Preclinical and Clinical Aspects of Taxane Responsiveness and Sensitivity in Castration Resistant Prostate Cancer

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Preclinical and Clinical Aspects of Taxane Responsiveness and Sensitivity in Castration Resistant Prostate Cancer

Preklinische en klinische aspecten van taxaan gevoeligheid in castratie-resistent prostaatcarcinoom

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Contents

1

General introduction

Taxane-based chemotherapy in mCRPC

Approximately 10-25% of men diagnosed with prostate cancer will present with or develop metastatic disease, that eventually confers in metastatic castration resistant prostate cancer (mCRPC) (1, 2). In contrast with localized disease that can be treated with radiotherapy, prostatectomy or watchful waiting, metastatic disease cannot be cured. In the past decade several treatments have become available that extend overall survival in mCRPC patients. Docetaxel plus prednisone became available in 2004 as first line of chemotherapy for mCRPC, following the results of the pivotal TAX327 and SWOG 99-16 studies in which docetaxel significantly improved survival and quality of life as compared with mitoxantrone (3, 4). Clinical response to first line treatment with docetaxel shows a large inter-patient variability and early resistance as well as late resistance is common. Cabazitaxel, a second generation taxane with proven activity in chemotherapy-naïve and docetaxel-resistant preclinical models (5), was approved by the FDA as a second line of chemotherapeutic treatment of docetaxel-progressive mCRPC patients, based on the phase 3 TROPIC study that showed survival benefit when compared with mitoxantrone in patients previously treated with docetaxel (6).

Docetaxel and cabazitaxel belong to the group of taxanes and function by inhibiting depolarization of the microtubules, thereby blocking microtubule dynamics, which leads to cell cycle arrest in the G2/M phase and eventually tumor cell death (7, 8). In addition to taxanes, bone-targeting radiotherapy Radium-223 (9), and AR targeting drugs such as abiraterone and enzalutamide (10-13), have come available for mCRPC patients. With these multiple new treatment options, potential interactions between treatments has increased. This has already been signified by the reduction in efficacy of docetaxel in some patients after treatment with novel AR targeted agents, while this reduction in efficacy is not seen in patients treated with cabazitaxel (14-16). In order to optimize treatment of individual patients, biomarkers are needed that will select patients who will have poor response to docetaxel, but will remain sensitive to cabazitaxel. To contribute to this aim, more insight into the development of docetaxel resistance and factors that influence taxane response is needed.

This thesis focuses on the prediction of docetaxel resistance and cabazitaxel sensitivity and the identification of potential biomarkers. We aimed to investigate clinically observed inter-individual variability in docetaxel and specific pathways underlying docetaxel resistance with the use of patient-derived prostate cancer xenografts and presented a candidate biomarker of taxane response in prostate cancer.

Chapter 2 describes underlying causes for the large inter-individual variability in relation with the pharmacokinetics (PK) of docetaxel. PK involves the absorption, distribution, metabolism and elimination of a drug in the body. Drug-drug interactions, for example on the level of induction or inhibition of docetaxel clearance, could lead to variable systemic

concentrations of docetaxel. As docetaxel has a small therapeutic window, it is of high importance to select patients who will likely respond to the therapy with manageable side effects. In this thesis, we describe the significant influence of patient factors such as gender, hormonal status (castration) and interactions with therapies that change the clearance of docetaxel via drug-drug interactions on the level of CYP3A4.

Besides PK influences on docetaxel response, potentially also the total number of docetaxel cycles may contribute. Thus far, the optimal total number of docetaxel cycles has not been investigated yet in mCRPC patients. The standard treatment regimen of docetaxel has been set on 10 cycles every 3 weeks, based on the registration trial TAX 327 (4). Ten cycles was however arbitrarily chosen, and docetaxel treatment is frequently halted at 6 cycles in clinical practice (17). Reasons for this may be either for convenience, or to avoid cumulative toxicity in the light of subsequent treatment with other available treatments.

Insight into the optimal total number of docetaxel would facilitate decision making when to stop treatment. In the Mainsail study, a phase 3 study, the combination of docetaxel, prednisone and lenalidomide (DPL) versus docetaxel, prednisone and placebo (DPL) was studied (18). Overall survival was significantly worse in the DPL versus the DP arm. As a result of increased toxicity with the combination treatment of docetaxel and lenalidomide, the DPL group received less treatment cycles of docetaxel (median of n=6), compared to the DP arm (median of n=8). In **chapter 3** we investigate whether the number of docetaxel cycles and the cumulative dose is an independent predictor on overall survival in mCRPC patients, using a posthoc analysis of the Mainsail study.

Tumor-specific characteristics may further determine the individual patient response to docetaxel treatment. Tumors can be intrinsically resistant to docetaxel or may acquire resistance during treatment. Mechanisms of docetaxel-resistance are probably multifactorial, and may include adaptions to the docetaxel-specific target of action, which is tubulin. Class III beta-tubulin overexpression in prostate cancer samples has shown decreased docetaxel response and survival (19). In **chapter 4,** the protein expression of Eg5 a kinesin spindle protein, that cross-links microtubules during cell division and which may be linked to the working mechanism of docetaxel, was tested for its potential as biomarker of docetaxel response. In this chapter we describe the role of Eg5 as a predictive marker of docetaxel response and a marker for tumor aggressiveness.

Pretreatment with other drugs such as novel AR-targeted agents may also influence the response to docetaxel in tumors by interacting on a molecular level. Cross-resistance of docetaxel with novel AR targeted agents such as abiraterone and enzalutamide has been reported to cause reduced efficacy of docetaxel (20-24). We previously identified a

mechanism of cross-resistance through their shared mechanism on androgen signaling: both taxanes and AR targeted agents block AR nuclear translocation via microtubules (21). Interestingly, a difference seems to exist between docetaxel and cabazitaxel. In **chapter 5** we identify enzalutamide-resistant tumors with decreased efficacy for docetaxel, but not cabazitaxel and further elaborate on a potential mechanism via the AR pathway.

Besides molecular pathways such as Eg5 expression and interaction on the level of the AR, drug concentrations in tumors are crucial, as the efficacy of chemotherapy is determined by the actual drug concentrations that can be achieved and maintained in tumor tissue (25). In non-small lung cancer patients uptake and accumulation of [C^{11}]-docetaxel was related to response: a high tumor uptake of [C^{11}]-docetaxel corresponded with improved tumor response in patients (26). Also, intratumoral retention of paclitaxel was related to progression-free survival and overall survival in gynecological cancers such as cervical, endometrial and ovarian carcinoma (27). These studies indicate that intratumoral taxane concentrations and its retention are directly linked to drug efficacy and suggest that drug transporters may play an important role in therapy response for taxanes. In **chapter 6** we investigate the relation between intratumoral concentrations of taxanes and efficacy in newly established docetaxel-resistant PDX models of mCRPC.

As intratumoral concentration profiles of docetaxel and cabazitaxel were different between chemo-naïve and docetaxel-resistant PDX models, we hypothesized that drug transporters play a role in the uptake and or efflux of these taxanes. Several members of the two major drug transporter families, the Solute Carriers (SLCs) and the adenosine triphosphate binding cassette (ABC) transporters, have been previously linked to docetaxel transport and/or resistance, such as, ABCB1, ABCC4, ABCC10 and SLCO1B1, SLCO1B3 and SLCO1A2 (28-32). In **chapter 7** we describe our search for drug transporters that relate to taxane sensitivity in our newly developed docetaxel resistant PDX models with the use of next generation sequencing. From this study downregulation of the Solute Carrier Organic Anion-transporting polypeptide 1B3 (SLCO1B3 / OATP1B3) was identified to strongly reduced intratumoral concentrations and hence the development of docetaxel resistance. In this chapter, the role of SLCO1B3 in taxane resistance is further investigated.

The finding of SLCO1B3 as a potential candidate biomarker of response is highly interesting and very wanted to select patients who may benefit from treatment with taxanes. In order to use this biomarker in clinical practice, validation in patients will be needed. In **chapter 8** the clinical and preclinical aspects of taxane responsiveness described here, are discussed. Especially our approach to develop a preclinical candidate biomarker is discussed and interpretation is given with focus on the translation of these data towards a potential biomarker predicting docetaxel resistance in patients.

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21

12

Inter-patient variability in docetaxel pharmacokinetics: a review

Abstract

Docetaxel is a frequently used chemotherapeutic agent in the treatment of solid cancers. Because of the large inter-individual variability (IIV) in the pharmacokinetics (PK) of docetaxel, it is challenging to determine the optimal dose in individual patients in order to achieve optimal efficacy and acceptable toxicity. Despite the established correlation between systemic docetaxel exposure and efficacy, the precise factors influencing docetaxel PK are not yet completely understood. This review article highlights currently known factors that influence docetaxel PK, and focuses on those that are clinically relevant. For example, liver impairment should be taken into account when calculating docetaxel dosages as this may decrease docetaxel clearance. In addition, drug-drug interactions may be of distinct clinical importance when using docetaxel. Particularly, drugs strongly inhibiting CYP3A4 such as ketoconazole should not be concurrently administered without dose modification, as they may decrease the clearance of docetaxel. Gender, castration status, and menopausal status might be of importance as potential factors influencing docetaxel PK. The role of pharmacogenetics in predicting docetaxel PK is still limited, since no polymorphisms of clinical importance have yet been established.

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Introduction

Docetaxel is approved for the treatment of several solid malignancies, including non-small cell lung cancer (NSCLC), metastatic castration resistant prostate cancer (mCRPC), breast cancer and head and neck cancer (1,2). Most of these cancers typically occur in elderly people, who may have comorbidities, organ dysfunction and are using various medications. The pharmacokinetics (PK) of docetaxel are highly variable, ranging from 30-45% (3). Therefore, it is challenging to predict toxicity and antitumor activity of docetaxel in individual patients. Ideally, patients would be individually dosed to prevent toxicity and improve the efficacy of docetaxel.

It is believed that the systemic exposure to a drug like docetaxel is related to its efficacy (4). This was also shown by Bruno et al., who found that the area under the plasma-concentration time curve (AUC) of the initial course of docetaxel was a predictor of time to progression in NSCLC patients (5). Also, a decreased clearance (CL) increased the risk of grade 4 and febrile neutropenia (6). Knowledge of factors that are of importance for PK variability could therefore lead to the optimization of docetaxel therapy.

Several studies have focused on determining factors that may influence docetaxel PK, aiming for better prediction of toxicity and exposure to docetaxel (see Figure 1). This excessive sum of studies makes it difficult to extract clinically relevant findings for usage in daily clinical practice. Hence, no label changes for docetaxel dosing have been made in the last decade although the current dosing strategy using body surface area (BSA) has been criticized, as this dosing strategy does not reduce the inter-individual variability (IIV) in docetaxel PK to an absolute minimum, since it does not account for other factors influencing docetaxel PK. This review article gives a comprehensive summary on the currently available and clinically relevant factors influencing docetaxel PK that can aid in individualizing docetaxel therapy in current clinical practice.

Drug transporters involved in docetaxel pharmacology

Drug transporters and docetaxel pharmacokinetics

The activity of docetaxel-transporters could be altered due to drug-drug interactions, which potentially influences the PK of docetaxel. The largest family of drug transporters consists of passive transporters: the solute carriers (SLCs), which cover 48% of the total amount of transporters. Docetaxel is a known substrate of SLC22A7 (7), SLCO1B1 (8), SLCO1B3 (9), SLC22A7 (7) and possibly of SLCO1A2 (7,10). Besides SLCs, members of the ATP-binding cassette (ABC) transporters are extensively studied with regard to multidrug resistance and the PK of several anticancer drugs. Docetaxel is known to be transported by ABCB1 (ATP-binding cassette transporter B1, p-glycoprotein (p-gp)) [11], ABCC2 (canalicular multispecific organic anion transporter 1 (*cMOAT*), MRP2) (12) and ABCC10 (multidrug resistance-associated protein 7 (MRP7)) (13).

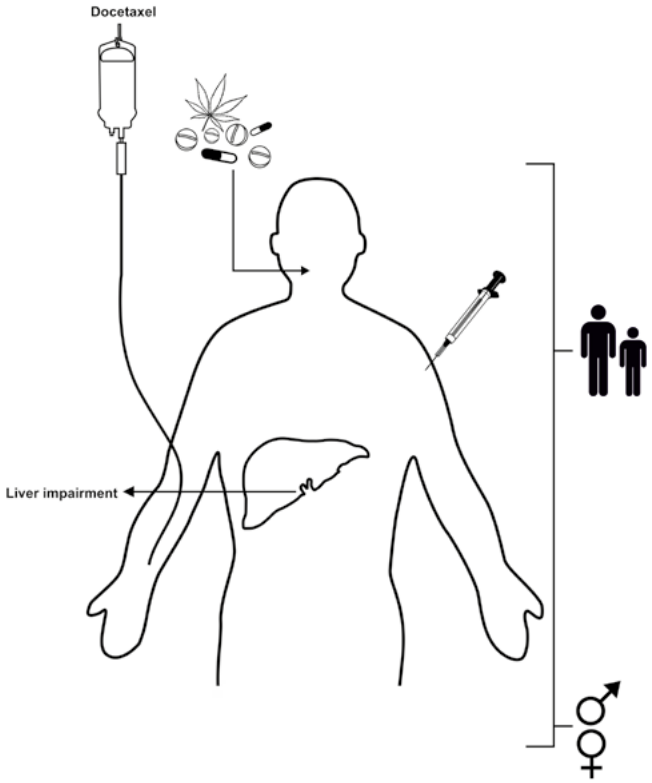

Figure 1. Factors influencing docetaxel pharmacokinetics. Co-medication and the use of complementary alternative medicines (CAMs) impact docetaxel PK in a clinically relevant way and should be taken into account when optimizing docetaxel treatment. In addition, patient related factors such as liver impairment, gender and hormonal status could potentially influence docetaxel PK.

Absorption

The gastro-intestinal absorption of docetaxel is limited. This is because ABCB1 directly excretes docetaxel into the intestinal lumen or bile (14). Moreover, docetaxel' s bioavailability is greatly reduced by the liver's first pass effect (15). Docetaxel is currently only being administered intravenously. As oral administrations of docetaxel could be more patient friendly, research is ongoing to improve the bioavailability of docetaxel (16,17).

Tissue distribution and accumulation

Over ninety percent of docetaxel is bound to plasma proteins (1). Because of its lipophilic properties, docetaxel has a large distribution volume, indicating accumulation in several tissues (1). Based on a bio-distribution study in cancer patients, a high uptake of [^{11}C]-docetaxel in the liver and gall bladder was seen, while there was fewer uptake in the small

intestines, kidney, bone marrow, lungs and bladder (18). Uptake of docetaxel in the brain was limited, resulting from an effective blood brain barrier containing efflux transporters like ABCB1 and ABCC2 (19).

Docetaxel metabolism and excretion

Hepatic uptake

Docetaxel is metabolized in the liver (Figure 2). Uptake is facilitated via uptake transporters such as Organic Anion Transporting Peptides (OATP) 1B1 and OATP1B3, which belong to the SLC family. These transporters mediate the uptake of docetaxel from sinusoidal blood into the hepatocytes (8-10). Iusuf and colleagues recently found that OATP1A2 was also involved in the *in vivo* uptake of docetaxel (10). Animal studies with the OATP1B3/OATP1B1 orthologue OATP1B2 showed that the CL of docetaxel is substantially decreased in OATP1B2 knockout mice (8, 10, 20, 21) in a manner that resembles drug phenotypes observed in mice with a deficiency of metabolic Cyp3a activity (22). Therefore, co-medication that inhibits both OATP1B1 and 1B3 should only be used with caution in combination with docetaxel. Also, we previously found that docetaxel's formulation vehicle polysorbate 80 could inhibit the uptake of docetaxel via interaction with OATP1B1 and OATP1B3 (8, 21).

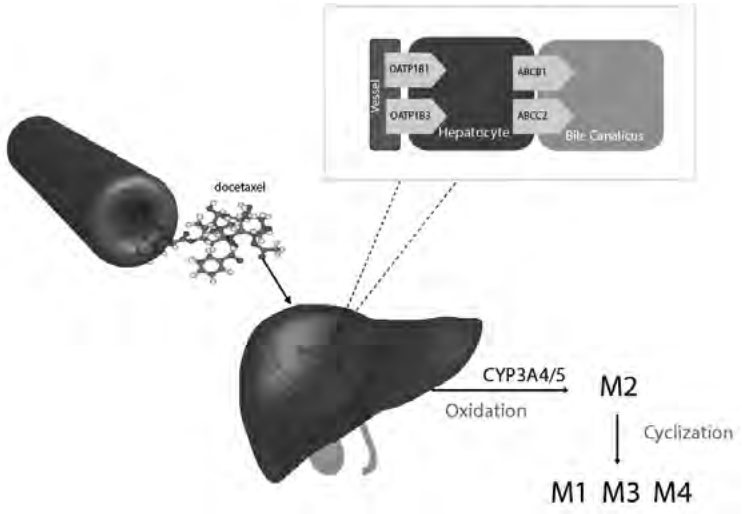

Figure 2. A schematic overview of docetaxel metabolism. Docetaxel is transported from the blood into the hepatocytes by OATP1B1 and OATP1B3. CYP3A4, and to a lesser extend CYP3A5, are responsible for the metabolism of docetaxel. ABCB1 and ABCC2 are accountable for the transport from hepatocyte into the bile canalicus.

Metabolism of docetaxel

Docetaxel is mainly metabolized via CYP3A4 and to a lesser extent by CYP3A5, and is processed into four metabolites (Figure 2) (23). A methyl group of docetaxel is oxidized into a primary alcohol forming metabolite M2. Further oxidation of M2 leads to formation of unstable metabolites of the alcohol that will lead to diastereoisomers (M1/M3) and a ketone metabolite (M4) (24). There are no indications that docetaxel-metabolites undergo phase II metabolism. All four metabolites showed limited anti-tumor activity (25), which suggests that the metabolism of docetaxel is the main contributor in the inactivation of the drug.

Excretion of docetaxel

Docetaxel and its metabolites are mainly excreted into bile via ABCB1 and ABCC2 mediated transport (11,12). Tumor cells can also express ABCB1 what will cause efflux of docetaxel and possibly leads to docetaxel-resistance. Therefore, clinical studies were designed to combine ABCB1-inhibitors in combination with docetaxel therapy. The first studies focused on PK interactions between ABCB1 inhibitors and docetaxel but did not show any interactions on PK level (26-28), indicating that the role of the ABCB1 transporter in the elimination of docetaxel is probably not the most dominant.

