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Novel actions of next-generation taxanes benefit advanced stages of prostate cancer

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Abstract

Purpose—To improve the outcomes of patients with castrate resistant prostate cancer (CRPC), there is an urgent need for more effective therapies and approaches that individual specific treatments for patients with CRPC. The current studies compared the novel taxane, cabazitaxel with the previous generation docetaxel, and aimed to determine which tumors are most likely to respond.

Experimental design—Cabazitaxel (CBTX) and docetaxel (DCTX) were compared via in vitro modeling to determine molecular mechanism, biochemical and cell biological impact, and cell proliferation, which was further assessed ex vivo in human tumor explants. Isogenic pairs of RB knockdown and control cells were interrogated in vitro, and in xenograft tumors for cabazitaxel response.

Results—The data herein show that i. CBTX exerts stronger cytostatic and cytotoxic response compared to DCTX, especially in CRPC; ii. CBTX induces aberrant mitosis, leading to pyknotic and multinucleated cells; iii. taxanes do not act through the androgen receptor (AR); iv. Gene expression profiling reveals distinct molecular actions for CBTX v. tumors that have progressed to castration resistance via loss of RB show enhanced sensitivity to CBTX.

Conclusions—CBTX not only induces improved cytostatic and cytotoxic effects, but also impacts distinct molecular pathways, compared to DCTX, which could underlie its efficacy after DCTX treatment has failed in CRPC patients. Finally, RB is identified as the first potential biomarker that could define the therapeutic response to taxanes in metastatic CRPC. This would suggest that loss of RB function induces sensitization taxanes, which could benefit up to 50% of CRPC cases.
Keywords
prostate adenocarcinoma; chemotherapy; retinoblastoma; docetaxel; androgen receptor

Introduction
Prostatic adenocarcinoma (PCa) is the most frequently diagnosed non-cutaneous malignancy amongst men in Western civilization and a leading cause of death by cancer(1, 2). The first line of therapeutic intervention for disseminated disease targets the androgen receptor (AR) through androgen ablative strategies (androgen deprivation therapy, ADT), frequently complemented with AR antagonists(3). Although initially effective, castration-resistant PCa (CRPC) develops within a median of 2–3 years after initiating primary androgen ablation therapy(4).

PCa generally responds poorly to standard cytotoxic regimens, and modest clinical benefit has been achieved with the chemotherapeutic docetaxel in metastatic CRPC (mCRPC)(5). Since 2010, several novel therapeutic agents, with distinct mechanisms of action, have been approved for treatment of CRPC. These include: i) the anti-androgen enzalutamide(6, 7), ii) the CYP17A1 inhibitor abiraterone acetate(8), iii) the alpha-emitting radiopharmaceutical radium-233(9), iv) the immunotherapeutic sipuleucel-T(10), and v) the new generation taxane cabazitaxel(11, 12). While radium-233, sipuleucel-T, abiraterone and enzalutamide can be used pre- or post-docetaxel, cabazitaxel is currently approved for patients with mCRPC who have progressed on docetaxel therapy(13). Despite the recent increase in treatment options, and potential combination or sequential regimens, patients ultimately succumb to the disease, with a median survival of mCRPC patients of 48 months in a SWOG III trial (14), and 15–18 months post-docetaxel(15). The limited therapeutic success emphasizes the need for more efficacious drugs and a patient-tailored approach towards cancer therapy to improve disease outcome.

Cabazitaxel is a second generation taxane, belonging to a class of compounds that stabilize the microtubules by binding β-tubulin, and promote their polymerized state. Taxanes attenuate the microtubule dynamicity, impairing the dynamics of the mitotic spindle and inducing mitotic arrest, microtubule nucleation, and apoptosis(16, 17). While cabazitaxel and docetaxel both target β-tubulin, the TROPIC clinical trial (NCT00417079) in mCRPC patients presenting with progressive disease after docetaxel treatment demonstrated that cabazitaxel still has therapeutic efficacy after docetaxel failure(11). Critical gaps in our knowledge of cabazitaxel include understanding of: i) the molecular mechanisms underlying the differential response to cabazitaxel versus docetaxel; ii) the optimal clinical state for administering cabazitaxel, i.e. the novel taxane may outperform docetaxel when administered to patients earlier in disease progression; and iii) a clinical biomarker to identify patients that will most likely benefit from cabazitaxel treatment.

The current studies assessed the molecular and cellular response to cabazitaxel. In vitro analyses showed that cabazitaxel is superior to docetaxel in its antitumor activity. These effects were most pronounced in CRPC model systems, wherein taxanes are preferentially utilized. While it has been suggested that taxanes may impinge on AR subcellular
localization (18–20), this does not appear to be a major facet of taxane activity, since at the low nanomolar concentrations, which show cytostatic and cytotoxic effects, no effect on AR was observed. These studies further identified a key biomarker of cabazitaxel responsiveness. In vitro and in vivo analyses of isogenic-paired models with and without RB demonstrated that PCA cells that have progressed to castration resistance through loss of RB are hypersensitive to cabazitaxel. Finally, the molecular activities of cabazitaxel were discerned using gene expression analyses that showed that the genomic response of cabazitaxel was distinct from docetaxel, and was strongly influenced by the hormonal milieu.

