCaP, prostate cancer cells exhibit multipolar spindle, after treatment with CBZ (72hrs); PC3 cells overexpressing the AR variant V7 and VCaP cells exhibit extensive multi-nucleation and multipolar spindle in response to CBZ (100μM). Scale bars are 10 μM. Panel B reveals expression profile of KIF2C (MCAK) and KIF1C (HSET) proteins in prostate cancer cells. Panel C, Expression of HSET, MCAK, and β-

tubulin in LNCaP cells treated with CBZ. GAPDH used as loading control. Panel D, RT-PCR analysis of mRNA expression of KIF2C in LNCaP cells. Panel E, Detection of pericentrin (green), actin (phalloidin-red) and DAPI (blue) in PC3v56es cells after CBZ treatment. Panel F, VCaP cells exhibit multi-nucleation and centrosome amplification in response to CBZ; scale bars, 10 μ M. Panel E, Detection of pericentrin (red), tubulin (green) and DAPI (blue) in VCaP cells in response to CBZ and/or MDV (1 μ M). 40 $\times$ oil immersion, scale bars, 10 μ M.

Figure 4. *In Vivo* Effect of Cabazitaxel in an Androgen-Responsive Prostate Cancer Model of Progression to Lethal Disease

Panel A, Histopathological appearance of prostate tumors from transgenic mouse model of prostate tumor progression TRAMP/DNTGF β R11 (400 $\times$ magnification, scale 50 μ M). Serial sections from tumors from control mice (VHC), castrated for 14 days (CSTR), or treated with CBZ for 14 days alone (CBZ) or in combination with castration (CSTR+CBZ), were subjected to apoptosis detection (TUNEL) and cell proliferation (Ki-67 and phospho-H3 nuclear staining). Lower panel (A) indicates cytokeratin-18 immunoreactivity in tumor sections from VHC, CBZ-treated, castrated and combination treated transgenic mice.

Prostate tumors (from same littermates) were subjected to immunostaining for CK-18, a marker of luminal glandular differentiation and visualized under 400 $\times$ (insert at $\times$ 1,000 magnification), scale bars, 50 μ M. Panel B, phospho-histone H3 expression identifying endoreduplication of prostate tumor nuclei after CBZ treatment; Magnification 100 $\times$, scale bars 50 μ M. Panels C and D, Mean number of apoptotic and proliferating prostate tumor cells (respectively) $\pm$ SEM; * indicates $P < 0.05$.

Figure 5. Cabazitaxel Impairs Advanced Prostate Cancer by Inducing MET and Targeting Kinesins

Panel A & B, Immunoreactivity profile of E-cadherin, N-cadherin, AR and MCAK expression in prostate tumor sections from control (VHC), castrated (CSTR), CBZ-treated, or castration and CBZ treated TRAMP/DNTGF β RII transgenic mice. CBZ induces EMT changes and reduces AR and kinesin expression. Magnification 400 $\times$; scale bars are 50 μ M. Panel C Quantification of AR nuclear staining (3+) in respective sections; mean numerical values (% nuclear AR) $\pm$ SEM; * indicates $P < 0.05$. Panels D, E and F, RT-PCR analysis of mRNA expression for E-cadherin (CDH1), Vimentin (VIM) and Twist1 (TWIST1) in VCaP

cells after CBZ treatment. Panels G, H and I, mRNA profiling of EMT genes in LNCaP cells in response to CBZ. Data represent mean of three independent experiments $\pm$ SEM; *indicates $P < 0.05$ as determined by a two way ANOVA.

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Figure 6. Effect of Cabazitaxel and Antiandrogen (Enzalutamide) on CRPC Xenograft Growth
Male nude mice inoculated with CRPC 22Rv1 cells, were treated with vehicle (VHC), Cabazitaxel alone (CBZ), Enzalutamide (MDV) or combination (CBZ+MDV) for 2wks. Panel A, gross appearance of prostate tumors after various treatments. Panel B, indicates the tumor mass (g) of 22Rv1 CRPC xenografts in response to treatments. Panel C, immunohistochemical assessment of serial sections of 22Rv1 tumors, for apoptosis (TUNEL), CD31 (vascularity) and cell proliferation (Ki-67); scale bars, 5 μ M. Panels D, E and F, Quantitative analysis of apoptosis, vascularity and proliferative index (as described in

“Materials and Methods” mean $\pm$ SEM); * indicates $P < 0.05$. Panel G, Protein analysis of AR (N-20), MCAK and E-cadherin in 22Rv1 cells treated with CBZ. GAPDH, loading control. Panel H, Action of CBZ against AR and kinesins causing EMT to MET reversal and multi-nucleation. The inhibitory effect of CBZ on pre-mitotic kinesins across the microtubules compromises AR nuclear export. Therapeutic response to Cabazitaxel proceeds via reversal of EMT to MET and multi-nucleation.

Figure 7. Impact of Cabazitaxel in CRPC via MET, Kinesins and Nuclear AR

Panel A, Profiling of the EMT landscape in 22Rv1 prostate xenografts in response to CBZ and antiandrogen (MDV) treatment. Serial tumor sections from VHC and treated mice were subjected to immunostaining for E-cadherin, N-cadherin and Zeb-1; Magnification 400 $\times$, scale bars are 50 μ M. Panel B, Effect of CBZ on MCAK and HSET protein expression. Lower panel indicates H&E staining of serial sections of CRPC tumors. Panel C, CRPC prostate 22Rv1 tumors from VHC and after treatment with MDV3100, Cabazitaxel as single agents or in combination were evaluated for AR. Magnification 400 $\times$; CBZ reduces AR

levels but retains its nuclear localization. Lower panel, mean numerical values of cellular distribution of AR (N-20) in prostate tumors $\pm$ SEM; * indicates $P<0.05$.

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