Pharmacokinetic drug interactions

Cancer patients use numerous drugs for the treatment of chemotherapy related side effects, comorbidities, and the management of cancer related pain. Therefore, studying pharmacokinetic drug-drug interactions is clinically important. As docetaxel has a narrow therapeutic window, pharmacokinetic interactions with drugs for supportive care as well as with complementary alternative medicines (CAMs) are of great clinical relevance for its pharmacodynamics. According to FDA guidelines, drug-drug interactions are generally considered clinically relevant when the difference in exposure after the addition of the co-medication of subject is 25% or more (29). As the IIV of docetaxel already ranges from 30-45%, it is therefore challenging to identify clinically relevant drug-drug interactions.

Interactions with anti-cancer agents

The concurrent use of anti-cancer drugs is common in the treatment of many tumor types. Current regulations regarding the clinical implementation of new anti-cancer regimens oblige extensive Phase I studies looking into synergistic effects and pharmacokinetic interactions. Here, we will not focus on possible synergistic effect of combination strategies, but only review pharmacokinetic effects and adverse events. In Table 1, an overview of studied anti-cancer drug combinations is given (30-51).

Docetaxel CL decreased with 50% when topotecan was administered on day 1-4 preceding the administration of docetaxel (42). This resulted in increased neutropenia. The combination

docetaxel and everolimus was associated with severe neutropenia and wide variation in the CL of both drugs (48). Authors state that concomitant treatment with these drugs is unpredictable due to a large variability in the CL of both drugs (48).

Table 1: Drug-drug interactions with anti-cancer drugs

Drugs	Interaction*	Effect	Ref
Cytostatics			
Cisplatin	no		[30]
Estramustine	no		[31]
5-FU	no		[32]
Capecitabine	no		[33]
Irinotecan	no		[34]
Carboplatin	no		[35]
Gemcitabine	no		[36]
Methotrexate	no		[37]
Cisplatin and 5-FU	no		[38]
Vinorelbine	no		[39]
Doxorubicin	yes	DTX followed by doxorubicin: duration grade 4 neutropenia	[40]
Ifosfamide	yes	DTX preceding ifosfamide: ↓ AUC ifosfamide	[41]
		On DTX AUC: no effect	
Topotecan	yes	Topotecan 1-4 days before DTX: DTX CL 50% ↓	[42]
Paclitaxel	yes	No effect DTX on paclitaxel	[43]
		DTX before paclitaxel: nadir ANC ↓	
Protein kinase inhibitors			
Lapatinib	no		[44]
Sunitinib	no		[45]
Imatinib	no	Inhibits CYP3A4, no effect on DTX CL	[46]
Erlotinib	yes	Substantial toxicity, not related to PK	[47]
Everolimus	yes	Substantial neutropenia and highly variable CL	[48]
Monoclonal antibodies			
Pertuzumab	no		[49]
Other			
Amifostine	no		[50]
Bortezomib	no		[51]

**clinically relevant interaction, DTX=docetaxel, AUC= area under the curve, CL=clearance, ANC= absolute neutrophil count, PK=pharmacokinetics, Ref=reference*

The combination docetaxel-erlotinib was associated with severe toxicity, without a significant change in PK (47). In contrast, in a phase I and PK study on the combination of docetaxel and pazopanib, a lower docetaxel CL was found due to pazopanib co-treatment (52). This probably results from OATP1B1 and CYP3A4 inhibition. For doxorubicin holds that when given before the administration of docetaxel instead of after the administration, a longer duration of grade 4 neutropenia was seen (40). The AUC of ifosfamide decreased when the administration was preceded by docetaxel (41).

Interactions with supportive medication

Patients may receive co-medication for treatment-associated side-effects such as nausea and vomiting. These toxicities can often be well treated with anti-emetic prophylaxis. Aprepitant was shown to inhibit CYP3A4 and induce CYP2C9 (53). Therefore, this drug could hypothetically decrease docetaxel CL (54). However, neither aprepitant nor other studied antiemetic drugs showed a clinically relevant interaction with docetaxel so far (see Table 2) and can therefore be safely used in docetaxel-treated patients (55-57). Besides regular drugs for the management of nausea and vomiting, cannabis was demonstrated to be effective and was approved by the FDA (58). No effects on docetaxel PK were demonstrated (59).

Interactions with Complementary Alternative Medicines (CAMs)

It is estimated that 40% of cancer patients seek relieve from anticancer therapy related adverse events by using complementary alternative medicine (CAMs) (60). Herbal and dietary supplements mostly influence the PK of docetaxel via CYP3A4, drug transporters and other metabolic pathways, thereby again potentially influencing toxicity and therapeutic efficacy (61). Patients should thus be well counseled if preference to CAMs is given in supportive care. Here, we discuss frequently used CAMs in cancer patients with regard to docetaxel PK (Table 2).

In breast cancer patients, a trend towards reduced docetaxel CL was found for patients using 600 mg of garlic twice daily for 12 consecutive days (62). In addition, St. John's wort was found to decrease docetaxel AUC from 3,035 ± 756 to 2,682 ± 717 ng h/mL, indicating that concomitant use of docetaxel and St. John's wort could diminish clinical efficacy and should thus be avoided (63). *Echinacea purpurea* also induces CYP3A4 activity, but does not influence docetaxel PK (64). This is consistent with earlier observations that the administration of other CYP3A4-inducing medications, such as dexamethasone, does not substantially alter the clearance of docetaxel (14). These collective observations are congruent with the supposition that, since >90% of the docetaxel dose is already metabolized by CYP3A enzymes in a normal (uninduced) state, induction of this route is unlikely to result in a further substantial increase in the extent to which the drug undergoes metabolic inactivation.

Preclinical studies suggested that components in grape seed, green tea and milk thistle potentially inhibit CYP3A4 activity, which could alter docetaxel PK. This however needs further validation in clinical setting (29). To note, caution is warranted when interpreting the results of studies on CAMs, as various (non-standardized) formulations with different concentrations of the active compound are available and used (65). Concentrations of the active compound in these varying formulations could differ and similarities in study outcome could be masked.

Table 2: Drug-drug interactions with co-administered medication

Subject	Co-administration	Endpoint	Interaction*	Effect	Ref
Co-medication	Dexamethason	CL	no		[6]
	premedication				
	Ketoconazole	CL	yes	50% CL ↓	[66]
		PK	yes	40% CL ↓, no difference in AUC	[3]
	Polysorbate 80	CL and Fu	yes	Fu P80 treated samples > Fu pretreatment samples	[81]
		CL	yes	↑ P80 AUC associated with ↓ unboud DTX CL	[79]
Supportive therapy	Aprepitant	PK	no		[55]
	Granisetron	PK	no		[56]
	Cannabis	PK	no		[59]
	Casopitant	PK	no		[57]
CAMs	Echinacea purpurea	PK	no		[64]
	St. John's worth	PK	yes	increases AUC and decrease CL	[63]
	Garlic	CL	no	trend towards decreased CL	[62]

**clinically relevant interaction, CL=clearance, PK=pharmacokinetics, AUC=area under the curve, Fu=unbound docetaxel, P80=polysorbate 80, DTX=docetaxel, Ref=reference*

Interactions with co-medication

Ketoconazole, used for the treatment of fungal infections, is a strong inhibitor of CYP3A4. Co-administration of ketoconazole decreased docetaxel CL with 40-50% (Table 2) (3, 66). Also, ketoconazole co-administration increased the IIV of docetaxel CL around 8% (3) and should therefore be avoided. Pre-medication with dexamethasone did not show an association with docetaxel PK (6).

To sum, for safe and optimal care, clinicians should be aware of drug-drug interactions and take these into account when administering docetaxel to patients as these interactions influence both PK and pharmacodynamics.

Patient factors

In addition to drug-drug interactions, patient related factors might play a role in the large pharmacokinetic IIV of docetaxel. Some factors have been studied extensively and are discussed below.

Gender, age and ethnicity

The effects of gender on docetaxel metabolism have been investigated in multiple studies and the results are indistinct (8, 9, 67). A previous study found that females had a 35% lower docetaxel CL than males and a gender effect on docetaxel metabolism was suggested (9) while others observed no clear effect of gender on docetaxel PK (8, 67). This might be due to underlying and masking factors, as hormonal factors such as menopausal status and castration status may play a role in the discrepancy regarding the effect of gender, masking potential clinically relevant effects.

Age is of insignificant influence on docetaxel PK (9, 67-71). Docetaxel CL and its variability was not altered in elderly patients compared to younger patients (70).

Regarding ethnicity, Japanese patients are usually treated with a lower dose than patients in Western countries. This resulted from different recommended phase II doses during early drug development, due to differences in (dose-limiting) toxicity between Asian and non-Asian patients (72). However, no statistically significant differences in docetaxel CL were seen when races were compared, suggesting that ethnicity does not substantially contribute in explaining the large docetaxel IIV (73, 74).

Hormonal status

The influence of castration status on docetaxel PK was investigated in 30 men with mCRPC (Table 3) (7). It was shown that castration status did not modify CYP3A levels, confirming earlier findings (75). However, castrated males showed increased docetaxel CL and a 2-fold decrease in AUC compared to non-castrated patients. These findings were further supported by studies in rodents, where castrated rats had reduced docetaxel peak concentrations (7). The increased expression of hepatic rOatp2 (slc22a7) was reported as a potential explanation for this finding (7). This increase in rOatp2 expression was hypothesized to result in increased hepatic docetaxel uptake and thus in increased metabolism.

Menopausal status was shown to affect docetaxel PK with premenopausal woman having a lower AUC (4124 µg h/l, n=53) than postmenopausal woman (4598 µg h/l, n=33) [76]. This study also showed that docetaxel AUC was significantly different in 40 pre-menopausal and post-menopausal women carrying the same C3435T genotype (CC), with a lower AUC in premenopausal woman (76). No effect was seen in woman with other genotypes. Castration status and menopausal status could thus potentially be part of the underlying mechanisms explaining the discrepancy in the influence of gender on docetaxel PK. At

this point, the influence of menopausal and castration status has to be validated in larger cohorts and is not yet usable in a clinical setting.

Obesity

When separating patients into quartiles based on their BSA, the mean docetaxel CL was highest in the highest BSA quadrant and lowest in the lowest BSA quadrant (69). In patients with a BSA > $2m^2$, a 33% increase in docetaxel CL was seen compared to patients with a BSA ≤ $2m^2$ (68). A BMI of ≥ 30 kg/m^2 was not associated with higher docetaxel CL (68, 77). Thus, no dose adaptations need to be made for obesity. However, extensively obese patients with a BSA > 2 m^2 had an increased docetaxel CL and may need a higher dose than patients with a BSA ≤ 2 m^2. This hypothesis however needs validation in larger cohorts.

Liver impairment

Liver impairment was shown to decrease docetaxel CL (Table 3) (78-80). Minami and colleagues demonstrated that patients with grade 2 and 3 elevations of transaminases at baseline together with alkaline phosphatase elevation had around a 30% decrease of docetaxel CL (78). Their advice was to consider a 20-40% dose reduction for patients with a grade 2 and 3 transaminase increase in combination with alkaline phosphatase elevation.

Plasma proteins

Plasma proteins are seen as possible determinants for docetaxel PK, as docetaxel is highly bound to proteins. Some studies looked into the relation between α 1-acid glycoprotein (AAG) and docetaxel PK (54, 79, 81, 82) (see Table 3). Ambiguous results were found. This discrepancy could possibly be caused by the fact that unbound docetaxel CL was used to test a possible correlation with AAG concentrations, which eliminates the effect of protein binding as a confounder (79). Also, AAG is an acute phase reactant, which could mask a potential effect. It is also known that in critically ill patients, albumin levels are low due to altered distribution between intravascular and extra vascular compartments (83). Decreased albumin levels thus might make up for the increase of AAG, explaining why no effect is seen in some of the studies that focus on AAG only. As also the expression of CYP3A4 is decreased during inflammatory response, the question rises whether AAG is mechanistically responsible for changes in PK or that increased levels of AAG are only a sign of ongoing inflammatory response, decreasing CYP3A4 activity (84). Currently, no definite clinical actions can be taken based on baseline plasma protein values.

Environmental factors

In addition to patient related factors, environmental factors may play a role in docetaxel PK. Smoking has been studied as such and demonstrated to have no effect on docetaxel PK (85). However, the incidence of grade 4 neutropenia was lower in smokers who were treated with docetaxel (35%) than in non-smokers (52%) (85). One of the supposed mechanisms for this effect is that patients inhale small particles when smoking, which could result in IL-6 and granulocyte macrophage colony stimulating factor release, that encourages the proliferation of pre-cursors in the bone marrow (86-88). Thus, the effect of smoking on docetaxel PK seems to be limited and at this point, the advantages of quitting smoking still seem to offset the possible protective effect on hematological toxicity.

Table 3: Patient and environmental factors influencing pharmacokinetics

Subject	Factor	Endpoint	Effect PK	Effect description	Ref
Patient factors	Liver impairment	CL	yes	Moderate and severe liver impairment ↓ CL	[78-80]
	α1-acid glycoprotein	CL	yes	↑ AAG leads to ↓ DTX CL	[82]
			no		[54,79,81]
	Menopausal status	PK	yes	AUC: premenopausal < postmenopausal	[76]
				woman with genotype C3435T (CC)	
	Castration status	CL, AUC	yes	100% ↑CL and 2-fold ↓ AUC in castrated	[7]
				vs. non-castrated patients	
	Ethnicity	CL	no		[73, 74]
	Gender	CL	yes	Woman 35% ↓ CL than men	[9]
			no		[8,67]
	Age	CL	no		[9,67,68,69,70,71]
	BSA >2.0 m^2	CL	yes	33% CL ↑	[68]
	BMI ≥30 kh/m^2	CL	no		[68]
Environmental factors	Smoking	PK	no	↓ grade 4 neutropenia in smokers	[85]

PK=pharmacokinetics, CL=clearance, AAG=α1-acid glycoprotein, AUC=area under the curve, DTX=docetaxel, Ref=reference

Current alternatives for BSA-based dosing

The BSA-based formula does not account for the factors described in the previous paragraph that potentially influence the PK of docetaxel, such as obesity, gender, hormonal status and liver impairment. To improve individualized dosing of docetaxel, other strategies have been studied and will be discussed here.

Therapeutic drug monitoring

A priori therapeutic drug monitoring (TDM) is a tool for calculating the optimal dose of a drug (4). Generally, drugs with a narrow therapeutic window and an existing correlation between toxicity and exposure may be suited for such an approach. Since docetaxel matches these criteria, docetaxel dosing could hypothetically be individualized by using TDM. To investigate this hypothesis, a TDM strategy was developed using a validated limited sampling model based on Bayesian analysis. Using TDM, the IIV in PK decreased significantly with 39% (89). Unfortunately, the incidence of hematological toxicity was not different in TDM dosed patients from patients that had been dosed using BSA. Despite the fact that relatively cheap immuno-assays for determining docetaxel plasma concentrations are currently (commercially) available, a problem of TDM is that it is still time-consuming for both patients and professionals.

BSA dose banding

To improve the current BSA strategy, it was recently suggested that dose-banding could be an alternative (90). A limited amount of predefined BSA ranges was used to determine an initial docetaxel dose and for adaptation of the dose in patients with extreme BSA values. This strategy was feasible, since the difference in the calculated docetaxel dosage was marginal compared to regular BSA dosing. This strategy has the potential to simplify pharmacy processes and to improve patient safety (90).

Probe-drug phenotyping

As an alternative for accounting for individual factors influencing docetaxel PK, researchers tried to predict CYP3A4 activity as a measure for docetaxel CL with the use of probes, such as antipyrine, midazolam and erythromycin (9, 54, 67, 82, 91). The erythromycin breath test, antipyrine CL, dexamethasone CL and midazolam exposure tests were demonstrated to be successful in predicting CYP3A4 activity and thereby docetaxel PK (9, 54, 67, 92, 93). To add, urinary 6-beta-hydroxy cortisol was used in a formula for the estimation of docetaxel CL (91). Compared with BSA-based dosing, using this method these researchers were able to reduce docetaxel's IIV significantly. However, the complexity of such methods, the interaction with several other mechanisms such as docetaxel transport and for example the interference with polysorbate 80, currently obstruct clinical application of these strategies (8, 79, 81, 94).

Pharmacogenetics

The effect of genetic variation on docetaxel PK has been studied extensively (see Table 4, refs. 8, 9, 62, 67, 73, 75, 95-100). Some of the studied single nucleotide polymorphisms (SNPs) have been associated with docetaxel PK alteration. For example, the SNP rs12762549

in *ABCC2* resulted in a significantly decreased docetaxel CL (98). A 50% increase in docetaxel CL was seen in patients carrying one *1A allele (rs776746) in *CYP3A5* (9). When carrying both *CYP3A4* *1B and *CYP3A5* *1/*3 alleles an increase in docetaxel CL was seen, as well as for carrying both *CYP3A4* *1B and *CYP3A5* *1A alleles (9, 95). Contradictory results have been shown for SNPs in *ABCB1* (rs1128563, 1236C>T) and *SLCO1B3* (rs11045585, IVS12-5676A>G) (8, 9, 97-99). For clinical applicability, these SNPs have to be validated in larger cohorts, possibly using genome wide association studies next to the usual candidate gene approach.

Recommendations

The high IIV in the PK of docetaxel renders it difficult to accurately choose an individual dose resulting in optimal docetaxel exposure, leading to efficacy at the cost of acceptable toxicity. Today, only BSA is used for calculating docetaxel dosages. However, this method does not fully reduce the high IIV of docetaxel PK to an absolute minimum. Unfortunately, no superior alternatives for the current dosing strategy are presently available. A new dosing strategy could therefore use some additional, and clinically applicable, tools for decreasing IIV and individualizing docetaxel treatment. Tools for such a strategy could thus be demographic factors partly explaining docetaxel's high IIV in PK.

From current knowledge, several recommendations can be given on factors influencing docetaxel PK in order to optimize docetaxel dosing. Liver impairment may decrease docetaxel CL, and should be taken into account. Also, hormonal status and gender may be of clinical relevance in future dosing strategies for docetaxel.

Drug-drug interactions have been established and some are distinctly relevant. These interactions are most probably mediated by drug transporters and cytochrome P450 iso-enzymes. Therefore, notice should be taken when using CYP3A4 inhibiting drugs in combination with docetaxel. Interactions at the level of uptake transporters may also be of relevance, as these are likely influencing hepatic uptake of docetaxel and thus drug elimination. The role of pharmacogenetics is currently still limited, and special recommendations on preemptive genotyping cannot be given.

