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 castration-resistant prostate cancer (CRPC), there is an urgent need for more effective therapies and approaches that individualize specific treatments for patients with CRPC. These 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 and docetaxel were compared via in vitro modeling to determine the molecular mechanism, biochemical and cell biologic 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) cabazitaxel exerts stronger cytostatic and cytotoxic response compared with docetaxel, especially in CRPC; (ii) cabazitaxel 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 cabazitaxel; and (v) tumors that have progressed to castration resistance via loss of RB show enhanced sensitivity to cabazitaxel.

Conclusions: Cabazitaxel not only induces improved cytostatic and cytocidal effects, but also affects distinct molecular pathways, compared with docetaxel, which could underlie its efficacy after docetaxel treatment has failed in patients with CRPC. 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 to taxanes, which could benefit up to 50% of CRPC cases. Clin Cancer Res; 21(4); 795–807. © 2015 AACR.

Introduction

Prostatic adenocarcinoma is the most frequently diagnosed noncutaneous malignancy among 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 prostate cancer (CRPC) develops within a median of 2 to 3 years after initiating primary androgen ablation therapy (4).

Prostatic adenocarcinoma generally responds poorly to standard cytotoxic regimens, and modest clinical benefit has been achieved with the chemotherapeutic docetaxel in metastatic CRPC (mCRPC; ref. 5). Since 2010, several novel therapeutic agents, with distinct mechanisms of action, have been approved for treatment of CRPC. These include: (i) the antiandrogen 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). Although 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 patients with mCRPC of 48 months in a SWOG III trial (14), and 15 to 18 months post-docetaxel (15). The limited therapeutic success emphasizes the need for more efficacious drugs and a patient-tailored approach toward 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). Although cabazitaxel and docetaxel both target β-tubulin, the TROPIC clinical trial (NCT00417079) in patients with mCRPC 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
Translational Relevance

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 affect overall survival in this stage of disease, and although the underlying mechanisms are unknown, the most effective chemotherapeutics are taxanes. Furthermore, the next-generation taxane cabazitaxel is effective in patients in whom docetaxel has failed, but the molecular basis for this has not been discerned. Via modeling in vitro, in vivo, and in human tumor explants, studies herein identify distinct molecular signatures of the taxanes, revealing novel molecular functions of cabazitaxel associated with enhanced efficacy in CRPC. Furthermore, loss of the RB tumor suppressor 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.

administering cabazitaxel, that is, 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.

These 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 used. Although 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, because 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 prostatic adenocarcinoma 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 revealed unique functions for cabazitaxel in CRPC, and identify the first potential biomarker for selecting patients who might most benefit from early chemotherapy.

Flow cytometry

Prostatic adenocarcinoma cells were treated in triplicate with a taxane for 16 or 48 hours, or vehicle, adherent and nonadherent cells were combined, gently resuspended in 100% ethanol, and fixed overnight at –20°C. Proliferation was measured by bivariate flow cytometry using a 2-hour pulse-label of bromodeoxyuridine (BrdUrd; Amersham, RPN201) before harvest and cell-cycle position by propidium-iodide (PI) staining (22). A BD Biosciences FACSCaliber was used to capture 10,000 BrdUrd/PI events. FlowJo software (TreeStar) was used to gate for the percentage of BrdUrd incorporation or cell cycle.

Immunoblotting

Control and taxane-treated cells were harvested to evaluate cleaved PARP-1 (Cell Signaling Technology) levels. Total protein was extracted by sonication in RIPA buffer, separated by SDS-PAGE, transferred to polyvinylidene difluoride, and immunoblotted overnight at 4°C. Immunoblots were quantified using a Bio-Rad 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 Biotechnology 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; ×40 objective, ×2 digital zoom). Quantification of phenotypes was done on a fluorescent microscope (Leica DMi3000B, ×20 objective) on at least four coverslips per condition, counting 200 cells per replicate, calculating the percentage of cells displaying specific morphologies.