Materials and Methods

Cell culture

Androgen-dependent (LNCaP, LNCaP shCon1) and CRPC (C4-2, LNCaP shRB1) cells were maintained as previously described (21). Unless otherwise indicated, cells were plated overnight in IMEM (Corning Cellgro) using standard serum (5% FBS) and treated with 1nM CBTX or DCTX.

Trypan blue exclusion assay

Cells were treated in duplicate with a dose range of 0.1–2nM of taxane for 48h, followed by 48h (LNCaP, C4-2) or 96h (LNCaP shCon1/shRB1) in fresh media. Cell samples were trypsinized, counted twice on a hemacytometer using the trypan-blue exclusion method, and normalized to a drug-free control. Experiments were performed at least twice.

Flow cytometry

PCA cells were treated in triplicate with a taxane for 16 or 48h, or vehicle, adherent and non-adherent cells were combined, gently re-suspended in 100% ethanol, and fixed overnight at −20°C. Proliferation was measured by bivariate flow cytometry using a 2hr pulse-label of BrdU (Amersham, RPN201) prior to harvest and cell cycle position by propidium-iodide (PI) staining (22). A BD Biosciences FACSCaliber was used to capture 10,000 BrdU/PI events. FlowJo software (TreeStar) was used to gate for %BrdU incorporation or cell cycle.

Immunoblotting

Control and taxane-treated cells were harvested to evaluate cleaved PARP-1 (Cell Signaling) levels. Total protein was extracted by sonication in RIPA buffer, separated by SDS-PAGE, transferred to PVDF, and immunoblotted overnight at 4°C. Immunoblots were quantified using a BioRad Chemidoc MP Imaging System.

Immunofluorescent microscopy

Cells were seeded overnight in 6-wells plates on poly-L-lysine-coated coverslips, treated, fixed with 4% paraformaldehyde (Acros Organics, 416780250), and permeabilized with Triton-X100 (Amresco, 0694-1L), blocked in 2% goat serum, and stained with rabbit-α-AR (Santa-Cruz N-20) and α-rabbit Alexa-fluor-546 (red). To stain F-Actin, cells were incubated with Alexa-Fluor-488 Phalloidin (Life Technologies). Coverslips were mounted
with Prolong Gold Antifade reagent with DAPI (Invitrogen, P36931). Images were taken with a confocal laser microscope (Zeiss 510 Meta, 40x objective, 2x digital zoom). Quantification of phenotypes was done on a fluorescent microscope (Leica DMI3000B, 20x objective) on at least 4 coverslips per condition, counting 200 cells per replicate, calculating % cells displaying specific morphologies.

**Human prostate tumor explants**

Fresh primary tumor tissue was obtained from PCa patients who underwent radical prostatectomy at Thomas Jefferson University Hospital in accordance with Institutional Review Board standards and in compliance with federal regulations governing research on de-identified specimens and/or clinical data (45 CFR 46.102(f)). Tumors were dissected by a clinical pathologist and collected in culture media: IMEM [5% FBS, 0.01 mg/ml insulin (Invitrogen, 12585-014), 30μM hydrocortisone (Sigma, H-0888), and pen/strep]. Tissue was subdivided into ~1mm³ pieces and placed (2–3 pieces/well) in a 24-well plate on pre-soaked 1cm³ dental sponges (Novartis, Vetspon) submerged in 0.5ml culture media in presence or absence of drugs (control, 50nM CBTX or DCTX). Treatments were refreshed every 48h, and explants were harvested after 6 days, formalin-fixed, paraffin-embedded, and analyzed by standardized immunohistochemistry (IHC) methods. Control- and taxane-treated explants (n=3 patients) with glandular epithelial tissue, as determined by a certified clinical pathologist, were stained for Ki-67 (Invitrogen, 180191Z) or AR (Santa-Cruz, N-20) using clinically approved protocols by the Thomas Jefferson University Hospital. Cleaved caspase-3 (catalog#9661L; Cell Signaling Technology) staining was outsourced to the Comparative Pathology and Mouse Phenotyping Shared Resource at the Ohio State University (Columbus, OH).

**Mouse xenografts**

Xenograft studies were performed in accordance with NIH Guidelines and animal protocols were approved by Thomas Jefferson University. LNCaP shCon1 or shRB1 cells (3 × 10⁶) were combined 1:1 with Matrigel (BD Biosciences, 354234) and injected subcutaneously into the flanks of 6-weeks-old, intact-male athymic nude mice (NCI-Frederick, Frederick, MD, USA). Mice were castrated when tumors reached 100–150mm³. After 1-week recovery, the mice were treated 2x/week with 8mg/kg cabazitaxel by intraperitoneal (IP) injections, and tumor volumes were monitored with calipers 3x/week. Tumors were harvested and processed after 3 weeks of treatment, or when the tumor exceeded 800mm³. Hematoxylin and eosin stained sections of shCon1 and shRB1 LNCaP tumors (n=6 tumors/group) were evaluated using a Motic BA400 microscope (Motic, Richmond, British Columbia, Canada). For each tumor section, the number of mitotic figures in ten randomly selected 400X (i.e., high-power) fields was determined by a board certified veterinary anatomic pathologist (LDBB). Additional tumor sections were stained for Ki-67 (1:250; Invitrogen, Carlsbad, CA, USA; 18-0191Z) and AR (Santa-Cruz, N20).