Table 4: SNPs associated with docetaxel pharmacokinetics

Gene	SNP	rs number	Endpoint	Effect PK	Effect	Ref
ABCB1	3435C>T, CC genotype only	rs2032582	AUC	yes	↓ AUC in premenopausal vs. postmenopausal woman	[76]
	3435C>T		CL	no		[9,67,95-97]
	1236C>T	rs1128503	CL	yes	25% ↑ CL	[97]
			CL	no		[9]
	2677G>T/A	rs2032582	CL/AUC	no		[9,95-97]
ABCC2	101620771C>G, 52425235C>G	rs12762549	CL	yes	↓ CL	[98]
	-1019A>G	rs2804402	CL	no		[9]
	-24C>T	rs717620	CL	no		[9]
	1249G>A	rs2273697	CL	no		[9]
	IVS26G>A	rs8187698	CL	no		[9]
	3972C>T	rs3740066	CL	no		[9]
	4544G>A	rs8187710	CL	no		[9]
SLCO1B1	-1187G>A	rs4149015	CL	no		[8]
	c.3386G>A	rs2306283	CL	no		[8]
	c.521T>C	rs4149056	CL	no		[8]
SLCO1B3	IVS12-5676A>G	rs11045585	AUC	yes	↑ AUC if genotype GG	[99]
			CL	no		[8,98]
	334T>G	rs4149117	CL	no		[8,9]
	439A>G	rs57585902	CL	no		[9]
	699G>A	rs7311358	CL	no		[8,9]
	767G>A	rs60140950	CL	no		[9]
	1559A>C	N/A	CL	no		[9]
	1679T>C	rs12299012	CL	no		[9]
CYP3A4	-392A>G>G (*1B)	rs2740574	CL	trend	62% ↑CL with one *1B allele	[9]
				no		[62,73,97]
	878T>C (*18 allele)	rs28371759	Vmax	yes	Vmax ↓	[100]
CYP3A5	6986A>G	rs776746	CL	yes	49% ↑ CL with one *1A allele	[9]
	22893G>A (*3)	rs776746	PK	no		[67,74,96,97]
	27289C>A (*2)	rs28365083	PK	no		[97]
	CYP3A4*1B and CYP3A5*1/*3		PK	yes	↑CL and ↓AUC	[95]
	CYP3A4*1B and CYP3A5 *1A		CL	yes	64% ↑CL	[9]

PK=pharmacokinetics, CL=clearance, AUC=area under the curve, Vmax=maximum velocity, Ref=reference

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31

31

Association of Survival Benefit With Docetaxel in Prostate Cancer and Total Number of Cycles Administered

Abstract

IMPORTANCE: The optimal total number of docetaxel cycles in metastatic castration resistant prostate cancer patients (mCPRC) has not been investigated yet. It is unknown whether it is beneficial for patients to continue treatment upon 6 cycles. **OBJECTIVE:** We investigated whether the number of docetaxel cycles administered to patients deriving clinical benefit was an independent prognostic factor for OS in a posthoc analysis of the Mainsail trial. **DESIGN, SETTING, AND PARTICIPANTS:** Mainsail was a multinational randomized phase 3 study in 1059 metastatic castration resistant prostate cancer (mCRPC) patients, receiving docetaxel, prednisone and either lenalidomide (DPL) or placebo (DP). Study patients were treated until progressive disease (PD), or unacceptable adverse effects occurred. Median Overall Survival (OS) was found to be inferior in the DPL arm when compared to DP alone. As a result of increased toxicity with the combination, patients on DPL received fewer docetaxel cycles, median 6, vs 8 cycles in the control group. As the dose intensity was comparable in both treatment arms, we investigated whether the number of docetaxel cycles administered to patients deriving clinical benefit on Mainsail was an independent prognostic factor for OS. We conducted primary univariate and multivariate analyses containing the ITT Population. Additional sensitivity analyses were done, excluding patients who stopped for reasons of disease progression and those who received ≤4 cycles of docetaxel for other reasons, minimizing the effect of confounding factors. **MAIN OUTCOMES AND MEASURES:** The total number of docetaxel cycles delivered was an independent factor for OS. **RESULTS:** Treatment with ≥8 cycles of docetaxel was associated with superior OS (Hazard Ratio (HR: <8 vs ≥8) 1.909 95% CI 1.660 - 2.194, P<0.0001), irrespective of lenalidomide treatment (HR 1.060 95% CI 0.924 - 1.215, p=0.4071). Likewise, in the sensitivity analysis, patients who received a greater number of docetaxel cycles had superior OS; patients who received >10 cycles had a median OS of 33.0 months compared to 26.9 months in patients treated with 8-10 cycles and 22.8 months for patients treated with 5-7 cycles of docetaxel (P<0.0001). **CONCLUSIONS AND RELEVANCE:** These findings suggest that continuation of docetaxel chemotherapy contributes to the survival benefit. Prospective validation is warranted.

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Introduction

Docetaxel combined with prednisone is the current first line chemotherapy in metastatic castration resistant prostate cancer (mCRPC). In the TAX 327 registration trial, the number of 10 cycles of docetaxel every 3 weeks was arbitrarily chosen and the median number actually delivered was 9.5[1]. In study SWOG 99-16 patients were scheduled to receive a maximum of 12 cycles[2]. To date, the optimal number of docetaxel cycles has not been established. Prospective clinical trials to improve upon docetaxel have generally focused on the addition of a second active agent. In these trials the number of cycles has been arbitrarily set at 10-12 cycles, or until disease progression, or unacceptable adverse effects occurred. Outside the context of clinical trials, especially following the recent advent of novel androgen receptor (AR)-targeted agents, including abiraterone and enzalutamide, docetaxel chemotherapy, either for convenience, or to avoid cumulative side effects, is quite often and increasingly halted at 6 cycles[3].

The Mainsail study investigated the safety and efficacy of addition of lenalidomide, an anti-angiogenic agent with immunomodulatory properties, to docetaxel plus prednisone in a randomized double-blind placebo controlled phase 3 clinical trial[4] (NCT00988208). The study was stopped early due to a futility analysis, in which the median Overall Survival (OS) of docetaxel/prednisone plus lenalidomide (DPL) was inferior to docetaxel/prednisone plus placebo (DP). The addition of lenalidomide to docetaxel increased the toxicity of the regimen, including increased myelotoxicity, that caused more frequent docetaxel dose reductions and eventually fewer cycles administered. The dose adjustment protocol for myelotoxicity specified that reductions were primarily made in the docetaxel dose. The study protocol mandated continuation of treatment (docetaxel and lenalidomide, or placebo), until radiographic disease progression, or unacceptable adverse effects occurred. The median number of cycles delivered in the experimental arm was 6, whereas the patients in the control arm received a median of 8 cycles. Since the dose intensity per cycle was comparable in both treatment arms (94.4% in the DPL arm and 95.6% in the DP arm), we investigated whether the difference in OS could be attributed to the cumulative dose as reflected by the total number of docetaxel cycles administered.

Patients and Methods

Study design and patients

Mainsail was a randomized, double-blind, placebo-controlled phase 3 study, conducted at 223 centers in the US, Canada, Europe, Russia, Australia, South Africa, Israel, and Mexico, accruing 1059 patients. The study was initiated in November 2009 and was ended early in November 2011 because of futility. Full details are provided in the original report[4]. Chemotherapy-naïve mCRPC patients were eligible for inclusion if they met the following

criteria: Eastern Cooperative Oncology Group (ECOG) performance status score of ≤2; hemoglobin level >9 g/dL; absolute neutrophil count >1·5×10^9/L; platelet count >100×10^9/L; creatinine clearance level >50 mL/min; total bilirubin level <1·0×upper limit of normal (ULN); serum aspartate transaminase and alanine transaminase levels <1·5×ULN; alkaline phosphatase level <2·5×ULN. Effective castration was defined as serum testosterone levels <50ng/dL. Patients were randomized 1:1 to docetaxel (75 mg/m^2) and prednisone, plus either lenalidomide 25 mg/day (DPL) or placebo (DP) on day 1-14. Patients were stratified by baseline ECOG performance status, geographic region and type of progressive disease (rising PSA versus tumor progression). Patients were kept on protocol treatment until disease progression, or until unacceptable adverse effects occurred. In case of hematologic toxicity (e.g. febrile neutropenia or grade 4 neutropenia lasting more than one week) and certain non-hematologic toxicities (e.g. grade >3 cutaneous reactions or moderate neurosensory symptoms), dose reductions were primarily made for docetaxel. The primary endpoint of the study was OS, defined as time from randomization to death.

Statistical analyses

Our primary analysis was an intention to treat (ITT) analysis on overall survival for the entire dataset updated by 15 March, 2016, using Kaplan Meier method and Cox proportional hazard model. We conducted univariate and multivariate analyses including the following parameters: treatment group (DPL or DP); baseline PSA; baseline LDH; baseline total testosterone; number of treatment cycles; duration of lenalidomide/placebo; baseline hemoglobin; baseline albumin; age; baseline ECOG performance status; baseline BMI; prior treatments; baseline creatinine clearance; geographic region; race group. In order to reduce the potential bias of stopping docetaxel due to disease progression and associated potential confounding impact on survival, we performed additional sensitivity analyses. The sensitivity analyses excluded patients who had stopped docetaxel due to disease progression, or had received less than a minimum of 5 cycles, since it was felt that patients who had been exposed to docetaxel for only a few cycles were not likely to obtain a meaningful survival benefit from the chemotherapy. Final multivariate model was selected by stepwise procedure from the proportional hazard model.

Results

Baseline characteristics

The ITT analysis comprised all 1059 randomized patients. The baseline characteristics are shown in **table 1**. In the DPL arm 244 subjects received ≥8 cycles of docetaxel and 289 subjects received <8 cycles of docetaxel.

Table 1: Baseline patient demographics and characteristics

	Statistic	DPL (N=553)		DP (N=526)		
		Cycle ≥8 (N=275)	Cycle <8 (N=258)	Cycle ≥8 (N=332)	Cycle <8 (N=194)	Total (N=1059)
Age (years)	n	275	258	332	194	1059
	Mean(SD)	67.5 (7.39)	70.5 (8.30)	68.3 (7.17)	68.8 (8.77)	68.7 (7.89)
	Median	67.9	71.3	68.1	69.8	69.0
	Min,Max	43, 88	45, 89	51, 87	47, 90	43, 90
	Q1, Q3	62.5, 73.0	65.9, 76.1	63.6, 73.6	63.5, 74,5	63.8, 74.4
	IQR	10.5	10.2	10.0	11.0	10.6
Age Categorized (years)						
<65	n (%)	107 (38.9)	56 (21.7)	109 (32.8)	62 (32.0)	334 (31.5)
65 ≤ Age ≤75	n (%)	128 (46.5)	116 (45.0)	156 (47.0)	90 (46.4)	490 (46.3)
>75	n (%)	40 (14.5)	86 (33.3)	67 (20.2)	42 (21.6)	235 (22.2)
Race						
American Indian or Alaska Native	n (%)	2(0.7)	1 (0.4)	2(0.6)	3(1.5)	8 (0.8)
Asian	n (%)	3 (1.1)	3 (1.2)	4 (1.2)	4 (2.1)	14 (1.3)
Black or African American	n (%)	8 (2.9)	13 (5.0)	12 (3.6)	13 (6.7)	46 (4.3)
White	n (%)	223 (81.1)	213 (82.6)	275 (82.8)	158 (81.4)	869 (82.1)
Other or no answer	n (%)	39 (14.2)	28 (10.9)	39 (11.7)	16 (8.2)	122 (11.5)
Gender Male	n (%)	275 (100)	258 (100)	332 (100)	194 (100)	1059 (100)
ECOG-PS						
O to 1	n (%)	268 (97.5)	240 (93.0)	321 (96.7)	183 (94.3)	1012 (95.6)
=0	n (%)	142 (51.6)	110 (42.6)	163 (49.1)	94 (48.5)	509 (48.1)
=1	n (%)	126 (45.8)	130 (50.4)	158 (47.6)	89 (45.9)	503 (47.5)
=2	n (%)	6 (2.2)	18 (7.0)	11 (3.3)	10 (5.2)	45 (4.2)
=3	n (%)	0	0	0	1 (0.5)	1(0.1)
not specified	n (%)	1 (0.4)	0	0	0	1 (0.1)
Region						
US or Canada	n (%)	64 (23.3)	76 (29.5)	75 (22.6)	61 (31.4)	276 (26.1)
EU or Australia	n (%)	180 (65.5)	150 (58.1)	215 (64.8)	114 (58.8)	659 (62.2)
Rest of World	n (%)	31 (11.3)	32 (12.4)	42 (11.7)	19 (9.8)	124 (11.7)
Type of Previous Disease progression – CRF						
Rising PSA only	n (%)	80 (29.1)	79 (30.6)	94 (28.3)	52 (26.8)	305 (28.8)
Radiographic Progression	n (%)	195 (70.9)	179 (69.4)	238 (71.7)	142 (73.2)	754 (71.2)
Prior Radiation Therapy						
Yes	n (%)	153 (55.6)	159 (61.6)	196 (59.0)	112 (57.7)	620 (58.5)
No	n (%)	122 (44.4)	99 (38.4)	136 (41.0)	82 (42.3)	439 (41.5)
Prior Cancer Surgeries						
Yes	n (%)	190 (69.1)	168 (65.1)	209 (63.0)	126 (64.9)	693 (65.4)
No	n (%)	85 (30.9)	90 (34.9)	123 (37.0)	68 (35.1)	366 (34.6)
Prior Hormonal Anti-Cancer Therapies						
Yes	n (%)	261 (94.9)	250 (96.9)	323 (97.3)	189 (97.4)	1023 (96.6)
No [2]	n (%)	14 (5.1)	8 (3.1)	9 (2.7)	5 (2.6)	36 (3.4)

Table 1: Baseline patient demographics and characteristics (Continued)

	Statistic	DPL (N=553)		DP (N=526)		
		Cycle ≥8 (N=275)	Cycle <8 (N=258)	Cycle ≥8 (N=332)	Cycle <8 (N=194)	Total (N=1059)
Other Prior Anti-Cancer Therapies						
Yes	n (%)	34 (12.4)	37 (14.3)	52 (15.7)	27 (13.9)	150 (14.2)
No	n (%)	241 (87.6)	221 (85.7)	280 (84.3)	167 (86.1)	909 (85.8)
Baseline PSA levels (ng/ml)	n	274	257	330	192	1053
	Mean (SD)	302.421 (810.7726)	331.512 (738.6567)	282.001 (599.0856)	304.552 (752.7563)	303.542 (720.2895)
	Median	98.200	114.000	84.000	87850	95.200
	Min, Max	0.21, 10759	0.10, 8665.0	0.33, 6807.0	0.01, 5715.0	0.01, 10759
	Q1, Q3	32.200, 264.000	34.900, 339.000	31.000, 275.000	33.650, 253.000	32.800, 283.000
	IQR	231.800	304.100	244.000	219.350	250.200
Metastatic sites – other than prostate						
Bone only	n (%)	84 (30.5)	85 (32.9)	100 (30.1)	57 (29.4)	326 (30.8)
Soft tissues only	n (%)	52 (18.9)	52 (20.2)	58 (17.5)	36 (18.6)	198 (18.7)
Both Bone and Soft Tissues	n (%)	138 (50.2)	121 (46.9)	173 (52.1)	100 (51.5)	532 (50.2)
None	n (%)	1 (0.4)	0	1 (0.3)	1 (0.5)	3 (0.3)

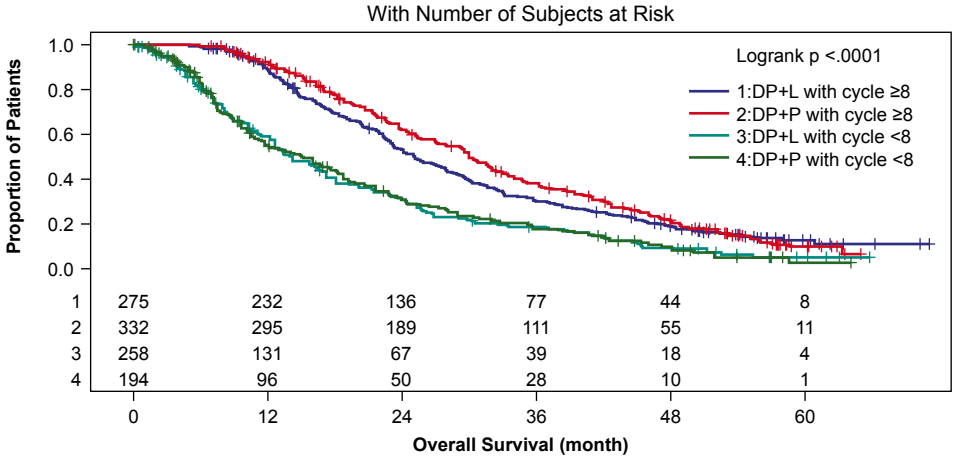
Notes: Any subject enrolling in the study with evidence of radiographic progression will be stratified into the radiographic progression strata regardless of their PSA status.
[1] BMI= Body mass index, defined as weight in kg divided by height in meters squared.
[2] All patients had either prior bilateral orchiectomy or ongoing androgen blockage.

In the DP arm, 296 patients received ≥8 cycles of docetaxel and 230 patients received fewer than 8 cycles docetaxel. For the sensitivity analysis, 250 patients were excluded since they had received ≤4 cycles of docetaxel, and 264 patients were excluded who had stopped docetaxel due to disease progression (of which 60 also received ≤4 cycles). Data were analyzed using several cut-off points; 5-7 vs 8-10, and ≤10 vs >10 cycles. In the sensitivity analysis patients in the DPL and the DP arm were tested separately as well as grouped together. Hence, 605 patients, who had not stopped docetaxel due to disease progression and who had a minimum exposure of 5 cycles were included in this analysis **(Supplementary figure 1)**.

Overall survival based on number of docetaxel cycles

The analysis on the ITT Population showed a robust superior OS for patients treated with a greater number of cycles. We examined the number of docetaxel cycles by using 6, 8 and 10 or more, as cut-off points, as well as the number of cycles as continuous variable. **Figure 1** shows the OS for patients for the DPL and the DP arm in the subgroups of receiving ≥8 cycles vs those receiving <8 cycles (P<0.0001). Identical findings were obtained for the

comparisons ≥6 cycles, versus <6 cycles and ≥10 cycles versus <10 cycles **(supplementary figure 2 and supplementary figure 3).**



	No. of Subjects	Event	Censored	Median	Survival (95% CL)
1:DP+L with cycle ≥8	275	221 (80%)	54 (20%)	25.0	(23.0-27.9)
2:DP+P with cycle ≥8	332	264 (80%)	68 (20%)	29.8	(27.8-32.1)
3:DP+L with cycle <8	258	213 (83%)	45 (17%)	14.2	(12.7-16.8)
4:DP+P with cycle <8	194	159 (82%)	35 (18%)	15.0	(11.5-18.3)

Figure 1: Kaplan-Meier plots of overall survival for the DP (docetaxel, prednisone) and the DPL (Docetaxel, prednisone + lenalidomide) arm in the subgroups of number of docetaxel cycles <8 and ≥8 (the ITT Population).