Human prostate tumor explants

Fresh primary tumor tissue was obtained from patients with prostatic adenocarcinoma 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 deidentified 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), 30 μmol/L hydrocortisone (Sigma; H-0888), and penicillin/streptomycin]. Tissue was subdivided into approximately 1-mm³ pieces and placed (2–3 pieces/well) in a 24-well plate on presoaked 1-cm³ dental sponges (Novatis; Vetshop) submerged in 0.5 mL culture media in presence or absence of drugs (control, 50 nmol/L cabazitaxel or docetaxel). Treatments were refreshed every 48 hours, and explants were harvested after 6 days, formalin-fixed, paraffin-embedded, and analyzed by standardized IHC methods. Control- and taxane-treated explants (n = 3 patients) with glandular epithelial tissue, as determined by a certified clinical pathologist, were stained for Ki67 (Invitrogen; 180191Z) or AR (Santa Cruz Biotechnology;
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 x 10^6) were combined 1:1 with Matrigel (BD Biosciences, 354234) and injected s.c. into the flanks of 6-weeks-old, intact-male athymic nude mice (NCI-Frederick, Frederick, MD). Mice were castrated when tumors reached 100 to 150mm^3. After a 1-week recovery, the mice were treated 2× per week with 8 mg/kg cabazitaxel by i.p. injections, and tumor volumes were monitored with calipers 3× per week. Tumors were harvested and processed after 3 weeks of treatment, or when the tumor exceeded 800mm^3. Hematoxylin and eosin (H&E) stained sections of shCon1 and shRB1 LNCaP tumors (n = 6 tumors/group) were evaluated using a Motic BA400 microscope (Motic). For each tumor section, the number of mitotic figures in 10 randomly selected ×400 (i.e., high power) magnification fields was determined by a board certified veterinary anatomic pathologist (L.D. Berman-Booty). Additional tumor sections were stained for Ki67 (1:250; Invitrogen) and AR (Santa Cruz Biotechnology; N20).

Immunohistochemistry quantification

Sections of shCon1 and shRB1 LNCaP xenograft tumors (n = 6 tumors/group) and 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 ×400 (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 cells 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 16 hours with 1 nmol/L cabazitaxel, docetaxel, or vehicle (EtOH). The expression data were RMA normalized, and filtered to remove low-expressing genes. Differential gene expression with corresponding P values (Student t test) was determined of drug treated over control. Gene lists with P < 0.05 were compared on the basis of the corresponding AffymetrixIDs, and plotted in a Venn diagram (http://bioinfoogg.cnb.csic.es/tools/venny/). Gene ontology (GO) analyses were performed on selected clusters by separating up- and downregulated genes (http://david.abcc.ncifcrf.gov/), and the resulting GO terms were cut-off at P < 0.05.

Statistical analyses

To determine statistical significance, P values were calculated by a standard unpaired Student t test, unless otherwise noted. Significant effects: *, P < 0.05; **, P < 0.01; and ***, P < 0.001.

Results

Cabazitaxel shows enhanced antiproliferative and procytotoxic effects in CRPC

To assess the relative impact of cabazitaxel and docetaxel on prostatic adenocarcinoma 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 (0.1–1 nmol/L) for 48 hours, followed by a washout of 48 hours in the absence of drug, and subsequent quantification of cell viability through cell counting. As shown (Fig. 1A), cabazitaxel and docetaxel showed relatively equivalent effects in hormone therapy–sensitive cells (left), with IC_{50} values of 0.220 nmol/L for cabazitaxel, and 0.319 nmol/L for docetaxel. However, cabazitaxel (IC_{50} = 0.142 nmol/L) resulted in markedly enhanced antitumor effects compared with docetaxel (IC_{50} = 0.269 nmol/L) 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 BrdUrd) revealed reduced S-phase entry after 16 and 48 hours of cabazitaxel treatment in both cell types, although less pronounced in CRPC cells (Fig. 1B, top, quantified in Fig. 1C). Docetaxel (Supplementary Fig. S1 and Fig. 1C) had a similar effect on proliferation of hormone therapy–sensitive cells, but did not affect S-phase entry of cells from the same lineage that have achieved castration resistance. Thus, cabazitaxel demonstrates an enhanced antiproliferative effect in CRPC cells compared with docetaxel. This enhanced effect was reiterated in ADT-sensitive (LAPC4, VCaP) and CRPC (LCaP-abl) cell models (Supplementary Fig. S2A). In vivo, cabazitaxel successfully induced C4-2 xenograft tumor remission, thereby demonstrating effectiveness of cabazitaxel in a CRPC model (Supplementary Fig. S2B).