**Immunohistochemistry quantification**

Sections of shCon1 and shRB1 LNCaP xenograft tumors (n=6 tumors/group) and prostate tumor explants (n=3 tumor explants/treatment) were immunostained for Ki67 (Invitrogen, 180191Z). The prostate tumor explants (n=3 explants/group) were also immunostained for
cleaved caspase-3 (catalog#9661L; Cell Signaling Technology). Three random images from each slide were obtained at 400X (i.e., high-power) magnification. The cell counter feature of the ImageJ64 (NIH, Bethesda, MD) analysis software was used to determine the percentage of immunopositive over all neoplastic cells.

**Gene expression array**

A genome-wide expression array (GSE63479) was performed on a GeneChip Human Gene 2.0ST Array (Affymetrix, 902112) with LNCaP cells infected with a control plasmid (MSCV-LMP) and C4-2 cells (23), treated in duplicate for 16h with 1nM CBTX, DCTX or vehicle (EtOH). The expression data were RMA normalized, and filtered to remove low-expressing genes. Differential gene expression with corresponding p-values (student’s t test) was determined of drug-treated over control. Gene lists with p<0.05 were compared based on the corresponding AffymetrixIDs, and plotted in a Venn diagram ([http://bioinfogp.cnb.csic.es/tools/venny/](http://bioinfogp.cnb.csic.es/tools/venny/)). GO analyses were performed on selected clusters by separating up- and down-regulated genes ([http://david.abcc.ncifcrf.gov/](http://david.abcc.ncifcrf.gov/)), and the resulting GOterms were cut off at p<0.05.

**Statistical analyses**

To determine statistical significance, p-values were calculated by a standard unpaired student’s t-test, unless otherwise noted. Significant effects: *p<0.05, **p<0.01, ***p<0.001.

**Results**

**Cabazitaxel shows enhanced anti-proliferative and pro-cytotoxic effects in CRPC**

To assess the relative impact of cabazitaxel (CBTX) and docetaxel (DCTX) on PCa cell growth and survival, analyses were performed in both hormone-therapy sensitive and CRPC model systems. Cells were treated with an increasing dose of each agent (0.1–1nM) for 48h, followed by a washout of 48h in the absence of drug, and subsequent quantification of cell viability through cell counting. As shown (Figure 1A), CBTX and DCTX showed relatively equivalent effects in hormone-therapy sensitive cells (left), with IC50 values of 0.220nM for CBTX, and 0.319nM for DCTX. However, CBTX (IC50=0.142nM) resulted in markedly enhanced anti-tumor effects compared to DCTX (IC50=0.269nM) in CRPC cells (right). Concordantly, flow cytometric analyses monitoring both cell cycle position (via propidium iodide, PI) and progression through S-phase (via uptake of bromodeoxyuridine, BrdU) revealed reduced S-phase entry after 16 and 48 hours of CBTX treatment in both cell types, although less pronounced in CRPC cells (Figure 1B, top, quantified in 1C). DCTX (Supplemental Figure 1, and Figure 1C) had a similar effect on proliferation of hormone-sensitive cells, but did not affect S-phase entry of cells from the same lineage that have achieved castration resistance. Thus, CBTX demonstrates an enhanced anti-proliferative effect in CRPC cells compared to DCTX. This enhanced effect was reiterated in ADT-sensitive (LAPC4, VCaP) and CRPC (LCaP-abl) cell models (Supplemental Figure 2A). In vivo, cabazitaxel successfully induced C4-2 xenograft tumor remission, thereby demonstrating effectiveness of cabazitaxel in a CRPC model (Supplemental Figure 2B).
In addition to the observed effects on cell cycle, the sub-2N content was enhanced in CRPC cells treated with CBTX compared to hormone-therapy sensitive models, eliciting almost double the effect of DCTX after 48h in C4-2 cells (Figure 1B, bottom; Figure 1D). Moreover, CBTX effectively induced apoptosis in C4-2 cells, as measured by PARP cleavage (Supplemental Figure 3). Together, these data suggest that CBTX shows enhanced anti-proliferative and pro-cytotoxic effects as compared to DCTX.

**Cabazitaxel promotes defective mitosis**

To address the means by which CBTX exhibits enhanced anti-tumor effects, the impact on nuclear integrity was assessed. Cells were fixed after 16h of exposure, and nuclear features assessed after DAPI staining (Figure 2A). Visualization on a confocal microscope demonstrated a modest enrichment of mitotic figures after 16h (quantified in Figure 2C, left), but evidence of defective mitoses was apparent in the asymmetric appearance of segregating nuclei. Pyknotic nuclei were also observed (Figure 2A), as quantified in Figure 2C (right), demonstrating a significant increase after 48h CBTX exposure, further supporting the contention that CBTX exerts cytotoxic effects.