As previously reported, the DPL arm showed a significantly inferior survival compared with the DP arm. In the univariate analysis, the number of treatment cycles (as continuous variable) (P<0.0001), the cumulative dose of docetaxel (P<0.0001), the duration of lenalidomide (P<0.0001), and the allocated treatment arm (P=0.0322) were all significant **(table 2)**. In the multivariate model, not taking into account the number of cycles as a variable, the treatment arm was statistically significant (Hazard ratio (HR) 1.626, 95% CI 1.237 - 2.13, P=0.0005). However, when the number of cycles (<8 vs ≥8) was included in the multivariate analysis the number of docetaxel cycles was a statistically significant independent factor affecting OS (HR 1.909 95% CI 1.660 - 2.194, P<0.0001), but the treatment arm (DPL vs DP) was not retained (HR 1.060, 95% CI 0.924 - 1.215, P=0.4071). This implies that the cumulative dose of docetaxel, as reflected by the total number of cycles administered, is an independent factor for overall survival. Other well-known predictors such as baseline LDH (P<0.0001), baseline albumin (P<0.0001), baseline hemoglobin (P<0.0001) and baseline ECOG Performance Status (P=0.0004) were significant independent contributors of OS following docetaxel treatment.

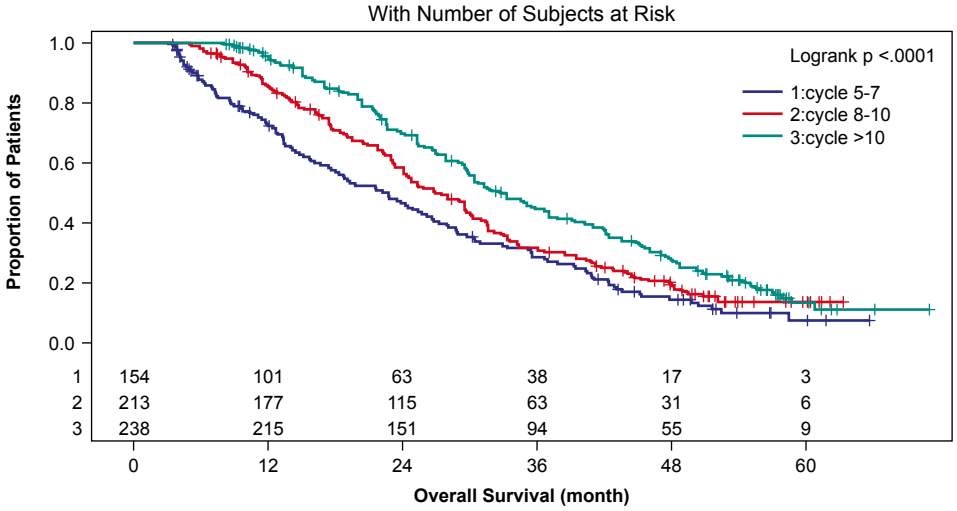
Table 2. Multivariate Cox Regression Model on OS (including number of treatment cycles <8 vs ≥8) (The ITT Population).

Variables	Univariate		Multivariate	
	Hazard Ratio (95% CI)	p-value	Hazard Ratio (95% CI)	p-value
Treatment group (DP+Lenalidomide vs DP+Placebo)	1.158 (1.013, 1.324)	0.0322	1.060 (0.924, 1.215)	0.4071
Baseline PSA (for every 100 ng/ml increase)	1.015 (1.008, 1.021)	<.0001		
Baseline LDH (for every 50U/L increase)	1.102 (1.089, 1.116)	<.0001	1.077 (1.063, 1.092)	<.0001
Number of treatment cycles (for each cycle increase)	0.930 (0.917, 0.943)	<.0001		
Number of treatment cycles (<8 vs >=8)	1.933 (1.687, 2.214)	<.0001	1.909 (1.660, 2.194)	<.0001
Duration of Lenalidomide/Placebo (for each week increase)	0.985 (0.981, 0.989)	<.0001		
Cumulative dose of docetaxel (for each 10mg/m2 increase)	0.990 (0.988, 0.993)	<.0001		
Baseline HGB (for each g/dL increase)	0.789 (0.753, 0.826)	<.0001	0.887 (0.842, 0.935)	<.0001
HGB (<=10 vs >10)	2.270 (1.771, 2.910)	<.0001		
Baseline value of Albumin (for each g/L increase)	0.906 (0.888, 0.924)	<.0001	0.947 (0.926, 0.968)	<.0001
Age category (<65 vs >75)	0.779 (0.648, 0.935)	0.0074		
Age category (65-75 vs >75)	0.784 (0.660, 0.930)	0.0052		
Baseline ECOG group (high (2,3) vs low (0,1))	2.639 (1.944, 3.583)	<.0001	1.797 (1.300, 2.485)	0.0004
Baseline BMI (for each unit increase)	0.985 (0.971, 1.000)	0.0440		
Prior Cancer surgery (No vs Yes)	1.096 (0.952, 1.261)	0.2014		
Prior hormonal anti-cancer therapy (No vs Yes)	0.870 (0.584, 1.295)	0.4922		
Prior radiation therapy (No vs Yes)	0.999 (0.872, 1.145)	0.9901		
Baseline creatinine clearance (for each unit increase)	0.999 (0.996, 1.002)	0.6739		
Region (EU and Australia vs US/Canada)	1.000 (0.857, 1.167)	0.9980		
Region (Rest of World vs US/Canada)	1.105 (0.847, 1.441)	0.4613		
Race group (Black or African American vs White)	1.087 (0.774, 1.526)	0.6316		
Race group (Other vs White)	0.950 (0.782, 1.155)	0.6081		

BMI= Body Mass Index; CI= Confidence interval; DP=docetaxel, prednisone; ECOG; Eastern Cooperative Oncology Group; HGB= Hemoglobin; LDH= Lactate dehydrogenase; PSA= Prostate-specific antigen.

In the sensitivity analysis, we investigated whether the number of docetaxel cycles administered to patients continuing treatment beyond 4 cycles and not stopping due to disease progression was an independent prognostic factor for OS. Treatment arm was not a significant factor affecting survival in either the univariate or the multivariate analysis **(Table 3)**. Patients who had received >10 cycles had the greatest median OS of 33.0 months, compared to those who had 8-10 cycles (26.9 months), or 5-7 cycles of docetaxel (22.8

months) when treatment groups were combined (P<0.0001) **(figure 2)**. The same holds true when the arms were analyzed separately **(supplementary figure 4)**: in the DPL arm, median OS for patients receiving >10 cycles, 8-10 cycles, and 5-7 cycles was 31.6, 24.4, and 18.8 months, respectively; in the DP arm, median OS for patients receiving >10 cycles, 8-10 cycles, and 5-7 cycles was 34.7, 29.7, and 23.6 months, respectively (p = 0.0007). All comparisons for OS between the cohorts receiving 5 or 6, versus >6 cycles, 5-7 versus 8-10 cycles and 8-10 vs >10 cycles, and cumulative dose of docetaxel, were significant in the univariate model. The cut-off 5 or 6, vs >6 cycles had the strongest independent significance and was thus retained in the multivariate model. The established contributors for OS – baseline LDH (P<0.0001), baseline hemoglobin (P=0.0060), baseline albumin (P=0.0061) and baseline ECOG Performance Status (P=0.0290) – also had independent significance and were retained in the multivariate model **(table 3)**.



	No. of Subjects	Event	Censored	Median	Survival (95% CL)
1:cycle 5-7	154	122 (79%)	32 (21%)	22.8	(17.6-26.6)
2:cycle 8-10	213	169 (79%)	44 (21%)	26.9	(24.1-30.1)

Figure 2: Kaplan-Meier plots of overall survival in the subgroups of number of docetaxel cycles 5-7, 8-10 cycles and >10 cycles. DP (docetaxel, prednisone and placebo) and DPL (docetaxel, prednisone, lenalidomide) treatment arms are combined, patients with progressive disease and/or less than ≤ 4 cycles of docetaxel were excluded from the analysis.

Table 3. Multivariate Cox Regression Model on OS, excluding patients who had PD or ≤4 cycles of docetaxel

	Univariate		Multivariate	
Variables	**Hazard Ratio (95% CI)**	**p-value**	**Hazard Ratio (95% CI)**	**p-value**
Treatment group (DP+Lenalidomide vs DP+Placebo)	1.089 (0.908, 1.305)	0.3577	1.014 (0.843, 1.220)	0.8810
Baseline PSA (for every 100 ng/ml increase)	1.014 (1.006, 1.022)	0.0005		
Baseline LDH (for every 50 U/L increase)	1.134 (1.107, 1.162)	<.0001	1.113 (1.085, 1.142)	<.0001
Number of treatment cycles (for each cycle increase)	0.945 (0.924, 0.966)	<.0001		
Number of treatment cycles (5-6 vs >6)	1.447 (1.139, 1.839)	0.0025	1.383 (1.085, 1.763)	0.0089
Number of treatment cycles (5-7 vs 8-10)	1.279 (1.014, 1.615)	0.0382		
Number of treatment cycles (8-10 vs >10)	1.340 (1.085, 1.656)	0.0066		
Duration of Lenalidomide/Placebo (for each week increase)	0.991 (0.985, 0.997)	0.0020		
Cumulative dose of docetaxel (for each 10mg/m2 increase)	0.994 (0.991, 0.998)	0.0010		
Baseline HGB (for each g/dL increase)	0.815 (0.766, 0.868)	<.0001	0.902 (0.838, 0.971)	0.0060
HGB (<=10 vs >10)	2.348 (1.644, 3.353)	<.0001		
Baseline value of Albumin (for each g/L increase)	0.917 (0.892, 0.943)	<.0001	0.957 (0.927, 0.987)	0.0061
Age category (<65 vs >75)	0.728 (0.566, 0.936)	0.0135		
Age category (65-75 vs >75)	0.779 (0.617, 0.982)	0.0343		
Baseline ECOG group (high (2,3) vs low(0,1))	2.429 (1.447, 4.078)	0.0008	1.825 (1.063, 3.133)	0.0290
Baseline BMI (for each unit increase)	0.993 (0.973, 1.013)	0.4629		
Prior Cancer surgery (No vs Yes)	1.086 (0.897, 1.316)	0.3972		
Prior hormonal anti-cancer therapy (No vs Yes)	0.848 (0.478, 1.505)	0.5735		
Prior radiation therapy (No vs Yes)	0.988 (0.821, 1.188)	0.8966		
Baseline creatinine clearance (for each unit increase)	1.000 (0.996, 1.004)	0.9637		
Region (EU and Australia vs US/Canada)	1.028 (0.828, 1.275)	0.8039		
Region (Rest of World vs US/Canada)	1.264 (0.876, 1.822)	0.2100		
Race group (Black or African American vs White)	0.703 (0.396, 1.249)	0.2298		
Race group (Other vs White)	0.991 (0.759, 1.293)	0.9456		

BMI= Body Mass Index; CI= Confidence interval; DP=docetaxel, prednisone; ECOG; Eastern Cooperative Oncology Group; HGB= Hemoglobin; LDH= Lactate dehydrogenase; PSA= Prostate-specific antigen

Discussion

Mainsail is one of the largest phase 3 trials in the setting of mCRPC in the past decade that investigated the addition of a second active biological drug to standard docetaxel every 3 weeks plus prednisone. In Mainsail the greater myelotoxicity caused by the addition of lenalidomide to docetaxel resulted in a reduction of the number of cycles of docetaxel that patients were able to tolerate – median of 6 cycles in the DPL arm vs. 8 in the DP arm. Median OS was shorter in patients receiving lenalidomide, which could have attributed to either a direct adverse effect of lenalidomide on OS, or, alternatively because of the reduction in the number of docetaxel treatment cycles. In this study we investigated the impact of the cumulative dose of docetaxel as reflected by the total number of cycles of docetaxel on median OS, in univariate and multivariate analyses on the ITT Population, both dependent upon the treatment arm, as well as irrespective of the treatment arm. In subsequent sensitivity analyses we addressed potential confounding factors on the eventual survival outcome, such as disease progression as the main reason for stopping docetaxel treatment, and excluding patients from the analysis who received less than a minimum of 5 cycles for whom meaningful survival benefit due to docetaxel was questionable and could therefore bias the analysis.

We found that the total number of docetaxel cycles delivered was an independent and important contributor to the overall survival benefit provided by docetaxel chemotherapy, that was independent of known prognostic factors for survival, including performance (ECOG-score), baseline LDH, baseline hemoglobin and baseline albumin[5]. Patients in the Mainsail study had been treated according to a strict protocol, mandating continuation of the allocated treatment until documented disease progression, or until unacceptable adverse effects occurred. In the sensitivity analysis we corrected for confounding factors, including disease progression as the reason for stopping docetaxel. The main reason for stopping protocol treatment early, though, was adverse effects. Enhanced toxicity by the addition of lenalidomide to docetaxel in the experimental arm resulted in a lower cumulative dose of docetaxel, reflected by fewer docetaxel cycles administered and some more frequent dose reductions. Since the median dose achieved per cycle administered was only modestly affected (respectively of the planned dose: 94.4% in the DPL arm and 95.6% in the DP arm), the number of cycles delivered was the key contributor to the different survival outcome[4]. Our data strongly suggest that the difference in the cumulative docetaxel exposure caused the worse OS in the experimental arm. These findings imply that the total dose of docetaxel, as reflected in total the number of cycles achieved, contributes to the eventual survival gain by chemotherapy in the mCRPC patient population.

This finding has important ramifications for the optimal administration of docetaxel chemotherapy. In order to provide the greatest survival gain by docetaxel chemotherapy, those patients who appear to benefit by clinical or radiological evidence and who tolerate

the chemotherapy well should continue beyond 6, and perhaps even beyond 10 cycles, until disease progression occurs or unacceptable adverse effects dictate otherwise.

An obvious limitation of this study is the posthoc nature of the analysis. Although all patients were treated according to the strict Mainsail study protocol, some patients may have discontinued for reasons not fully reflected in the study case report file. Subtle changes in PSA that may influence treatment decisions in daily clinical practice are less likely to have occurred in the context of a strict protocol, as evidenced by the observation that more than 50% of the patients continued treatment beyond 8 cycles. In addition, such potential unrecognized cessation of docetaxel treatment for non-specified reasons, is not likely to have a meaningful confounding effect, given the sample size of the study and the robustness and consistency of the data. We conducted both an ITT analysis and sensitivity analysis and all analyses point in the same direction. Of note, the number of cycles was independent of the performance score and other known prognostic factors for survival. In 2010 and subsequent years additional treatment options have become available in the post-docetaxel setting, including cabazitaxel[6], abiraterone[7,8], enzalutamide[9,10], and radium-223[11]. It is conceivable that many patients after ending treatment in the Mainsail study received at least one additional line of treatment. Unfortunately, no information on post study treatment was collected in the Mainsail database. We have no reason to anticipate any meaningful imbalance in post-docetaxel treatments between the groups, since the gap between halting docetaxel chemotherapy at 6 or 8 cycles and continuing additional cycles will be limited to a time gap of only a few months.

A prospective study directly comparing 6 vs 10 cycles, or beyond 10 cycles would be required to prove a survival benefit of docetaxel continuation. Barriers to conducting such a study would include higher neurological and extra-medullary toxicity expected with higher cumulative doses, higher costs and the robust findings of this present retrospective analysis. A similar question is what is the optimal number of docetaxel cycles in patients with metastatic hormone sensitive prostate cancer (mHSPC). In the two pivotal studies the survival benefit by the early use of docetaxel has been obtained with 6 cycles[12,13], while in the GETUG trial 9 doses of docetaxel were mandated[14]. In the mHSPC setting a study of 6 vs 10 cycles would help to answer that question.

Conclusion

In conclusion, we found a robust and independent impact on overall survival by the number of docetaxel cycles administered in the setting of mCRPC. These data indicate that patients who appear to have clinical, radiological or biochemical benefit by docetaxel should continue beyond 6 cycles, as long as they tolerate their treatment well. A prospective study, potentially in the setting of mHSPC, may lend further prospective evidence.