In addition to the observed effects on cell cycle, the sub-2N content was enhanced in CRPC cells treated with cabazitaxel compared with hormone therapy–sensitive models, eliciting almost double the effect of docetaxel after 48 hours in C4-2 cells (Fig. 1B, bottom; and Fig. 1D). Moreover, cabazitaxel effectively induced apoptosis in C4-2 cells, as measured by PARP cleavage (Supplementary Fig. S3). Together, these data suggest that cabazitaxel shows enhanced antiproliferative and procytotoxic effects as compared with docetaxel.

Cabazitaxel promotes defective mitosis

To address the means by which cabazitaxel exhibits enhanced antitumor effects, the impact on nuclear integrity was assessed. Cells were fixed after 16 hours of exposure, and nuclear features assessed after DAPI staining (Fig. 2A). Visualization on a confocal microscope demonstrated a modest enrichment of mitotic figures after 16 hours (quantified in Fig. 2C, left), but evidence of defective mitoses was apparent in the asymmetric appearance of segregating nuclei. Pyknotic nuclei were also observed (Fig. 2A), as quantified in Fig. 2C (right), demonstrating a significant increase after 48 hours cabazitaxel exposure, further supporting the contention that cabazitaxel 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 (Fig. 2B), and allowed for clear definition of...
Figure 1.
Cabazitaxel shows enhanced antitumor effects in CRPC. A, dose-dependent response to 48 hours cabazitaxel (CBTX) and docetaxel (DCTX) treatment and 48 hours 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 1 nmol/L cabazitaxel or control for 16 or 48 hours. In the top graphs, the x-axis represents relative DNA content as indicated by PI staining; the y-axis shows cells undergoing active S-phase as indicated by 2 hours BrdUrd labeling. Inset values, the percentage of BrdUrd incorporation in viable cells (mean ± SD, from an experiment performed in biologic triplicate). The bottom graphs represent the corresponding PI traces only, showing a G2–M arrest, followed by cell death after cabazitaxel exposure. C, quantification of the percentage of BrdUrd+ cells in B and Supplementary Fig. S1. Significant reduction is observed in taxane over control (LNCaP, P < 0.05; C4-2, P < 0.0005 for all conditions). Cabazitaxel appears to have a mild effect on proliferation of C4-2, although not significant, whereas docetaxel does not. D, taxanes induce significant cell death after 48 hours of cabazitaxel or docetaxel 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 cabazitaxel and docetaxel at the same treatment duration, showing no significance in LNCaP, but improved efficacy for cabazitaxel in C4-2 cells (16 hours, P = 0.0042; 48 hours, P = 0.0092).

cell borders, revealing enhanced presence of multinucleate cells in CRPC cells treated with cabazitaxel, likely resulting from aberrant mitosis. Multinucleate LNCaP and C4-2 cells were quantified in at least four duplicates for cabazitaxel versus control-treated samples, which demonstrated a highly significant increase in polynuclear cells after 16 and 48 hours (Fig. 2C right). Together, these data support the hypothesis that cabazitaxel 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, supraclinical doses (µmol/L) levels of...
Taxanes were used, 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 (Fig. 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. 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 (Fig. 3B). Cabazitaxel or docetaxel had no impact on AR localization at a nanomolar level (1 nmol/L) in either cell line. Taken together, the enhanced cytotoxic and cytostatic effects of cabazitaxel appear to be independent of AR subcellular regulation.

**Cabazitaxel exerts enhanced antitumor effects in human tumors**

Given desirable cytostatic and cytotoxic effects of cabazitaxel in model systems, the antitumor 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; Fig. 4A; refs. 22, 26). 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 H&E staining confirmed retention of the tumor microenvironment (Fig. 4B left). IHC to assess the proliferative indices (using an antibody to Ki67) was performed in parallel, of which representative examples are shown in Fig. 4B (middle), and quantified (Fig. 4C, top). Strikingly, this direct comparison in fresh tumor explants confirmed that cabazitaxel exerts a markedly enhanced cytostatic response compared with docetaxel. Caspase-3 staining revealed an increasing trend, however nonsignificant, likely due to the limited sample number (n = 3; Fig. 4C right). Finally, assessment of AR localization in response to 50 nmol/L drug treatment revealed retention of nuclear AR in the tumor cells (Fig. 4D). Thus, similar to what was observed in vitro, the antitumor effects of nanomolar level cabazitaxel appear to occur independently of altered AR localization. Collectively, these data identify an enhanced capacity of cabazitaxel to elicit antiproliferative and proapoptotic events in primary human tumors.