Given the known function of taxanes in serving as microtubule stabilizers and perturbing cytoskeletal integrity, treated cells were also stained with fluorescently labeled phalloidin to visualize the cellular architecture. This revealed a reduction in cytoplasmic volume in all cells (Figure 2B), and allowed for clear definition of cell borders, revealing enhanced presence of multinucleate cells in CRPC cells treated with CBTX, likely resulting from aberrant mitosis. Multinucleate LNCaP and C4-2 cells were quantified in at least four duplicates for CBTX versus control treated samples, which demonstrated a highly significant increase in polynuclear cells after 16h and 48h (Figure 2C right). Together, these data support the hypothesis that CBTX fosters an enhanced antitumor capacity via disruption of the cell architecture and defective mitoses.

**Taxane action is independent of effects on AR localization**

Taxanes have been reported to affect AR localization, and could thereby potentially block prostate cancer proliferation(18–20). In these previous studies, supra-clinical doses (micromolar) levels of taxanes were utilized, whereas in vitro IC50-values for these drugs are in the nanomolar range(24, 25). Thus, it is imperative to determine whether the reported effects occur at doses that are robustly cytostatic and cytotoxic, and are clinically attainable. As shown, using doses and time points sufficient to both suppress proliferation and induce cell death, endogenous AR remains nuclear in the presence of androgen-replete media (Figure 3A). To determine whether these effects hold true under conditions that mimic castration, parallel studies were conducted in the presence of charcoal-dextran-treated serum (CDT). As expected, androgen deprivation alone resulted in loss of nuclear AR enrichment in hormone-therapy sensitive cells; however, in CRPC cells, AR is retained in the nucleus even under castrate conditions (Figure 3B). CBTX or DCTX had no impact on AR localization at a nanomolar level (1nM) in either cell line. Taken together, the enhanced cytotoxic and cytostatic effects of CBTX appear to be independent of AR subcellular regulation.
**Cabazitaxel exerts enhanced anti-tumor effects in human tumors**

Given desirable cytostatic and cytotoxic effects of CBTX in model systems, the anti-tumor effects were further assessed using next-generation, *ex vivo* tumor explants that allow for determination of effects on the complex 3D-tumor microenvironment. Fresh tumor material was obtained from radical prostatectomy of high volume disease, and tissue slices randomized into control or taxane-treated arms. As has been previously reported, these tumor slices retain the salient features of the tumor at the time of resection (including AR expression and proliferative capacity)\(^{(22, 26)}\) (Figure 4A). These studies allow for intrinsically controlled analyses of taxane effects within the same tumor. Tumors were harvested after 6 days of treatment, formalin-fixed and paraffin-embedded. Standard hematoxylin and eosin (H&E) staining confirmed retention of the tumor microenvironment (Figure 4B left). Immunohistochemistry (IHC) to assess the proliferative indices (using an antibody to Ki67) was performed in parallel, of which representative examples are shown in Figure 4B (middle), and quantified (Figure 4C, top). Strikingly, this direct comparison in fresh tumor explants confirmed that CBTX exerts a markedly enhanced cytostatic response compared to DCTX. Caspase-3 staining revealed an increasing trend, however non-significant, likely due to the limited sample number (n=3; Figure 4C right). Finally, assessment of AR localization in response to 50nM drug treatment revealed retention of nuclear AR in the tumor cells (Figure 4D). Thus, similar to what was observed *in vitro*, the anti-tumor effects of nanomolar level CBTX appear to occur independently of altered AR localization. Collectively, these data identify an enhanced capacity of CBTX to elicit anti-proliferative and pro-apoptotic events in primary human tumors.

**Tumors that progress to CRPC by RB loss show hypersensitivity to cabazitaxel**

While the above studies suggest that CBTX harbors properties that are highly desirable in the clinical setting, a major hurdle is to identify tumor subtypes that would most benefit from treatment with the agent. It has been previously demonstrated that loss of retinoblastoma tumor suppressor protein (RB) or function occurs with high frequency in CRPC, and that this event alone can promote bypass of hormone therapy\(^{(21)}\). Conversely, we and others have shown that RB loss compromises selected DNA damage checkpoints, and can result in sensitization to a subset of chemotherapeutics, including docetaxel\(^{(23, 27)}\). These findings put forward the provocative hypothesis that prostate cancers that achieve castration-resistance via loss of RB may be exquisitely responsive to taxanes. To assess this, isogenic pairs of LNCaP cells with control shRNA or shRNA directed against RB (shCon1 and shRB1 cells)\(^{(21)}\), were initially assessed *in vitro* for differential response to CBTX. In these studies, shRB1 cells demonstrated a modest sensitization to CBTX as compared to shCon1 (Figure 5A). To challenge this *in vivo*, cells were subcutaneously injected into male athymic nude mice; when the xenograft tumors (n=5 per group) reached a size of 100–150mm\(^3\), the mice were surgically castrated, allowing a week of recovery before starting 8mg/kg cabazitaxel treatment by intraperitoneal (IP) injections twice a week (Figure 5B schematic). Tumor volumes were monitored three times a week with caliper measurements, and as shown, a remarkably enhanced tumor-suppressive effect was observed in the shRB1 tumors (Figure 5B left). Normalization of individual tumor volumes at 14 days after start of treatment compared to their respective volumes at t=0 shows a significant difference in
cabazitaxel response between the shCon1 tumors (n=4) and the shRB1 tumors (n=5) (Figure 5B right). Notably, the fifth mouse growing an LNCaP shCon1 tumor had to be sacrificed before this time point, because the tumor volume had already exceeded 800 mm$^3$, despite cabazitaxel administration. These in vivo findings robustly support the concept that RB-deficient tumors are hypersensitive to treatment with CBTX.