Acknowledgements
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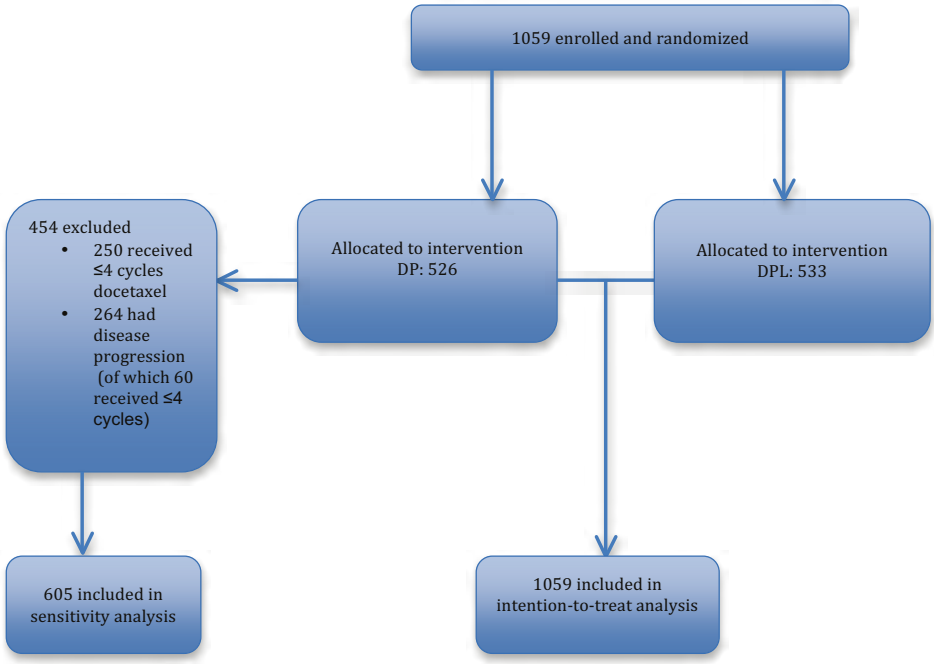
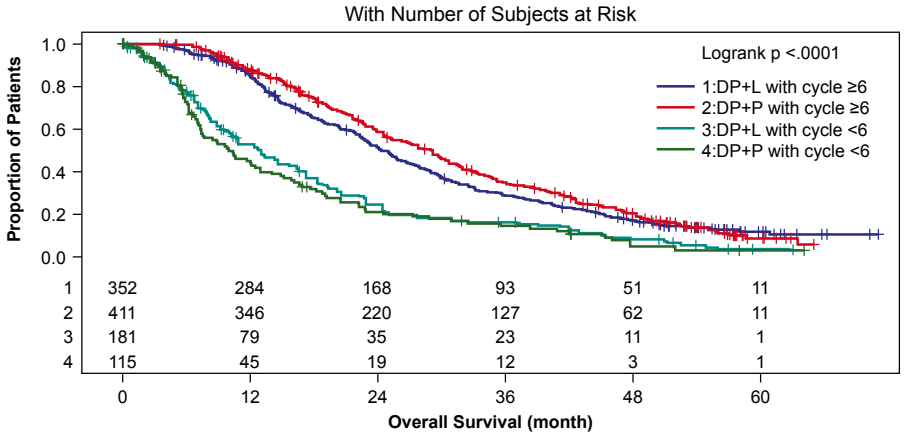
Supplementary data

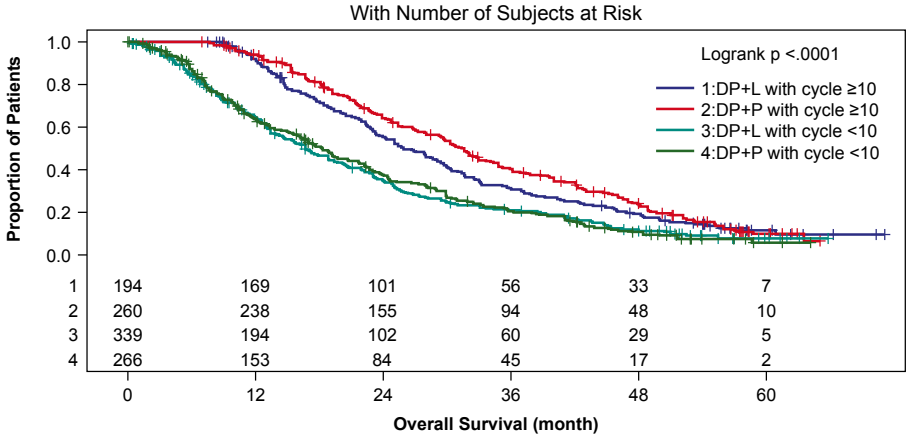


Supplementary figure 1: Overview of the patients included in the analysis



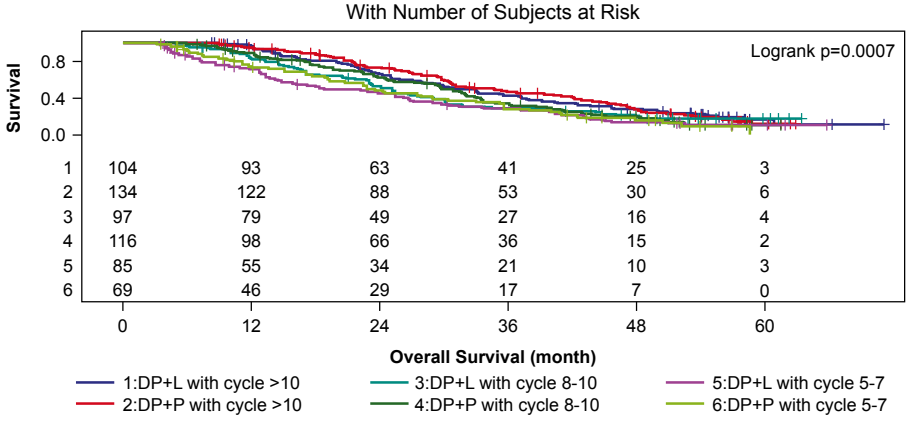
	No. of Subjects	Event	Censored	Median	Survival (95% CL)
1:DP+L with cycle ≥6	352	286 (81%)	66 (19%)	24.4	(22.8-26.6)
2:DP+P with cycle ≥6	411	329 (80%)	82 (20%)	29.1	(25.4-30.5)
3:DP+L with cycle <6	181	148 (82%)	33 (18%)	13.0	(10.2-16.4)
4:DP+P with cycle <6	115	94 (82%)	21 (18%)	10.0	(7.4-13.0)

Supplementary figure 2: Kaplan-Meier plots of overall survival for the DP and the DPL arm in the subgroups of number of docetaxel cycles <6 and ≥6.



	No. of Subjects	Event	Censored	Median	Survival (95% CL)
1:DP+L with cycle ≥10	194	160 (82%)	34 (18%)	25.8	(23.2-29.6)
2:DP+P with cycle ≥10	260	208 (80%)	52 (20%)	31.4	(29.1-34.0)
3:DP+L with cycle <10	339	274 (81%)	65 (19%)	16.6	(14.5-19.1)
4:DP+P with cycle <10	266	215 (81%)	51 (19%)	18.2	(16.0-21.6)

Supplementary figure 3: Kaplan-Meier plots of overall survival for the DP and the DPL arm in the subgroups of number of docetaxel cycles <10 and ≥10.



	No. of Subjects	Event	Censored	Median	Survival (95% CL)
1:DP+L with cycle >10	104	78 (75%)	26 (25%)	31.6	(25.8-37.2)
2:DP+P with cycle >10	134	100 (75%)	34 (25%)	34.7	(30.0-41.9)
3:DP+L with cycle 8-10	97	77 (79%)	20 (21%)	24.4	(22.2-28.1)
4:DP+P with cycle 8-10	116	92 (79%)	24 (21%)	29.7	(24.8-33.0)
5:DP+L with cycle 5-7	85	68 (80%)	17 (20%)	18.8	(14.2-26.6)
6:DP+P with cycle 5-7	69	54 (78%)	15 (22%)	23.6	(18.9-30.3)

Supplementary figure 4: Kaplan-Meier plots of overall survival for patients in the docetaxel prednisone and placebo (DP) and docetaxel prednisone and lenalidomide (DPL) arm. (Subgroups 5-7 cycles, 8-10 cycles and > 10 cycles of docetaxel).

Supplementary file: protocol summary

The present study contains a posthoc analaysis of the Mainsail study, which was a large phase trial that investigated the addition of lenalidomide, an anti-angiogenic agent with immunomodulatory properties, to docetaxel plus prednisone in a randomized double-blind placebo controlled phase 3 clinical trial. The primairy analysis of Mainsail was previously published in Lancet Oncology[1]. Please find a summary of the most important characteristics of the study below that are cited from the original publication.

Selection of patients

- Inclusion criteria
- Men aged 18 years or older at the time of consent;
- Life expectancy of 12 weeks or more;
- Eastern Cooperative Oncology Group (ECOG) performance status score of 2 or lower;
- Haemoglobin concentration more than 9 g/dL;
- Absolute neutrophil count more than 1·5 × 109 cells per L;
- Platelet count more than 100 × 109 cells per L;
- Creatinine clearance level more than 50 mL/min;
- Total bilirubin concentration less than 1·0 × upper limit of normal (ULN);
- Serum aspartate aminotransferase and/or alanine aminotransferase concentrations less than 1·5 × ULN concomitant with alkaline phosphatase concentration less than 2·5 × ULN.

Non-taxane-based adjuvant or neoadjuvant treatment completed more than 3 years before randomisation was allowed. Castration was defined as: effective castration as serum testosterone concentrations less than 50 ng/dL. Patients without previous bilateral orchiectomy continued treatment with luteinising hormone-releasing hormone agonists. Otherwise, concurrent anti-androgen therapy was only acceptable per investigator decision, if a 4 week or 6 week delay for anti- androgen washout would not compromise the patient's health and safety.

Exclusion criteria

- A history of clinically significant disease that places subject at an unacceptable risk for study entry
- Prior Therapy with thalidomide, lenalidomide or pomalidomide
- Prior chemotherapy for prostate cancer
- Use of any other experimental drug or therapy within 28 days prior to randomization

1 Petrylak DP, Vogelzang NJ, Budnik N, et al: Docetaxel and prednisone with or without lenalidomide in chemotherapy-naive patients with metastatic castration-resistant prostate cancer (MAINSAIL): a randomised, double-blind, placebo-controlled phase 3 trial. Lancet Oncol 16:417-25, 2015

- Prior radiation to ≥ 30% of bone marrow or any radiation therapy within 28 days prior to randomization
- Prior use of Strontium-89 at any time or Samarium-153 within 56 days prior to randomization
- Surgery within 28 days prior to randomization
- Concurrent anti-androgen therapy
- Abnormal serum chemistry or hematology laboratory values
- Significant active cardiac disease within the previous 6 months:
- Thrombotic or thromboembolic events within the past 6 months:
- History of peripheral neuropathy of ≥grade 2
- History of severe hypersensitivity reaction to drugs formulated with polysorbate 80
- Paraplegia
- History of Central nervous system (CNS) or brain metastases
- History of malignancies other than prostate cancer within the past 5 years, with the exception of treated basal cell/squamous cell carcinoma of the skin
- Concurrent use of alternative cancer therapies

Schema and treatment plan

Treatment was given in 21 day cycles of intravenous docetaxel (75 mg/m2) on day 1 of each cycle, plus oral prednisone 5 mg twice daily on days 1–21. Patients were pretreated with dexamethasone or corticosteroids, as per docetaxel label. In the lenalidomide group, lenalidomide was given orally at 25 mg/day on days 1–14 of each cycle; placebo was given on days 1–14 in the placebo group.

Rules for dose modification

We permitted dose modifications or discontinuation of study drug for treatment-related toxic effects; patients remained eligible to continue study treatment with the remaining other two drugs. However, complete withdrawal of both drugs resulted in study discontinuation. The lenalidomide dose could be reduced due to adverse events to a minimum of 10 mg/day, with one dose reduction allowed during any cycle. The docetaxel dose was reduced to 60 mg/m2 in case of febrile neutropenia, neutrophil count less than 500 cells per µL for more than 1 week, severe cumulative cutaneous reactions, or moderate neurosensory symptoms. If the adverse event did not resolve at a docetaxel dose of 60 mg/m2, treatment was discontinued. We did not allow re-escalation of lenalidomide or docetaxel after dose reductions. No dose reduction of prednisone was recommended in the protocol.

Measurement of treatment effect

Treatment continued until disease progression per Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1, with the exception of progression of bone lesions (non-target lesions) or discontinuation for other reasons. Treatment follow-up occurred every 90 days until death or up to 5 years after discontinuation.

The primary endpoint was overall survival, defined as the time from randomisation to death. Secondary endpoints were progression-free survival (defined as the time from randomisation to disease progression or death); the proportion of patients who achieved an objective response (defined as the proportion of patients with complete response or partial response); and safety. Progression-free survival and the proportion of patients with an objective response were determined by investigators according to RECIST version 1.1 criteria, except bone lesions. Assessments were done with either radiography, CT, and MRI, rather than clinical examination, except when lesions could not be imaged but were assessable by clinical exam; these assessments were done at screening (within 28 days before the first treatment dose, cycle 1 day 1) and then every subsequent third cycle day 1 (ie, every 9 weeks). If no death was reported for a patient before the cutoff date for overall survival analysis, we censored overall survival at the last date at which the patient was known to be alive. For analysis of progression-free survival, we censored patients who had progression or died more than 21 days after study treatment on the date of their last adequate tumour assessment before the last treatment date, plus 21 days. Each tumour assessment was assigned to one of the following categories: complete response, partial response, stable disease, progressive disease, and not evaluable. Patients who received another anti-tumour therapy before progression were censored on the last adequate tumour assessment before receiving the other anti-tumour therapy. Patients who progressed or died immediately after two or more consecutive missed visits for tumour assessment were censored at the date of the last adequate tumour assessment before progression or death. Patients who were still active at data cutoff and who had not progressed were censored on the date of their last adequate tumour assessment. Patients without baseline tumour assessments (or inadequate baseline tumour assessments) were censored on the date of randomisation.

Reasons for early cessation of trial therapy

Treatment continued until disease progression per Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1, with the exception of progression of bone lesions (non-target lesions) or discontinuation for other reasons. Additional reasons for treatment discontinuation included adverse events that, in the judgment of the investigator, could cause severe or permanent harm or could rule out continuation of study drug; disease progression, except progression attributable to a single new bone lesion; two or more new bone lesions, and for the first post-baseline reassessment only, a confirmatory scan done

at least 6 weeks later showing a minimum of two or more additional new lesions; patient withdrawal of consent; patient loss to follow-up; death; protocol violation; patient no longer able to adhere to the protocol (in investigator's opinion); or patient unwilling to comply with the lenalidomide counselling programme.

Safety

We assessed safety by evaluating adverse events (graded per National Cancer Institute Common Terminology Criteria for Adverse Events version 4.0), clinical laboratory data, physical examination, vital signs, concomitant medication and procedures, and electrocardiograms. These data were collected at screening, cycle 1 day 1, cycle 1 day 14, the first day of all subsequent cycles, and at treatment phase discontinuation. Measurements on cycle 1 day 14 were collected for the first 100 patients and any individual participating in the sparse pharmacokinetic sampling substudy, for evaluation during the initial scheduled DMC safety assessment. Additionally, information regarding adverse events and concomitant medications and procedures were also collected during the follow-up phase, at 28 days after last dose. ECGs were done at screening and at treatment phase discontinuation.

Objectives and entire statistical section (including endpoints)

The primary endpoint was overall survival, defined as the time from randomisation to death. Secondary endpoints were progression-free survival (defined as the time from randomisation to disease progression or death); the proportion of patients who achieved an objective response (defined as the proportion of patients with complete response or partial response); and safety. PSA was an exploratory endpoint, although it was not specified in the protocol.

Assuming that the median overall survival of placebo treatment in this trial would be similar to the published median overall survival (19·2 months) of placebo treatment of the TAX 327 study (docetaxel given every 3 weeks plus prednisone in men with metastatic hormone-resistant prostate cancer), the lenalidomide group had a targeted median overall survival of 25·0 months (30% improvement; targeted hazard ratio [HR] 0·77). This design allowed the demonstration of a significant difference in overall survival at a two-sided 5% significance level with at least 90% or power. We used the O'Brien- Fleming boundary to determine the nominal significance with an overall two-sided 5% significance level. On the basis of these assumptions, we planned to enrol around 1015 chemotherapy-naive patients with metastatic castration-resistant prostate cancer. An interim analysis for overall survival was planned when enrolment was complete and at least 468 events were observed. The final analysis was planned after 624 events and was done by Celgene. In addition to review of efficacy data, an independent data monitoring committee (composed of medical oncologists and a statistician, all of whom were not involved in the study as investigators)

also reviewed safety data on a predetermined schedule. We assessed safety data after 100 randomly assigned patients had either completed two treatment cycles or withdrawn from study treatment, and every 6 months after this first review. Furthermore, an initial safety assessment on day 14 of cycle 1 (for adverse events; physical examination; vital signs; and laboratory results for haematology and serum chemistry) was mandatory for all patients unless the independent data monitoring committee recommended that this assessment was no longer required. Additional safety assessments were to be done by the independent data monitoring committee as appropriate. We did efficacy analyses in the intention-to-treat population, comprising all patients who were randomly assigned. Patients who received at least one dose of study drug were included in the safety analyses.

We analysed overall survival and progression-free survival by the Kaplan-Meier method and log-rank test. We used a Cox proportional hazards regression model with only treatment included in the model to obtain the point estimate for HR and two-sided 95% CIs. All statistical analyses were done with SAS version 9.1 or higher.

41

Nuclear Eg5 (kinesin spindle protein) expression predicts docetaxel response and prostate cancer aggressiveness

Abstract

Novel biomarkers predicting prostate cancer (PCa) aggressiveness and PCa docetaxel therapy response are needed. In this study the correlation between nuclear Eg5-expression, PCa docetaxel response and PCa aggressiveness was assessed. Immunohistochemical staining for nuclear Eg5 was performed on 117 archival specimens from 110 PCa patients treated with docetaxel between 2004 and 2012. Samples were histologically categorized as positive/negative.

Median follow-up time from diagnosis was 11.6 years. Nuclear Eg5-expression was significantly related to docetaxel response ($p=0.036$) in tissues acquired within three years before docetaxel initiation. Nuclear Eg5-expression was not related to Gleason-score ($p=0.994$). Survival of patients after docetaxel initiation did not differ based on nuclear Eg5-expression ($p=0.540$). Analyzing samples taken before hormonal therapy, overall survival and time to docetaxel use were significantly decreased in patients with nuclear Eg5-expressing tumors ($p<0.01$). Eg5-positive nuclei were found more frequently in T4-staged tumors ($p=0.04$), Gleason 8-10 tumors ($p=0.08$), and in metastasized tumors ($p<0.01$). Multivariate analyses indicated that nuclear Eg5-expression may be an independent parameter for tumor aggressiveness. Limitations of a retrospective analysis apply.

In conclusion, nuclear Eg5-expression may be a predictive biomarker for docetaxel response in metastatic castrate-resistant PCa patients and a prognostic biomarker for hormone-naive PCa patients. Prospective validation studies are needed to validate nuclear Eg5 as a biomarker.

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Introduction

Metastatic castrate-resistant prostate cancer (mCRPC) is the second deadliest cancer in men in the Western world.[1] Primary first-line therapy for most mCRPC patients consists of the taxane docetaxel with prednisone.[2, 3] although various other mCRPC therapies have recently been introduced.[4-8] About 48% of patients initially respond to docetaxel therapy;[2] eventually all patients progress during or after docetaxel therapy, usually within few months after their last cycle. As docetaxel inhibits depolarization of microtubules regardless of cell type,[9] toxicities may be severe, such as polyneuropathy and bone marrow suppression.[2] To prevent or restrict unnecessary docetaxel use, and to determine the optimal treatment sequence for individual mCRPC patients,[10] biomarkers predicting docetaxel response need to be identified and implemented in clinical practice.[11]

We hypothesized that nuclear Eg5 (Kindle Spindle Protein/KSP/KIF11/kinesin-5) may be such a marker. Eg5 separates spindle poles of a mitotic cell by crosslinking two antiparallel microtubules and moving to the plus-ends of both microtubules.[12] Due to its essential function in mitosis, multiple Eg5-inhibitors have been developed for anti-cancer therapy, such as ispinesib.[13] Two studies with ispinesib focused particularly on mCRPC patients, with ambiguous results. In a phase I study, six out of fourteen mCRPC patients had stable disease (SD) for ≥18 weeks and one patient had a prostate-specific antigen (PSA)-decrease of >50% when ispinesib was combined with docetaxel in mCRPC patients.[14] In a phase II study in which ispinesib was administered as monotherapy, no responses were reported.[15] Twenty out of 21 patients had been treated with docetaxel prior to ispinesib. Immunohistochemistry analysis on archival tumor tissue from sixteen patients indicated that only one tumor stained positive for Eg5. It was concluded that ispinesib is not effective in primary prostate cancer (PCa) due to their low mitotic index, resulting in low Eg5 expression. However, considering their similar mechanism of action, an alternative explanation could be that cross-resistance occurs between docetaxel and Eg5-inhibitors.