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

Although the above studies suggest that cabazitaxel 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 RB tumor-suppressor protein 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; ref. 21), were initially assessed in vitro for differential response to cabazitaxel. In these studies, shRB1 cells demonstrated a modest sensitization to cabazitaxel as compared with shCon1 (Fig. 5A). To challenge this in vivo, cells were s.c. injected into male athymic nude mice; when the xenograft tumors (n = 5/group) reached a size of 100 to 150mm3, the mice were surgically castrated, allowing a week of recovery before starting 8 mg/kg cabazitaxel treatment by i.p. injections twice a week (Fig. 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 (Fig. 5B left). Normalization of individual tumor volumes at 14 days after start of treatment compared with their respective volumes at \( t = 0 \) shows a significant difference in cabazitaxel response between the shCon1 tumors (\( n = 4 \)) and the shRB1 tumors (\( n = 5 \); Fig. 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 mm³, despite cabazitaxel administration. These in vivo findings robustly support the concept that RB-deficient tumors are hypersensitive to treatment with cabazitaxel.

To further probe the underlying basis of the observed enhanced effect in RB-deficient tumors, histopathologic 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...
Figure 5.
Tumors that progress to CRPC by RB loss show hypersensitivity to cabazitaxel (CBTX). A, RB knockdown LNCaP cells (shRB1) show a modest sensitization in vitro to 48 hours cabazitaxel and 96 hours in fresh media (no cabazitaxel) in culture conditions mimicking ADT, but not in presence of hormones (in serum), as measured by cell viability in a trypan blue exclusion assay. B, treatment schematic for nude athymic mice s.c. injected with LNCaP shRB1 or LNCaP shCon1 cells to obtain xenograft tumors in vivo (n = 5/group). Growth analysis of xenograft tumors was monitored over time of cabazitaxel 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 cabazitaxel (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 cabazitaxel treatment. AR remains nuclear after cabazitaxel exposure, irrespective of RB status. D, quantification of mitotic figures (top) and Ki67-positive cells (bottom) in 10 high power fields (HPF, ×400) per tumor show the elevated percentage of mitotic figures in shCon1 tumors, and a reduction in proliferating cells in shRB1 tumors. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
cabazitaxel treatment. By contrast, shRB1 tumors elicited fewer mitotic figures (Fig. 5C, quantified in Fig. 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 (Fig. 5D, bottom). Taken together, the in vivo data suggest that RB-deficient tumors are hypersensitive to cabazitaxel, 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**

Although the above studies identify novel antitumor effects of cabazitaxel 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 cabazitaxel function. Hormone therapy–sensitive LNCaP, and castration-resistant C4-2 cells were treated for 16 hours with cabazitaxel or docetaxel, 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 (cabazitaxel or docetaxel) were normalized to the corresponding values for control-treated samples either with or without steroids. Differentially expressed genes were selected by a Student t test (P < 0.05), and compared between treatments, as visualized by the two Venn diagrams representing the two cell models (Fig. 6A). Complete gene lists and fold alterations for each gene cluster are provided in Supplementary Table S1 (LNCaP) and Supplementary Table S2 (C4-2). Notably, distinct gene-expression outcomes were readily apparent between the two taxanes. Furthermore, 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 on the basis of the data above, that cabazitaxel exerts differential effects compared with docetaxel, and that the proliferative status of the tumor cell can alter downstream biologic effects.