To further probe the underlying basis of the observed enhanced effect in RB-deficient tumors, histopathological analyses were performed. Through H&E and Ki67 IHC analyses, shCon1 tumor cells showed expected accumulation of mitotic figures, indicating an appropriate cell cycle arrest in metaphase as a result of CBTX treatment. By contrast, shRB1 tumors elicited fewer mitotic figures (Figure 5C, quantified in 5D top), consistent with previously reported “mitotic slippage” in cells lacking cell cycle checkpoints(28). Quantification of Ki67 positive cells in three images per tumor slide confirmed a strong reduction in proliferative cells in the shRB1 tumors versus shCon1 (Figure 5D bottom). Taken together, the in vivo data suggest that RB-deficient tumors are hypersensitive to CBTX, and support the postulate that RB should be developed as a biomarker to identify tumors that may be most responsive to taxanes.

**Cabazitaxel displays novel actions in prostate cancer cells by expression profiling**

While the above studies identify novel anti-tumor effects of CBTX and putative biomarkers to identify tumors that would be most responsive to this taxane, additional studies were performed to further uncover the molecular basis of divergent CBTX function. Hormone therapy-sensitive LNCaP, and castration-resistant C4-2 cells were treated for 16h with CBTX or DCTX, in presence or absence of steroid hormones, and subjected to a genome-wide mRNA analysis. The resulting expression data were filtered to remove low-expressing genes, to reduce background and false positive hits. Triplicate expression values were averaged, after which samples from the individual taxanes (CBTX or DCTX) were normalized to the corresponding values for control treated samples either with or without steroids. Differentially expressed genes were selected by a student’s t-test (p<0.05), and compared between treatments, as visualized by the two Venn diagrams representing the two cell models (Figure 6A). Complete gene lists and fold alterations for each gene cluster are provided in Supplemental Table 1 (LNCaP) and 2 (C4-2). Notably, distinct gene expression outcomes were readily apparent between the two taxanes. Further, these analyses revealed divergent effects of the agents in cells that are cycling (steroid replete) versus those that were arrested via steroid depletion (androgen deprived). These findings suggest, as expected based on the data above, that CBTX exerts differential effects compared to DCTX, and that the proliferative status of the tumor cell can alter downstream biological effects.

To gain deeper understanding of the cellular response to CBTX and DCTX, gene ontology analyses were performed on both castrate (in blue and red, respectively, corresponding to the Venn clusters in Figure 6A) and steroid replete (yellow and green) gene lists (Supplemental Figure 4 LNCaP; Supplemental Figure 5 C4-2). For complete gene lists per GO term, see in Supplemental Table 3. These studies revealed enrichment of genes involved in cell cycle and chromosomal organization and regulation after cabazitaxel treatment exclusively in C4-2 cells (Figure 6B), consistent with the enhanced anti-proliferative effect of this agent in
CRPC cells (Supplemental Figure 3). In the ADT-sensitive LNCaP cells under androgen-deprived conditions, CBTX and DCTX share enrichment in gene transcription related pathways. However, these appear to be negatively regulated by CBTX, versus a positive impact by DCTX, which supports the hypothesis that these drugs have distinct downstream effects. In C4-2 cells, transcription was affected regardless of steroid conditions, however, chromosomal regulation appears to suffer a greater impact during steroid repletion, which supports the hypothesis that hormone conditions matter for chemotherapeutic response even in castration-resistant tumor cells. DCTX and CBTX may impact similar pathways, nonetheless, CBTX has a stronger effect on cell cycle and chromatin regulation, whereas DCTX appears to have a more significant impact on transcription and repair. These differences could be caused by differences in microtubule stabilization. While a general mechanism has been described for taxane action, these drugs may act with differing kinetics, or be less or more efficient at stabilizing the tubulin dimers, resulting in various degrees of aberrant mitotic events. Notably, CRPC cells have a more substantial taxane response in terms of chromatin organization and regulation, which downstream would ultimately lead to aberrant mitosis and cell death. As shown, genes most altered (up- or down-regulated) by cabazitaxel in absence or presence of steroids are displayed in tables (Figure 6C). Genes that are common between the two cell models are marked with an asterisk. Classical androgen receptor targets (KLK3, TMPRSS2, FKBP5, KLK2) are not affected by CBTX or DCTX, and are only altered by steroid deprivation (data not shown). Strikingly, the hormone conditions affect the molecular response of taxanes, demonstrated by enrichment of distinct pathways in hormone replete versus androgen-deprived media, even in castration-resistant tumor cells. This differential response to CBTX (or DCTX) will likely be of relevance for designing combination therapies with androgen receptor pathway targeting drugs, such as enzalutamide and abiraterone acetate. These unbiased analyses further illustrate the divergent effects of CBTX and DCTX, and provide a gene “signature” of response to CBTX in the presence and absence of androgen.