Recent studies indicate that Eg5 may also play a role in intracellular transport in the cytoplasm, suggesting that Eg5-inhibitors may target Eg5 expressing non-mitotic cells too.[16, 17]. Xing et al. analyzed archival specimens from 80 patients with clinically localized PCa; half stained positive for Eg5, while benign prostate cells did not express Eg5.[18] Considering the low mitotic index of PCa cells regardless of disease stage,[19] these data suggest that Eg5 may indeed be expressed in non-mitotic PCa cells too.[20]

Combining aforementioned findings, initial Eg5 expression of PCa may have been decreased once tumors have become docetaxel resistant.[14, 15, 18] This led to our hypothesis that Eg5 may be a predictive marker for docetaxel response. Based on recent findings that patients with high Gleason-scores respond better to taxane-based therapy,[21] we further hypothesize that Eg5 may be a prognostic marker for tumor aggressiveness and clinical outcome.

Materials and methods

Collection of patient material and data

Formalin fixed and paraffin embedded (FFPE) human PCa samples (biopsies, transurethral resections of prostate (TUR-P) or radical prostatectomies), stored at room temperature, were collected from pathology archives of Leiden University Medical Center, Reinier de Graaf Gasthuis and Erasmus Medical Center Rotterdam. mCRPC patients who had pathological material available taken before docetaxel therapy were included. The study was carried out in accordance with the Dutch code of conduct for the secondary use of human tissues; informed consent was therefore not required when enough material remained to serve the patient's and family's needs.[22] Additional patient information was collected anonymously in a database. Approval was obtained from the Medical Ethics Board (METC) of Leiden University Medical Center (P12.219).

Immunohistochemistry

Samples (3µm sections) were stained for Eg5 using a polyclonal Anti-Eg5 antibody (1:1500, HPA006916, Sigma-Aldrich) on an automated immunohistochemistry stainer (Ventana Benchmark Ultra) (Fig. 1). This stainer utilized the ultraView Universal DAB Detection Kit (760-500, Ventana) for visualization of antibodies. The kit consisted of various enzyme labeled secondary antibodies that bind to primary antibodies; the complex was visualized with hydrogen peroxidase substrate and a 3.30-diaminobenzidine tetrahydrochloride (DAB) chromogen. For antigen retrieval, ULTRA CC1, an EDTA-Tris pH 8.4 solution, was used (950-224, Ventana). Representative images were taken at 20x10 under an Olympus BX41 microscope (Olympus Optical Co., Ltd.) from each slide using a colorview IIIu camera (Olympus), and analyzed with Cell^B imaging software (version 2.4108-181207). If an image was representative for the whole slide, only one picture was taken; otherwise, three representative views were imaged per slide.

Data analysis

Images were examined and scored blindly and independently by two researchers (MDW, ESdM). A clear contrast between nuclear and cytoplasmic Eg5 staining was evident (Fig. 1). Recent studies have indicated that intracellular functions of Eg5 may differ based on its subcellular localization;[17] not all functions may be related to docetaxel response. Therefore, samples were scored for positive or negative staining of nuclei, cytoplasm or any cellular compartment (nucleus and/or cytoplasm).

Samples were considered positive when in one high-power field of view (20x10) at least four cancer cells were positive, regardless of intensity. This cut-off value ensured that random mitotic cells, infrequently found in the negative control too, were excluded. For analysis, average scores from both observers were calculated. If >50% of all scores per sample were positive for Eg5, the sample was considered Eg5-positive; otherwise it was considered Eg5-negative.

Clinical endpoints

Clinical endpoints used in this study include survival from docetaxel initiation, overall survival (OS), time to symptomatic mCRPC and best therapy response.

Time to symptomatic mCRPC was defined as time between PCa diagnosis and docetaxel initiation. OS was calculated as time between diagnosis and patient death. If patients had not died or were lost to follow-up, survival was censored at the day the patient was last known to be alive before July 20th, 2013.Tumor aggressiveness was based on OS, time to symptomatic mCRPC, Gleason-score, and TNM-classification. Determination of best disease response (progressive disease, partial response) followed PCa working group guidelines as described previously, and could indicate PSA response and/or response as viewed on imaging such as computer tomography.[23, 24]

Statistical analyses

Microsoft Excel 2003 was used for basic statistical analyses; student's t-tests were conducted for comparisons. SPSS (version 20) was used for the Kaplan-Meier analyses of survival and time to symptomatic mCRPC; log-rank tests were used to compare these parameters between groups. Multivariate analyses were performed using the Cox-regression model. P-values ≤0.050 were considered statistically significant.

Results

Patient and tissue characteristics

In total, 117 samples were collected from 110 mCRPC patients. These patients had been diagnosed with PCa between 1994 and 2011 and treated with docetaxel between July 15th, 2004 and December 24th, 2012. Median time to follow-up from date of PCa diagnosis was 11.6 years (interquartile range 8.7-14.2 years). Clinicopathological parameters are listed in Table 1 (Supplementary figure 1). Median age of patients when diagnosed with PCa was 64 years. Median Gleason-score of tumors was 8. About two-thirds of patients had ≥2 measured metastatic localizations when docetaxel was initiated. Of note, tumor imaging methods such as CT-scans were not performed in all patients, underestimating the number of metastatic lesions. All patients had been medically and/or surgically castrated. In general, patients had been heavily pretreated: patients had received up to five therapies before docetaxel therapy.

For immunohistochemistry, tonsil and healthy prostate tissue served as positive and negative controls, respectively (Fig. 1A-B). Obtained PCa tissue consisted primarily of biopsies (70.0%) (Table 1). In the tumor samples, a clear distinction was observed between samples with nuclear Eg5 staining (5.1%), cytoplasmic Eg5 staining (19.7%), and samples staining positive for Eg5 in both compartments (63.2%), irrespective of the samples' age (Fig. 1C-F). Samples were scored for nuclear or cytoplasm staining separately. Interobserver agreement of scoring was 98.1%.

Immunohistochemical Eg5 expression and docetaxel response

Eg5 expression varied in tumors from some patients who had multiple biopsies taken before docetaxel therapy. This variability always reflected a disappearance of Eg5 expression over time. It is unknown whether these changes occurred as the tumor evolved spontaneously or due to other therapies, such as androgen-deprivation therapy. Therefore, correlation between Eg5 expression and docetaxel response was evaluated for all patients (n=110) as well as for patients with samples taken within three years before docetaxel start (n=61). A clear trend was observed between nuclear Eg5 expression and a better response to docetaxel therapy (Fig. 2A, supplementary figure 2). This correlation was significant in patients from whom tissue was taken within three years before docetaxel initiation: 71.9% of these patients with nuclear Eg5 expression had a PR versus 36.4% of patients without nuclear Eg5 expression (p=0.036). Conversely, cytoplasmic or any Eg5 expression did not predict docetaxel response (Supplementary figure 3).

Table 1: Characteristics of mCRPC patients (n=110), their disease and treatment, and of the obtained tissue (n=117).

Patient Age		
At time of prostate cancer diagnosis [median (range)]	64	(43-84)
At time of tissue sampling [median (range)]	65	(43-86)
At time of start docetaxel [median (range)]	69	(46-87)
Disease characteristics (diagnostic imaging)		
Gleason-score [median (range)]		
All patients	8	(4-10)
Hormone-naive patients	8	(4-10)
Number of metastatic lesions [number of patients (%)]		
1	37	(33.6%)
2	49	(44.5%)
≥3	24	(21.8%)
Confirmed localization of metastases [number of patients (%)]		
Lymph node	71	(64.5%)
Bone	106	(96.4%)
Liver	10	(9.1%)
Lung/pleura	16	(14.5%)
brain	1	(0.9%)
Treatment characteristics		
Pretreatment [number of patients (%)]		
Androgen-deprivation therapy	109	(99.1%)
Radical prostatectomy	15	(13.6%)
TUR-P	25	(22.7%)
Surgical castration	4	(3.6%)
Lymph node dissection	34	(30.9%)
Radiotherapy prostate	34	(30.9%)
Radiotherapy metastases	40	(36.4%)
Other	4	(3.6%)
Docetaxel treatment		
# courses [median (range)]	1	(1-3)
# cycles [median (range)]	6	(1-20)
Best response [number of patients (%)]**:**		
progressive disease	22	(20.0%)
stable disease	38	(34.5%)
partial response	49	(44.5%)
Docetaxel rechallenge [number of patients (%)]	7	(6.4%)

Table 1: Characteristics of mCRPC patients (n=110), their disease and treatment, and of the obtained tissue (n=117).

Patient Age	
Treatment characteristics	
Posttreatment [number of patients (%)]	91 (82.7%)
Cabazitaxel	16 (14.5%)
Abiraterone	30 (27.3%)
Enzalutamide	6 (5.5%)
Radiotherapy	47 (42.7%)
Strontium-89	24 (21.8%)
Samarium-153	4 (3.6%)
Mitoxantrone	15 (13.6%)
Other	8 (7.3%)
Obtained pathological material	
Type of material [number of samples (%)]	
Biopsy	82 (70.0%)
TUR-P	24 (20.5%)
Radical prostatectomy	11 (9.4%)
Disease stage [number of samples (%)]	
hormone-naive	87 (74.4%)
pre-docetaxel	112 (95.7%)
within three years of start docetaxel	61 (52.1%)
mCRPC post-docetaxel	5 (4.3%)
OS in years [median (IQR)]	4.8 (2.6-9.3)
Lost-to-follow-up [number of patients (%)]	18 (16.4%)

IQR, interquartile range; mCRPC, metastatic castrate-resistant prostate cancer; OS, overall survival; TUR-P, transurethral resection of the prostate

As a previous report identified Gleason-scores as a predictive marker for docetaxel response, it was tested whether a correlation existed between Gleason-score and docetaxel response in our set of patient samples (Supplementary table 1). Gleason-score was not related to docetaxel response, neither in all patients (p=0.343) nor in patients with tissue available in the three years before docetaxel initiation (p=0.884). Furthermore, Gleason-score and nuclear Eg5 expression were not related in this latter subpopulation (p=0.994), suggesting that nuclear Eg5 expression was an independent marker of docetaxel response.

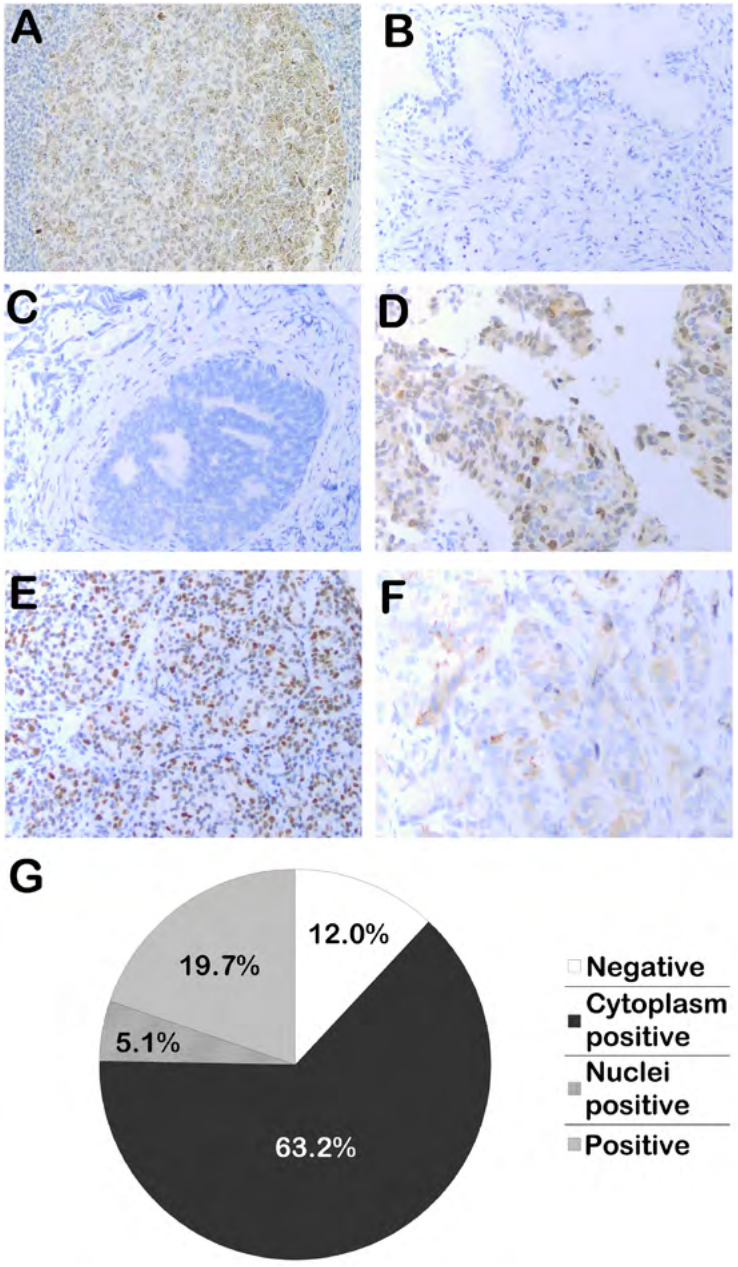

Figure 1. Immunohistochemical analysis of Eg5 expression in human clinical samples. A. Positive control: lymphatic tissue in a tonsil. B. Negative control: healthy prostate tissue. C. Prostate cancer (PCa) sample staining negative for Eg5. D. PCa sample with Eg5 expression in both the nuclei and cytoplasm. E. PCa sample with nuclear Eg5 expression. F. PCa sample with cytoplasmic Eg5 expression. G. Percentages indicate the frequencies samples with this subcellular staining pattern were found in our sample set (n=117).

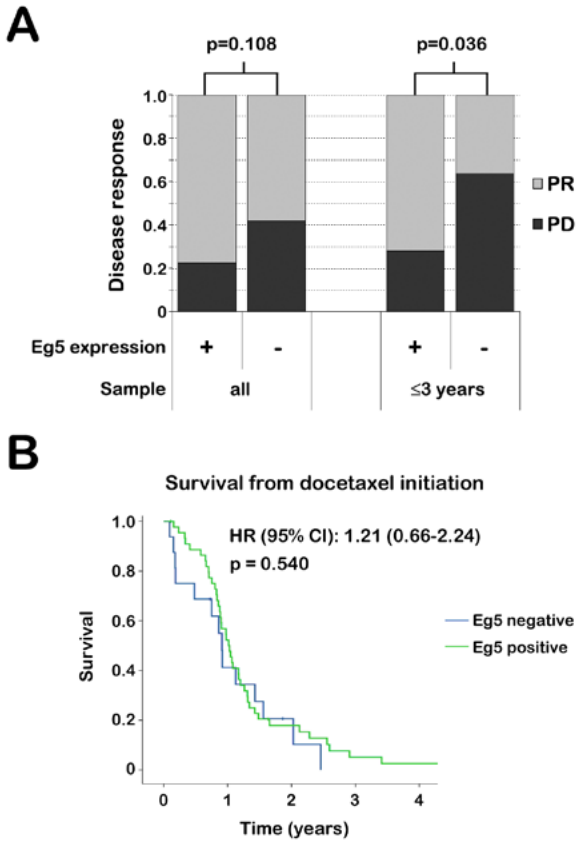

Figure 2. Nuclear Eg5 expression and docetaxel response in mCRPC patients. A. Best disease response to docetaxel therapy in mCRPC patients, grouped by nuclear Eg5 expression of their tumor. Patients with stable disease (SD) were excluded from this analysis. The most recent PCa tissue before docetaxel therapy was analyzed from all patients (left) or only from patients who had tissue available within three years before docetaxel therapy (right). In general, patients with nuclear Eg5 expression had a higher percentage of partial responses (PR). PD, progressive disease. B. Overall survival (OS) after docetaxel initiation. Patients were excluded when they only had PCa tissue available acquired more than three years before docetaxel therapy. Selected mCRPC patients were grouped based on nuclear Eg5 expression of their tumor. Median OS did not differ between patient groups, although initially there was more patient death in the group with Eg5-negative tumors.

We further explored the correlation between docetaxel response and Eg5 expression by investigating patients who had a PCa sample taken before and after docetaxel treatment. Only five patients matched these criteria. While cytoplasmic Eg5 expression did not alter in these patients, three out of four tumors with positive Eg5 nuclei before docetaxel therapy did not have nuclear Eg5 expression after docetaxel treatment (Supplementary figure 4). These three patients had progressive disease upon discontinuation of docetaxel. On the other hand, the patient whose tumor expressed nuclear Eg5 pre- and post-docetaxel

discontinued docetaxel therapy due to unacceptable toxicities. Despite the small patient number, these results suggested that loss of nuclear Eg5 expression may be related to docetaxel resistance.
Intriguingly, although patients with nuclear Eg5 expression had a better response to docetaxel (Fig. 2A), no difference in OS, calculated from the start of docetaxel therapy to death, was evident between tumors based on nuclear Eg5 expression (p=0.540) (Fig. 2B).

Immunohistochemical nuclear Eg5 expression and tumor aggressiveness

We evaluated whether tumors with nuclear Eg5 expression behaved more aggressively. Analyzing samples from all 110 patients, patients with tumors with nuclear Eg5 expression had a significantly decreased OS (median 6.6 versus 4.7 years, p=0.046) (Fig. 3A). Time from diagnosis to symptomatic mCRPC was also decreased (median 4.0 versus 2.8 years, p=0.037) (Fig. 3B). When selecting samples from hormone-naive patients (n=87), differences in OS and time to symptomatic mCRPC were even more pronounced (p=0.010 and p=0.006, respectively) (Fig. 3C-D). In this subset of patients, nuclear Eg5 expression was related to Gleason-score (p=0.014) and TNM classification (tumor stage, p=0.052; any metastases, p=0.007; distant metastases, p=0.021); no correlation existed between nuclear Eg5 expression and age (Fig. 4).

Multivariate analyses were performed to test whether the correlation between nuclear Eg5 expression and tumor aggressiveness (OS and time to symptomatic mCRPC) remained evident when correcting for potential confounding variables, such as Gleason-score (Table 2). When including all patients, addition of most covariates resulted in no statistically significant correlation between nuclear Eg5 expression and OS or time to symptomatic mCRPC. This included correction for age, while this variable was neither related to nuclear Eg5 expression nor to prognosis, suggesting the study was underpowered for such analyses. However, a trend towards positive nuclear Eg5 expression and aggressive tumors was evident. When assessing hormone-naive patients, a clearly positive trend existed between nuclear Eg5 expression and tumor aggressiveness regardless of the covariate added (hazard ratio >1.75), suggesting a potential independent prognostic value for nuclear Eg5 expression. The correlation between nuclear Eg5 expression and time to symptomatic mCRPC was significant in all subgroup analyses, except when metastases (N1 and/or M1) were added as a covariate (p=0.063).