To gain deeper understanding of the cellular response to cabazitaxel and docetaxel, GO analyses were performed on both castrate (in blue and red, respectively, corresponding to the Venn clusters in Fig. 6A) and steroid replete (yellow and green) gene lists (Supplementary Fig. S4, LNCaP and Supplementary Fig. S5, C4-2). For complete gene lists per GO term, see in Supplementary Table S3. These studies revealed enrichment of genes involved in cell cycle and chromosomal organization and regulation after cabazitaxel treatment exclusively in C4-2 cells (Fig. 6B), consistent with the enhanced antiproliferative effect of this agent in CRPC cells (Supplementary Fig. S3). In the ADF-sensitive LNCaP cells under androgen-deprived conditions, cabazitaxel and docetaxel share enrichment in gene transcription–related pathways. However, these appear to be negatively regulated by cabazitaxel, versus a positive impact by docetaxel, 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. Docetaxel and cabazitaxel may affect similar pathways, nonetheless, cabazitaxel has a stronger effect on cell-cycle and chromatin regulation, whereas docetaxel appears to have a more significant impact on transcription and repair. These differences could be caused by differences in microtubule stabilization. Although 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 downregulated) by cabazitaxel in absence or presence of steroids are displayed in tables (Fig. 6C). Genes that are common between the two cell models are marked with an asterisk. Classical AR targets (KLK3, TMPRSS2, FKBP5, and KLK2) are not affected by cabazitaxel or docetaxel, 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 cabazitaxel (or docetaxel) will likely be of relevance for designing combination therapies with AR pathway–targeting drugs, such as enzalutamide and abiraterone acetate. These unbiased analyses further illustrate the divergent effects of cabazitaxel and docetaxel, and provide a gene “signature” of response to cabazitaxel 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 (OS). One of the few options available for advanced disease is taxane-based chemotherapy, and the new-generation drug cabazitaxel has been shown to exert antitumor effects even after docetaxel has failed (11). This study presents first-in-field distinctions between these two agents, using clinically relevant conditions. Key findings are: (i) cabazitaxel induces improved cytostatic and cytotoxic response, especially in CRPC; (ii) cabazitaxel 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 cabazitaxel are identified by gene-expression profiling; and (v) tumors that have progressed to castration resistance via loss of RB show enhanced sensitivity to cabazitaxel.

These studies demonstrate that the cellular consequences of cabazitaxel are distinct from that of docetaxel 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 cabazitaxel. Cabazitaxel 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, cabazitaxel is...
Specific GOterm C4-2*

DNA repair

DNA-dependent DNA replication 0.014 0.013

Positive regulation of transcription factor activity 0.011

Transcription factor activity 0.011

Regulation of transcription 0.024

Positive regulation of DNA binding 0.046

Protein-DNA complex assembly 0.016

DNA replication

DNA replication 0.025 0.001

DNA-dependent DNA replication 0.054 0.013

DNA repair

Death

Induction of apoptosis by extracellular signals 0.007

B GO analysis comparison

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C GO pathways LNCaP C4-2

- GO pathway LNCaP
- GO pathway C4-2

*common genes
Impact of Cabazitaxel on CRPC

effective in patients who have failed docetaxel, which suggests that cabazitaxel could prolong OS when used as a first-line chemo-

therapeutic (13). In this study, cabazitaxel elicits stronger responses in CRPC models, as opposed to ADT-sensitive cells, and distinctly shows an improved response window versus doc-
etaxel. Moreover, metastatic disease may respond better to taxanes upon first diagnosis than after exposure to other treatment regi-

ments, 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 prostatic adenocarcinoma models, it has been previously suggested that taxanes may inhibit AR translocation to the nucleus and activity, and thus contribute to antitumor efficacy (18–20). However, these studies were performed in suprapharmacologic concentrations of taxane (50 nmol/L–1 μmol/L), whereas the IC50 values described for this class of chemotherapeutics are in the single nanomolar range (24, 25). AR and downstream targets are not affected by cabazitaxel at the nanomolar level, as demonstrated by the gene-expression analysis. Taken together, the studies herein provide evidence that taxanes do not affect AR localization and activity at the low nanomolar levels in vitro, or clinically relevant concentrations in vivo and ex vivo, whereas cytostatic and cytotoxic effects are observed. Although it is probable that at higher (μmol/L) levels AR transport is impaired, and thus its activity, this is likely a bystander effect of the disruption of the cellular highway affecting general cellular trafficking that depends on the microtubules.