Discussion

Currently, no durable cure exists for advanced CRPC, and only limited therapeutic success has been achieved in terms of improved overall survival. One of the few options available for advanced disease is taxane-based chemotherapy, and the new generation drug cabazitaxel has been shown to exert anti-tumor effects even after docetaxel has failed [de Bono 2010]. This study presents first-in-field distinctions between these two agents, using clinically relevant conditions. Key findings are: i. CBTX induces improved cytostatic and cytotoxic response, especially in CRPC; ii. CBTX induces aberrant mitosis, leading to pyknotic and multinucleated cells; iii. taxanes do not act through AR to induce cytostatic and cytotoxic effects at nanomolar concentrations; iv. novel molecular actions for CBTX are identified by gene expression profiling; v. tumors that have progressed to castration resistance via loss of RB show enhanced sensitivity to CBTX.

These studies demonstrate that the cellular consequences of CBTX are distinct from that of DCTX in CRPC. In general it is thought that taxanes inhibit mitosis by binding β-tubulin and stabilizing the microtubules. In CRPC, taxanes induced both cytostatic and cytotoxic effects, but these properties were enhanced with CBTX. CBTX induced an increase in
mitotic figures in vitro and in vivo, often asymmetrical in shape, suggestive of a prolonged, aberrant mitotic arrest, resultant in multinucleated cells likely due to mitotic checkpoint slippage. Previous studies in which lung carcinoma cells were treated with low molecular levels support this concept, as such that cells escaped from a prolonged mitotic arrest without a proper cell division, resulting in tetraploid cells (29). Clinically, CBTX is effective in patients who have failed DCTX, which suggests that CBTX could prolong overall survival when used as a first-line chemotherapeutic (13). In this study, CBTX elicits stronger responses in CRPC models, as opposed to ADT-sensitive cells, and distinctly shows an improved response window versus DCTX. Moreover, metastatic disease may respond better to taxanes upon first diagnosis than after exposure to other treatment regimens, which allows cells to evolve into even more aggressive disease.

Contrary to expectation, the effects of taxanes at clinically relevant (nanomolar) doses appear to be independent of AR regulation. In PCa models, it has been previously suggested that taxanes may inhibit AR translocation to the nucleus and activity, and thus contribute to anti-tumor efficacy (18–20). However, these studies were performed in supra-pharmacological concentrations of taxane (50nM-1uM), whereas the IC50s described for this class of chemotherapeutics are in the single nanomolar range (24, 25). AR and downstream targets are not affected by CBTX at the nanomolar level, as demonstrated by the gene expression analysis. Taken together, the studies herein provide evidence that taxanes do not impact AR localization and activity at the low nanomolar levels in vitro, or clinically relevant concentrations in vivo and ex vivo, while cytostatic and cytotoxic effects are observed. Although it is probable that at higher (micromolar) levels AR transport is impaired, and thus its activity, this is likely a bystander effect of the disruption of the cellular highway impacting general cellular trafficking that depends on the microtubules.

In addition to demonstrating the commonalities between CBTX and DCTX actions, gene expression analyses reveal a molecular basis for the divergent effects that CBTX exerts as compared to DCTX, even in hormone-therapy sensitive cells. Cabazitaxel elicits a stronger response than docetaxel across different model systems, which could be attributed to different downstream molecular pathways affected by the drug. A common pathway described for DCTX in a previous genome wide study is cell cycle (31), nonetheless, CBTX appears to have an elevated effect on cell cycle and mitosis as evident from related GO terms in the CBTX distinct gene clusters in the study presented here. Moreover, chromatin organization is exquisitely impacted by CBTX in CRPC cells, likely underlying the stronger response to the novel therapeutic, as well as its effectiveness in patients who have progressed on docetaxel. DCTX seems more enriched for transcription pathways, as previously reported [Li et al 2004]. It has been speculated that DCTX resistance can occur via bIII-tubulin overexpression or mutation, or by increased P-glycoprotein (13, 32, 33). Improved response to CBTX was proposed to be due to a lower binding affinity for this drug pump, but this has not been demonstrated. Conversely, it has been shown that P-glycoprotein regulates CBTX levels in the brain in vivo (34, 35). In sum, CBTX has an elevated impact on cell cycle pathways and chromatin organization compared to DCTX, resulting in stronger cytostatic and cytotoxic effects, and does not act via AR. Of note, the molecular impact of CBTX depends on the hormone conditions, which emphasizes the need to further explore the
optimal treatment conditions; for example, it should be determined whether this novel drug should be administered as a single therapeutic, or in combination with ADT.