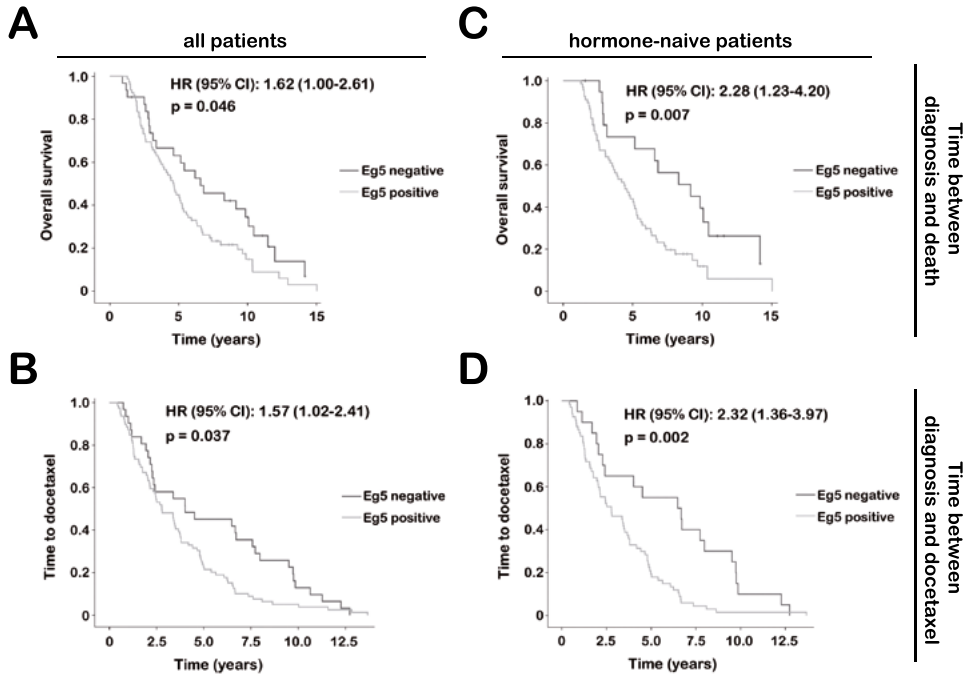

Figure 3: Tumor aggressiveness in mCRPC patients based on Eg5 expression. Patients were selected of whom PCa tissue acquired within three years (left) or three months (right) of diagnosis was available. Patients were divided in groups based on nuclear Eg5 expression. Median OS (top) and time to symptomatic mCRPC (bottom) were compared between patients groups.

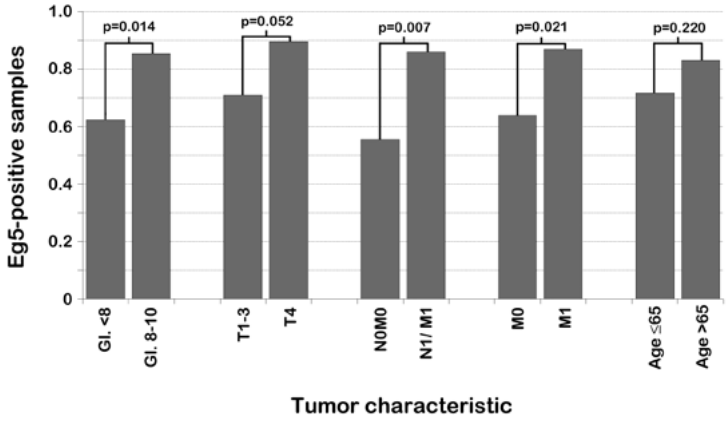

Figure 4: Correlation between PCa characteristics and nuclear Eg5 expression in hormone-naive PCa patients. Tumors were divided in groups based on Gleason-score and TNM-classification upon diagnosis; the percentage of tumors with Eg5 expressing nuclei was compared. Gl, Gleason; T, tumor stage according to TNM-classification; N, lymph node metastases (0, no metastases; 1, metastases); M, distant metastases (0, no metastases; 1, metastases), N1/M1, any metastases (lymph node and/or distant metastases).

Table 2: Multivariate analysis using the Cox-regression model exploring potential confounders for the correlation between nuclear Eg5 expression and tumor aggressiveness.

	All patients		Hormone-naive patients	
Covariate	**OS [HR (95% CI)]**	**TTD [HR (95% CI)]**	**OS [HR (95% CI)]**	**TTD [HR (95% CI)]**
Age	1.56 (0.95-2.54)	1.56 (1.00-2.42)	2.13 (1.13-4.02)	2.29 (1.31-3.98)
Gleason	1.48 (0.91-2.40)	1.45 (0.94-2.23)	1.76 (0.92-3.36)	1.78 (1.00-3.18)
Gleason <7 and ≥7	1.58 (0.95-2.63)	1.50 (0.95-2.38)	2.61 (1.35-5.07)	2.67 (1.50-4.75)
Gleason <8 and ≥8	1.48 (0.91-2.40)	1.46 (0.95-2.26)	1.84 (0.79-3.49)	1.93 (1.09-3.41)
T stage	1.75 (1.03-2.99)	1.59 (0.99-2.53)	2.56 (1.32-4.97)	2.64 (1.47-4.75)
Any metastases	1.52 (0.81-2.85)	1.28 (0.74-2.22)	2.25 (0.99-5.13)	1.97 (0.96-4.02)
Distant metastases	1.40 (0.79-2.49)	1.25 (0.75-2.08)	1.84 (0.87-3.87)	2.33 (1.14-4.79)
Number of metastases	1.64 (1.01-2.65)	1.59 (1.04-2.44)	2.61 (1.35-5.07)	2.67 (1.50-4.75)

CI, confidence interval; HR, hazard ratio; OS, overall survival; T stage, T stage according to TNM classification; TTD, time to docetaxel therapy.

Discussion

Research has been ongoing identifying prognostic biomarkers and biomarkers predictive for therapy response in PCa which have improved accuracy compared to established biomarkers such as serum PSA levels and Gleason-score, with some success.[11] Urokinase plasminogen activator and its inhibitor PAI-1, and Ki-67 have been identified as potential prognostic biomarkers of PCa.[25, 26] Cytoplasmic localization of the androgen-receptor and increased blood serum levels of Macrophage Inhibitory Cytokine 1 (MIC-1) have been identified as potential markers for PCa docetaxel response.[27, 28] PCa tumors expressing class III beta-tubulin were relatively insensitive to PCa therapy: class III beta-tubulin expression resulted in faster recurrence after radical prostatectomies, a decreased docetaxel response and decreased survival.[29] Unfortunately, none of these markers are available yet for use in clinical practice.[11] Additional studies, such as the one we present here, are needed to identify a biomarker to be used in clinical practice.

In the current study, we found that nuclear Eg5 expression in PCa was associated with improved antitumor efficacy of docetaxel, independently of patient's Gleason-score. Furthermore, we identified nuclear Eg5 as a prognostic marker in hormone-naive PCa patients: patients whose tumor expressed nuclear Eg5 had a decreased median OS and progressed more rapidly to mCRPC. Similar findings were reported in non-small lung cancer patients: patients with Eg5 expressing tumors had a better response to chemotherapy, but a lower OS.[30] Similarly, Eg5 expression was related to worse clinical outcome in renal cell carcinoma patients.[31]

Once docetaxel was initiated, survival of mCRPC patients was similar irrespective of nuclear Eg5 expression. This may indicate that nuclear Eg5 expressing tumors initially respond well to docetaxel, resulting in decreased patient mortality. However, once these Eg5 expressing tumors progress, these tumors behave more aggressively, increasing patient death. This trend could indeed be derived from the survival curve (Fig. 2B) and might explain why survival of patients with nuclear Eg5 expression is not increased after docetaxel treatment despite responding better to docetaxel therapy. Alternatively, other factors may have resulted in the similar survival curve, such as unequal patient and treatment characteristics between groups other than Eg5 expression.

Nuclear Eg5 expression could provide a useful tool for clinical practice. Interobserver agreement between researchers was very high (98.1%), as no subjective degrees of positive staining (mild/moderate/strong) were used. Positive/negative scoring requires little interpretation from the pathologist. Determination of Eg5 expression at the time of diagnosis would be non-invasive, as tissue material has already been acquired. Additional tissue sampling once the mCRPC stage has been reached, could aid the physician in deciding when to initiate docetaxel therapy. Patients whose tumor expresses nuclear Eg5 may benefit from early docetaxel treatment; patients with Eg5-negative tumors may be recommended to initiate other therapies first, as docetaxel response is more limited.

In the current study, a retrospective design was chosen, resulting in several limitations. FFPE PCa samples were collected from pathology archives; these samples were taken for diagnostic purposes (biopsies) and consisted of residual materials from surgical procedures such as TUR-P or radical prostatectomies. Therefore, the sample set we created was heterogeneous in origin. However, on the contrary to other tumors such as breast cancer, only limited tissue material is available from PCa patients during their disease, as many patients have a prostatectomy early in their disease and primarily suffer from bone metastases, which are not easily accessible. Furthermore, additional tissue sampling is often not needed, as it currently would not influence further therapy decisions. Therefore, although our initial patient number was relatively large, only limited tissue material was available from patients shortly before docetaxel initiation. Hence all samples taken within three years of docetaxel initiation were collected for analysis. This led to a heterogeneous cohort of samples (both biopsies and residual surgical material), representing various stages of PCa disease. Furthermore, patients may have received various treatments between tissue sampling and docetaxel initiation. E.g., antiandrogen treatment significantly changed gene expression profiles of prostate cancer.[32] It is unknown whether such treatment specifically affects nuclear Eg5 expression. To overcome these challenges, a prospective study will be needed in which tissue will be collected shortly before docetaxel initiation to confirm the correlation between nuclear Eg5 expression and docetaxel response. However, such a study

will need to overcome ethical and practical challenges as well.
In addition, our patient population was underpowered for multivariate analyses in hormone-naive patients. Further prospective studies are warranted to validate whether nuclear Eg5 expression may serve as an independent prognostic biomarker.

Previous studies found that PCa patients with aggressive tumors respond well to docetaxel, but also respond better to cabazitaxel, suggesting that aggressive tumors respond well to taxanes in general.[24, 33] Therefore, additional studies are needed to assess whether Eg5 predicts response to cabazitaxel too. Finally, our study results suggest that loss of Eg5 expression may be related to docetaxel resistance. Although ispinesib had limited antitumor efficacy after docetaxel, our study and previous phase I findings suggest that ispinesib may be effective when administered before or concomitantly with docetaxel, when up to 70% of tumors express nuclear Eg5.[14] However, combination therapy with docetaxel would need direct comparison to docetaxel monotherapy. Eg5-inhibitors may provide further clinical benefit when selecting mCRPC patients based on nuclear Eg5 expression (personalized medicine).

In conclusion, nuclear Eg5 expressing PCa is aggressive, but responds well to docetaxel. Loss of nuclear Eg5 expression may be associated with docetaxel resistance. Determining nuclear Eg5 expression in PCa samples may aid to improve timing to initiate docetaxel therapy in individual PCa patients. Additional prospective studies are needed to confirm the predictive and prognostic value of nuclear Eg5.

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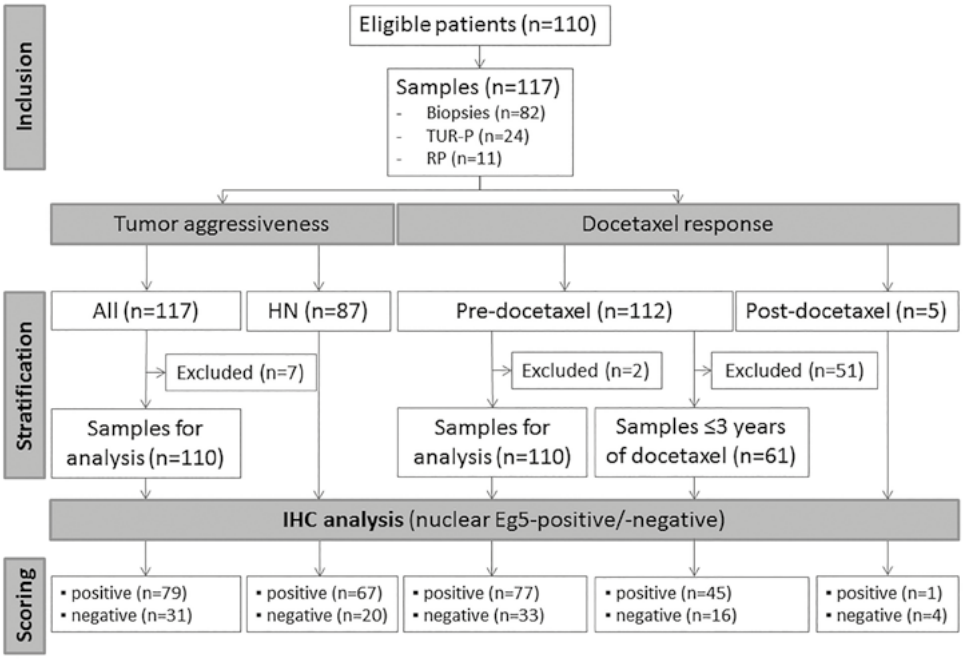
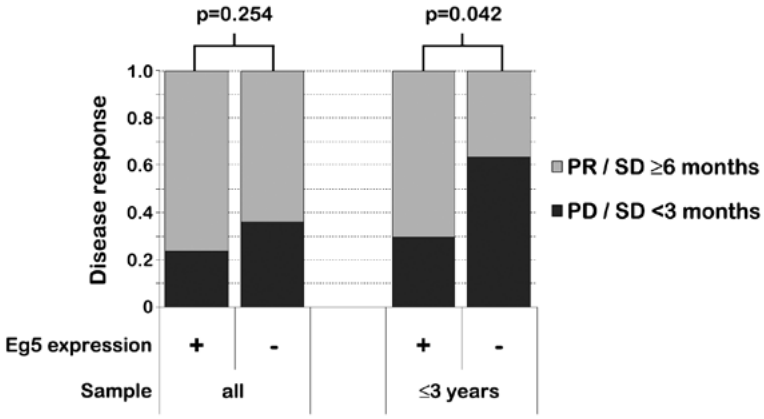
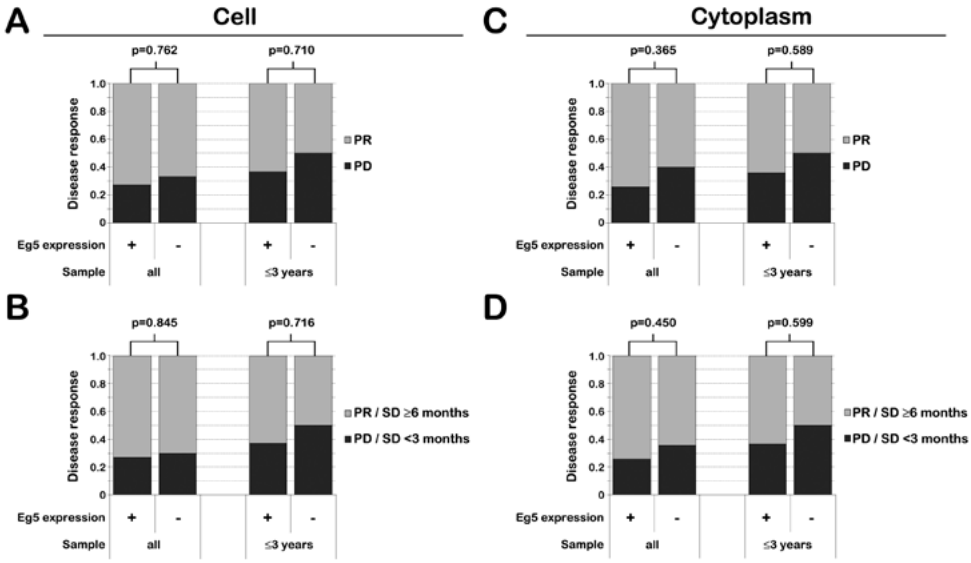
Supplementary data



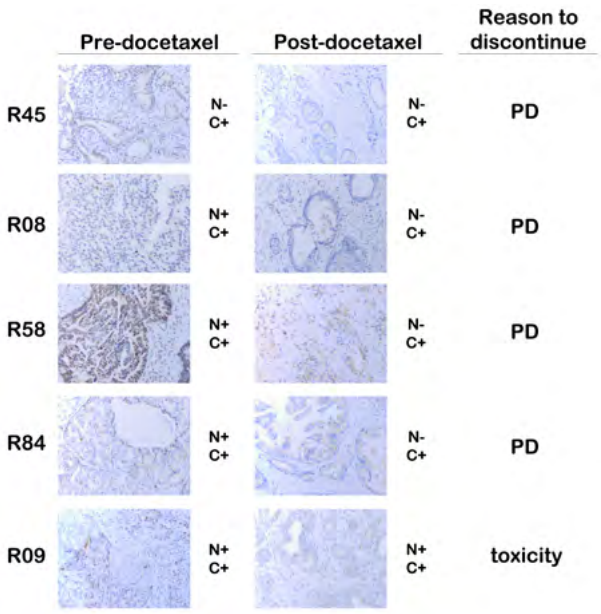
Supplementary figure 1. Diagram depicting sample size for all performed IHC analyses. Per patient, maximal one sample was included in each analysis: for tumor aggressiveness the patients' sample that had the shortest duration between diagnosis and acquisition of tumor material (resulting in 7 exclusions), for docetaxel response the sample that was taken shortest before docetaxel was started (resulting in 2 exclusions). HN, hormone-naive; IHC, immunohistochemistry; RP, radical prostatectomy; TUR-P, transurethral resection of prostate.



Supplementary Figure 2. Best disease response to docetaxel therapy in mCRPC patients, stratified by nuclear Eg5-expression of their tumor. Patients with a stable disease (SD) duration of three to six months were excluded from this analysis. The most recent PCa tissue before docetaxel therapy was analyzed from all patients (left) or only from patients who had tissue available obtained from the patient within three years before docetaxel therapy (right). In general, patients with nuclear Eg5-expression had a higher percentage of partial responses (PR) or extended SD (≥6 months). PD, progressive disease.



Supplementary Figure 3. Best disease response to docetaxel therapy in mCRPC patients, stratified by cellular (any compartment) (A-B) or cytoplasmic (C-D) Eg5-expression of their tumor. The most recent prostate cancer tissue before docetaxel therapy was analyzed from all patients (marked 'all' in each graph) or only from patients who had tissue available obtained from the patient within three years before docetaxel therapy (marked '≤3 years' in each graph). No relationship was evident between cellular or cytoplasmic Eg5 expression and docetaxel response (p≥0.365). A. and C. Patients with stable disease (SD) were excluded from this analysis. B. and D. Patients who had a partial response (PR) or SD ≥6 months (prolonged SD), as well as patients who had progressive disease (PD) or SD <3 months were selected for this analysis.