In addition to demonstrating the commonalities between cabazitaxel and docetaxel actions, gene-expression analyses reveal a molecular basis for the divergent effects that cabazitaxel exerts as compared with docetaxel, 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 docetaxel in a previous genome wide study is cell cycle (30), nonetheless, cabazitaxel appears to have an elevated effect on cell cycle and mitosis as evidenced from related GO terms in the cabazitaxel-distinct gene clusters in the study pre-

sented here. Moreover, chromatin organization is exquisitely affected by cabazitaxel in CRPC cells, likely underlining the stron-
ger response to the novel therapeutic, as well as its effectiveness in patients who have progressed on docetaxel. Docetaxel seems more enriched for transcription pathways, as previously reported (11 and colleagues; ref. 30). It has been speculated that docetaxel resistance can occur via βIII-tubulin overexpression or mutation, or by increased P-glycoprotein (13, 31, 32). Improved response to cabazitaxel 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 cabazitaxel levels in the brain in vivo (33, 34). In sum, cabazitaxel has an elevated impact on cell-cycle pathways and chromatin organization compared with docetaxel, resulting in stronger cytostatic and cytotoxic effects, and does not act via AR. Of note, the molecular impact of cabazitaxel depends on the hormone conditions, which empha-
sizes 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, cabazitaxel utilization is approved only in patients with pro-
gressive disease after docetaxel 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 cabazitaxel 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 prostatic adenocar-
cinoma is a leading cause of death in the United States and Europe, and currently few therapeutic options are available for CRPC, it is imperative to improve treatment. Loss of RB func-
tionality 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 patients with CRPC, 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 resis-
tance (35), and dysregulation of the RB pathway has been described to cause genomic instability and aneuploidy (36, 37), suggesting that RB loss would induce cabazitaxel resis-
tance, 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 (38). Other potential biomarkers for taxane sensitivity reported across different tumor types are BRCA1, negative Bcl2, negative SIRT2 protein (39–42). 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 toward patient-tailored treatment decisions and improved care of CRPC.

Overall, the data presented here support that cabazitaxel would improve therapeutic response compared with docetaxel, likely as applied to the docetaxel space of CRPC. This hypothesis is currently being tested in a phase III clinical trials [FIRSTIANA

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 16 hours 1 nmol/L cabazitaxel (CBTX) compared with docetaxel (DCTX), dependent on culture conditions: steroid-rich (+) versus -depleted (−) media, in triplicate. B, David Bioinformatic 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 cabazitaxel 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, common genes between LNCaP and C4-2 cells.

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NCT01308567]. Moreover, a recent report of an interim analysis on another trial in ADT-sensitive metastatic disease (CHARITED NCT00309985) shows a striking benefit from addition of docetaxel to standard ADT, underscoring the importance of testing chemotherapeutics in earlier disease states. The gene-expression data in these 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-naive patients presenting with mCRPC to retrospectively assess the potential impact of RB as a biomarker for cabazitaxel sensitivity (ABICABAZI NCT02218606), which will compare the effects of abiraterone alone versus abiraterone in combination with cabazitaxel. In summary, this article and recent clinical developments underpin that redesigning the optimal clinical space and approach for chemotherapeutics would likely improve OS 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 cabazitaxel 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 cabazitaxel therapy. Combined, these studies provide the basis for improving efficacy of taxane-based therapy in prostate cancer.

Disclosure of Potential Conflicts of Interest

A.P. Dicker reports receiving speakers bureau honoraria from Bayer and is a consultant/advisory board member for Merck, Merck EMD, and Vertex. W.K. Kelly is a consultant/advisory board member for Sanofi-Aventis. K.E. Knudsen is a consultant/advisory board member for and reports receiving commercial research support from Sanoﬁ. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. de Leeuw, L.D. Berman-Booasy, M.J. Schiewer, R.B. Den, A.P. Dicker, E.J. Trabulsi, K.E. Knudsen

Writing, review, and/or revision of the manuscript: R. de Leeuw, L.D. Berman-Booasy, M.J. Schiewer, R.B. Den, A.P. Dicker, W.K. Kelly, E.J. Trabulsi, C.D. Lallas, L.G. Gomella, K.E. Knudsen

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. de Leeuw, A.P. Dicker, L.G. Gomella, K.E. Knudsen

Study supervision: W.K. Kelly, C.D. Lallas, K.E. Knudsen

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