In addition to definition of the optimal treatment regimen, there is an urgent need for biomarkers in prostate cancer, to determine which patients are most likely to benefit. Currently, CBTX utilization is approved only in patients with progressive disease after DCTX has failed, without knowledge of tumor markers to indicate who might most benefit from taxane therapy. Striking data herein show that tumors that progressed to CRPC via loss of RB are hypersensitized to CBTX in vitro and in vivo, supporting the hypothesis that RB could be applied as a biomarker for treatment outcome predictions in patients. Given the fact that PCa is a leading cause of death in the US and Europe, and currently few therapeutic options are available for CRPC, it is imperative to improve treatment. Loss of RB functionality is associated with up to 60% of all CRPC cases (21, 23). This study provides preclinical evidence that RB loss sensitizes tumor cells to taxanes, supporting the hypothesis that RB status could be applied as a metric to determine treatment strategies for CRPC patients, as such that RB-deficient tumors would be treated with taxane-based chemotherapy. This approach would not only improve chances at longer recurrence free survival, but also limit unnecessary treatment with drugs that are less likely to be successful for an individual patient, thereby avoiding potential adverse side effects. Despite previous reports that in other tumor types a high level of chromosomal instability (CIN) is associated with taxane resistance (36), and dysregulation of the RB pathway has been described to cause genomic instability and aneuploidy (37, 38), suggesting RB loss would induce CBTX resistance, the opposite was observed here. This could mean that taxanes have an additive effect to the already existing high levels of CIN in RB-deficient cells, leading to catastrophic segregation errors and reduced cancer cell viability (39). Other potential biomarkers for taxane sensitivity reported across different tumor types are BRCA1, negative Bcl2, negative SIRT2 protein (40–43). However, the data herein present the first in vivo evidence of a single gene, RB, as a potential biomarker for treatment response in prostate cancer, which is a crucial step towards patient-tailored treatment decisions and improved care of CRPC.

Overall, the data presented here support that CBTX would improve therapeutic response compared to DCTX, likely as applied to the DCTX space of CRPC. This hypothesis is currently being tested in a phase III clinical trials [FIRSTANA NCT01308567]. Moreover, a recent report of an interim analysis on another trial in ADT-sensitive metastatic disease (CHAARTED NCT00309985) shows a striking benefit from addition of docetaxel to standard androgen deprivation therapy, underlining the importance of testing chemotherapeutics in earlier disease states. The gene expression data in the current studies suggest that the hormone conditions affect taxane action, not only in ADT-sensitive, but also in CRPC cells. The preclinical data strongly support the rationale behind a new phase II clinical trial that is currently recruiting chemotherapy-naïve patients presenting with metastatic CRPC to retrospectively assess the potential impact of RB as a biomarker for CBTX sensitivity (ABICABAZI NCT02218606), which will compare the effects of abiraterone alone versus abiraterone in combination with cabazitaxel. In summary, this paper and recent clinical developments underpin that redefining the optimal clinical space
and approach for chemotherapeutics would likely improve overall survival of patients with advanced prostate cancer.

In conclusion, these studies demonstrate the first molecular and cellular distinctions between cabazitaxel versus docetaxel in prostate cancer. The data provided not only demonstrate that these agents elicit differential molecular effects that are distinct from AR regulation, but show that the enhanced effects of CBTX in CRPC can be preclinically modeled. Moreover, the studies described put forth the first putative biomarker to select for patients that might most benefit from CBTX therapy. Combined, these studies provide the basis for improving efficacy of taxane-based therapy in prostate cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References


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Translational impact

There is an urgent need for more efficacious therapeutics to treat advanced, castration resistant prostate cancer (CRPC), which to date remains a uniformly fatal disease. Few chemotherapeutic options exist that impact overall survival in this stage of disease, and although the underlying mechanisms are unknown, the most effective chemotherapeutics are taxanes. Further, the next-generation taxane cabazitaxel is effective in patients that have failed docetaxel, but the molecular basis for this has not been discerned. Via modeling in vitro, in vivo, and human tumor explants, studies herein identify distinct molecular signatures of the taxanes, revealing novel molecular functions of cabazitaxel associated with enhanced efficacy in CRPC. Further, loss of the retinoblastoma tumor suppressor (RB) was identified as a biomarker of enhanced response to cabazitaxel in vivo. These findings reveal unique functions for cabazitaxel in CRPC, and identify the first potential biomarker for selecting patients who might most benefit from early chemotherapy.
Figure 1. Cabazitaxel shows enhanced anti-tumor effects in CRPC

A. Dose-dependent response to 48h cabazitaxel (CBTX) and docetaxel (DCTX) treatment and 48h fresh media without drugs was assessed in hormone therapy sensitive (LNCaP) and resistant prostate cancer cells (C4-2) by trypan blue exclusion, and cell numbers were normalized to EtOH treatment. B. Bivariate flow cytometry analyses of LNCaP and C4-2 cells treated with 1nM CBTX or control for 16 or 48h. In the top graphs, the x-axis represents relative DNA content as indicated by propidium iodide (PI) staining; the y axis shows cells undergoing active S-phase as indicated by 2h BrdU labeling. Inset values: % BrdU incorporation in viable cells (mean±s.d., from an experiment performed in biological triplicate). The bottom graphs represent the corresponding PI traces only, showing a G2M arrest, followed by cell death after CBTX exposure. C. Quantification of % BrdU+ cells in (B) and Suppl Fig 1, significant reduction is observed in taxane over control (LNCaP p<0.05; C4-2 p<0.0005 for all conditions). CBTX appears to have a mild effect on
proliferation of C4-2, although not significand, whereas DCTX does not. D. Taxanes induce significant cell death after 48h of CBTX or DCTX over CTRL in both LNCaP and C4-2 cells (p<0.01, detected as an increase in Sub-G1 content by flow cytometry (PI in B), with enhanced effects in the CRPC cells. Asterisks indicate significant differences between CBTX and DCTX at the same treatment duration, showing no significance in LNCaP, but improved efficacy for CBTX in C4-2 cells (16h 0.0042; 48h p=0.0092).
Figure 2. Cabazitaxel treatment results in increased multinucleated cells and aberrant nuclei

A. The nuclei of fixed LNCaP and C4-2 cells that were treated for 16hrs with taxanes were visualized with DAPI staining (cyan) on a confocal microscope (40x), demonstrating defective mitosis and pyknotic nuclei (yellow and orange arrows). B. F-Actin stained with Alexa Fluor 488 labeled phalloidin shows changes in cytoplasmic volume and cell morphology, as induced by taxanes after 16hrs compared to control. The pink arrows indicate multinucleate cells, which are quantified in the whisker plots in (C). C. Quantification of mitotic figures, pyknotic cells and multinucleate cells after CBTX treatment in at least quadruplicate shows significant elevation of multinucleate LNCaP and C4-2 cells after 16h (p=0.0055 and p=0.0042, respectively) and 48h (p<0.0001 in both cell lines), and pyknotic cells after 48h (p=0.0107; p=0.0096). C4-2 cells show a modest, however non-significant increase in mitotic figures after 16h.
Figure 3. The effects of taxanes are independent of altered AR localization
A. Immunofluorescence after 16h 1nM CBTX, DCTX or CTRL treatment demonstrates nuclear localization of AR (red) is unaffected in presence of steroids. DAPI staining (cyan) denotes cell nuclei. B. In androgen deprivation, AR resides in the cytoplasm of LNCaP cells, but remains nuclear in C4-2 CRPC cells (steroid depleted), even after 16h cabazitaxel exposure.
Figure 4. Cabazitaxel exerts enhanced anti-tumor effects in human tumors
A. Schematic overview of the \textit{ex vivo} explant assay. Fresh tissue obtained from radical prostatectomy is subdivided and cultured for 6 days on dental sponges submerged in cell culture media with different treatments (CTRL, 50nM CBTX or DCTX), after which tissue is formalin-fixed, paraffin-embedded, and analyzed by standardized immunohistochemistry (IHC) methods. B. H&E and IHC on explant tissues demonstrate that taxane treatment reduces tumor cell proliferation (Ki67) and induces cell death, (Caspase-3). C. Quantification of Ki67 (top) in three independent explant assays, performed on tissue from three patients, shows reduced proliferation in cabazitaxel treated samples compared to control (p<0.05 by Kruskal-Wallis non-parametric multiple comparison analysis). Cabazitaxel demonstrates improved drug efficacy compared to docetaxel \textit{ex vivo}. Quantification of Caspase-3 in the three explant assays (bottom) indicates a moderate, yet non-significant increase in cell death, as induced by taxane treatment. D. AR (IHC) remains nuclear in tumor specimens after 6 days of taxane treatment.
Figure 5. Tumors that progress to CRPC by RB loss show hypersensitivity to cabazitaxel

A. RB knockdown LNCaP cells (shRB1) show a modest sensitization in vitro to 48h cabazitaxel and 96h in fresh media (no CBTX) in culture conditions mimicking androgen deprivation therapy (ADT), but not in presence of hormones (in serum), as measured cell viability in a trypan blue exclusion assay. B. Treatment schematic for nude athymic mice, subcutaneously injected with LNCaP shRB1 or LNCaP shCon1 cells to obtain xenograft tumors in vivo (n=5 per group). Growth analysis of xenograft tumors was monitored over time of CBTX treatment (start t=0). The graph on the right denotes the sizes for each tumor at t=14 relative to the size at start of treatment (t=0), showing that only LNCaP shRB1 tumors respond to CBTX (p=0.014). C. RB proficient LNCaP xenograft tumors display
decreased Ki67 staining (unpaired t-test: \( p<0.0001 \)), and elevated numbers of mitotic figures after CBTX treatment. AR remains nuclear after CBTX exposure, irrespective of RB status.

D. Quantification of mitotic figures (top) and Ki67 positive cells (bottom) in 10 High Power Fields (HPF, 400x) per tumor show elevated % of mitotic figures in shCon1 tumors, and a reduction in proliferating cells in shRB1 tumors.
Figure 6. Expression profiling reveals novel action(s) of cabazitaxel in ADT-sensitive and CRPC prostate cancer cells

A. Venn diagrams of two microarray analyses of p<0.05 after background filtering reveal differential response of castrate-sensitive LNCaP cells and castrate-resistant C4-2 cells to 16hrs 1nM CBTX compared to DCTX, dependent on culture conditions: steroid rich (+) versus depleted (-) media, in triplicate. B. David Bioinformatics GO analyses with p<0.05 (p-values in table) reveal common and distinct pathways for the two taxanes, dependent on cell context and steroid conditions. C. Top 25 significant hits of CBTX unique in androgen deprived (blue) or steroid replete (yellow) conditions are displayed for each cell line, with the corresponding mean fold change over control treated samples. Asterisks highlight common genes between LNCaP and C4-2 cells.