Supplementary Figure 4. Comparison of Eg5 expression in prostate cancer samples pre- and post-docetaxel. Cytoplasmic Eg5 expression did not alter in these patients, three out of four tumors with positive Eg5 nuclei before docetaxel therapy did not have nuclear Eg5 expression after docetaxel treatment. N+, positive nuclear staining; N-, negative nuclear staining; C+, positive cytoplasmic staining; C-, negative cytoplasmic staining; PD, progressive disease.

Supplementary table 1: Correlation between Gleason-score, docetaxel-response and nuclear Eg5-expression

	Number of patients	Gleason [median (IQR)]	p-value
Patients with tissue available within 3 years before docetaxel			
Eg5-positive	11	8 (7-9)	0.994
Eg5-negative	32	8 (7-9)	
Progressive disease	16	8 (8-9)	0.884
Partial response	27	8 (7-9)	
All patients			
Progressive disease	21	8 (7-9)	0.343
Partial response	49	8 (7-9)	

51

Targeting the androgen receptor confers in vivo cross-resistance between enzalutamide and docetaxel, but not cabazitaxel, in castration-resistant prostate cancer

Abstract

Treatment options for metastatic castration-resistant prostate cancer (CRPC) have evolved with the established benefit of novel androgen receptor (AR)-targeted agents abiraterone and enzalutamide in the pre-chemotherapy setting. At the same time, concerns of cross-resistance between the taxanes (i.e. docetaxel and cabazitaxel) and these AR-targeted agents have risen, and the optimal drug treatment sequence is unknown. Here, we investigated the in vivo efficacy of docetaxel and cabazitaxel in enzalutamide-resistant CRPC, and mechanisms of cross-resistance between these agents. Castrated mice harboring enzalutamide-resistant tumors and enzalutamide-naïve tumors were treated with docetaxel and cabazitaxel. Tumor growth kinetics, AR nuclear localization, AR regulated gene expression, Ki67 expression, and serum levels of PSA, docetaxel, and cabazitaxel were analyzed. Docetaxel inhibited tumor growth, AR nuclear localization, and AR regulated gene expression in enzalutamide-naive tumors, but did not in enzalutamide-resistant tumors, demonstrating in vivo cross-resistance. In contrast, cabazitaxel remained highly effective in enzalutamide-resistant tumors and demonstrated superior anti-tumor activity as compared to docetaxel, independent of the AR pathway. These findings demonstrate that the AR pa;thway is able to confer in vivo cross-resistance between enzalutamide and docetaxel, but not cabazitaxel, in CRPC.

PATIENT SUMMARY: We found reduced efficacy of docetaxel, but not cabazitaxel, in enzalutamide-resistant prostate cancer.

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Eur Urol. 2015 Jun;67(6):981-5

For almost a decade docetaxel has been the standard first-line chemotherapy for metastatic castration-resistant prostate cancer (mCRPC). In recent years, treatment options for mCRPC have evolved with the introduction of cabazitaxel, abiraterone, and enzalutamide, that all prolonged survival in the post-docetaxel setting [1]. Recently, the treatment paradigm has changed with evidence that novel AR targeted therapies abiraterone and enzalutamide are effective when administered to men with mCRPC also before chemotherapy [2,3]. With these novel AR targeting therapies also available in the pre-chemotherapy setting, treatment sequencing has become increasingly challenging, especially since concerns have been raised regarding the efficacy of docetaxel when used after abiraterone [4,5]. Clinical cross-resistance has been suggested in retrospective studies that demonstrated reduced efficacy of docetaxel in men with mCRPC who had previously been treated with abiraterone [4,5]. Moreover, a preclinical study by our group identified inhibition of AR nuclear translocation as an overlapping working mechanism that potentially confers cross-resistance between taxanes and AR targeted agents abiraterone and enzalutamide [6]. Interestingly, retrospective clinical data suggested that cabazitaxel, in contrast to docetaxel, remains effective in men with mCRPC after prior abiraterone [7,8]. The efficacy of docetaxel and cabazitaxel after first-line enzalutamide is yet unknown.

With the availability of novel hormonal agents before chemotherapy, there is an urgent need to investigate the optimal treatment sequence, and potential mechanisms of cross-resistance between the current treatment options. Here, we investigated the in vivo efficacy of docetaxel and cabazitaxel in castration-resistant prostate cancer (CRPC) with acquired resistance to enzalutamide, and mechanisms of cross-resistance between these agents.

We performed in vivo studies including the patient-derived, enzalutamide-naïve PC346C [9] and enzalutamide-resistant PC346Enza tumors as described in the Supplementary Methods. Tumors were analyzed for AR nuclear localization, Ki67 expression, and AR regulated gene expression. Serum levels of PSA, docetaxel and cabazitaxel were measured.

We first confirmed that the PC346Enza xenograft was resistant to enzalutamide in vivo (Fig.1A-B). Docetaxel showed good tumor responses as compared with placebo in castrate male mice bearing enzalutamide-naïve PC346C tumors (-78% mean tumor volume change from baseline (TVC), SEM +/- 7%), whereas its efficacy was impaired in mice bearing enzalutamide-resistant PC346Enza tumors (+364%TVC, SEM +/- 69%) demonstrating cross-resistance between docetaxel and enzalutamide in vivo ($P<0.01$) (Fig.1C-D). Progression-free survival and tumor growth curves over time are shown in Fig.1E-F, and Fig.S1. Concordant with the observed tumor responses, docetaxel reduced serum PSA levels as compared to placebo in castrate mice bearing PC346C, while it did not in mice bearing PC346Enza tumors (Fig.S2A-B). Thus cross-resistance between docetaxel and enzalutamide was not only observed at the level of tumor growth, but also in terms of clinically relevant serum PSA response, which is directly related to tumor volume.

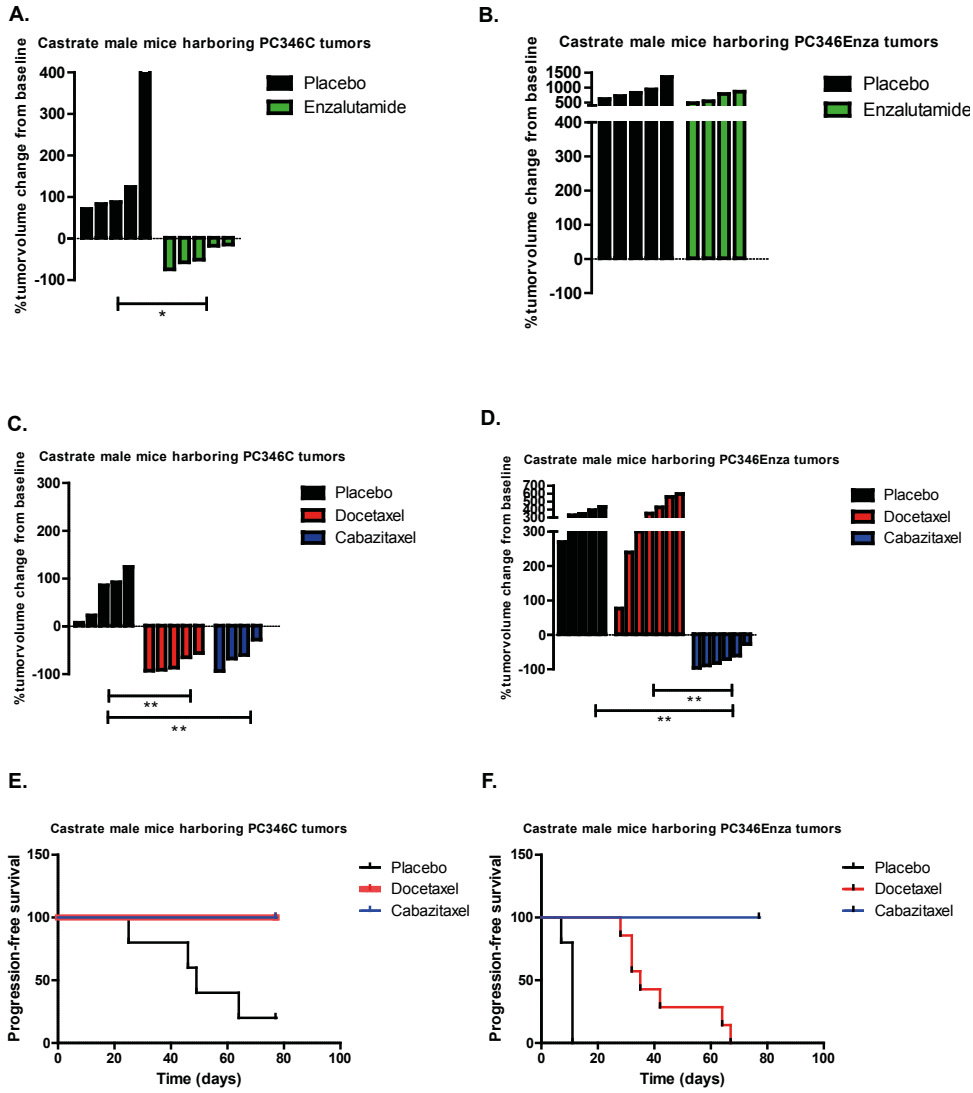

Figure 1. (**A and B**) Castrate male mice harboring enzalutamide-resistant PC346Enza tumors and the parental enzalutamide-naïve PC346C tumors were treated with daily oral enzalutamide (60 mg/kg) and placebo. (**C and D**) Castrate mice bearing PC346Enza and PC346C tumors were treated with docetaxel (33 mg/kg) and cabazitaxel (33 mg/kg) using a single intraperitoneal injection, or placebo. The percentage of tumor volume change form baseline was calculated after a cut-off of 77 days. Differences between groups were evaluated using an unpaired t-test. (*) represents $p<0.05$, (**) represents $p<0.01$. The exact p-values are quoted for comparisons with borderline significance ($0.05<p<0.10$), and the absence of a star indicates $p>0.10$. Bars represent individual mice. (**E and F**) Progression-free survival during 77 days was plotted with progression defined as a ≥50% increase in tumor volume.

Tumor responses for cabazitaxel were similar in PC346Enza and PC346C tumors, demonstrating that there was no cross-resistance between enzalutamide and cabazitaxel (Fig.1C-D). While docetaxel efficacy was impaired in mice bearing PC346enza tumors (+364%TVC, SEM +/- 69%), cabazitaxel remained very effective (-70%TVC, SEM +/- 10%) and demonstrated greater anti-tumor activity ($P<0.01$) and serum PSA declines (Fig.S2A-B) as compared to docetaxel.

Plasma concentrations of docetaxel and cabazitaxel were similar in enzalutamide pretreated versus non-pretreated control mice, indicating no effect of enzalutamide on the pharmacokinetics of both taxanes (Fig.S3, Table S1). Furthermore, plasma concentrations of docetaxel and cabazitaxel in mice were similar as compared to those reported in patients (Table S1) indicating that our observed cross-resistance occurs at clinical relevant concentrations.

While the expression of AR was similar among treatment groups (Fig.S4A-B), docetaxel was able to affect the downstream AR pathway by inhibiting intratumoral AR nuclear localization (Fig.2A,C) and the AR target gene PSA (Fig.S4C) in PC346C tumors. In contrast, while expressing lower baseline levels, docetaxel did not inhibit AR nuclear localization and PSA expression as compared to placebo in PC346Enza tumors (Fig.2A,C, and S4D), indicating a reduced anti-tumor activity via the AR pathway in these tumors. This impaired anti-AR effect in PC346Enza tumors was also observed for cabazitaxel (Fig.2A and S4C-D) and enzalutamide (Fig.S4G-H). Although the effects of cabazitaxel via the AR were impaired in enzalutamide-resistant tumors, it demonstrated stronger antiproliferative properties compared to docetaxel as depicted in Ki67 staining (Fig.2B-C), independent of the AR pathway.

In this study, we present the first evidence for in vivo cross-resistance between docetaxel and enzalutamide in CRPC. We showed that docetaxel efficiently impaired AR nuclear localization and consequently AR signaling in enzalutamide-naïve tumors, while it did not in enzalutamide-resistant tumors. These results indicate that the inhibiting effects of docetaxel on the AR represent part of its antitumor activity, which is impaired by previous AR targeted therapy such as enzalutamide. In this light, it could also explain the reduced efficacy of docetaxel when used after abiraterone that was observed in retrospective clinical studies [4,5]. Our findings are especially of interest with the increasing use of enzalutamide and abiraterone pre-chemotherapy.

In contrast to docetaxel, cabazitaxel demonstrated robust tumor and PSA responses in enzalutamide-resistant tumors, while the effects on AR signaling were reduced as compared to those in enzalutamide-naïve tumors. These observations indicate that cabazitaxel is less dependent on its inhibitory effects on the AR pathway, and exerts greater anti-tumor activity via AR independent mechanisms as compared to docetaxel. This is concordant with clinical observations [7,8], and is probably caused by a higher potency of cabazitaxel to suppress microtubule dynamics as compared to docetaxel, with faster drug uptake and

better intracellular retention[10]. This is further augmented by our observed lower Ki67 expression in enzalutamide-resistant tumors treated with cabazitaxel as compared to docetaxel, indicating stronger antiproliferative properties.

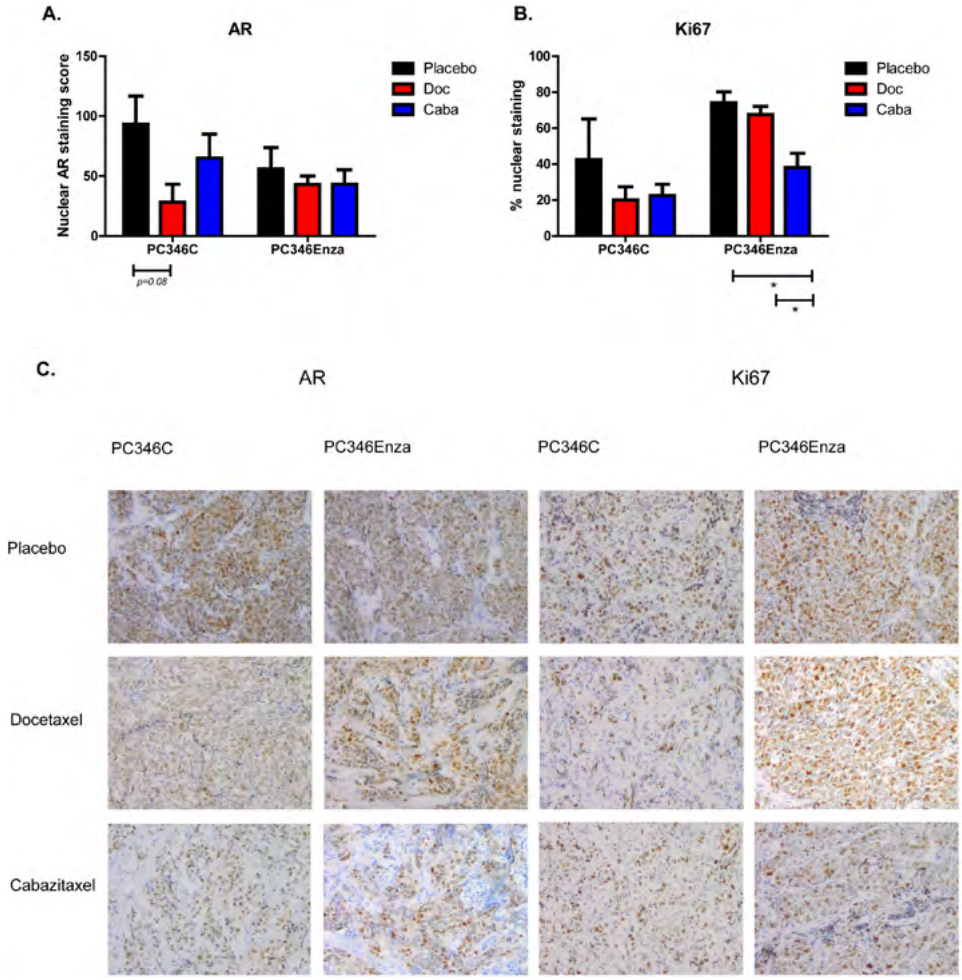

Figure 2. (**A and B**) AR nuclear localization and Ki-67 staining of enzalutamide-resistant PC346Enza tumors and the parental enzalutamide-naïve PC346C tumors. Immunostainings were scored by two readers, blinded for treatment and type of tumor. The score was composed using a sum of the nuclear AR score (0 for no stain, 1 for weak stain, and 2 for intense stain), each multiplied by the corresponding percentage of cells . Ki-67 score was calculated by estimating the percentage of positive cells in the whole tumor section. Differences in AR nuclear localization and Ki67 expression were tested using an unpaired t-test. (*) represents $p<0.05$, (**) represents $p<0.01$. The exact p-values are quoted for values with borderline significance ($0.05<p<0.10$), and the absence of a star indicates $p>0.10$.
(**C**) Representative pictures of AR nuclear localization and Ki67 staining in PC346Enza and PC346C tumors treated with docetaxel, cabazitaxel and placebo.

The greater potency of cabazitaxel after AR-targeted treatment might have clinical implications, as currently docetaxel is the standard first-line chemotherapy for men with mCRPC. Considering the superior efficacy of cabazitaxel over docetaxel in enzalutamide-resistant tumors, our results provide a rationale for clinical studies comparing cabazitaxel with docetaxel in men with mCRPC who progressed on first-line enzalutamide or abiraterone.

In summary, we demonstrated that a reduced inhibition of the AR pathway by docetaxel in enzalutamide-resistant CRPC confers cross-resistance between these drugs in vivo. Cabazitaxel remained highly effective in enzalutamide-resistant tumors, demonstrating greater antiproliferative properties independent of the AR pathway. This merits further clinical evaluation of cross-resistance and the optimal treatment sequence for patients with mCRPC.

Acknowledgements

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Supplementary methods

Supplementary Table 1: Plasma concentrations of docetaxel and cabazitaxel in mice with and without enzalutamide pretreatment

	Enzalutamide		*p* value (*t* test)	Patient plasma levels [a]	*p* value vs mice
	Pretreated	Not pretreated			
Plasma docetaxel (ng/ml)	1667 ± 639	1451 ± 1113	0.63	2180 (170)	0.07
Plasma cabazitaxel (ng/ml)	871 ± 634	1306 ± 931	0.30	535 (305)	0.14

Data are presented as mean ± SD.

[a] Patient plasma levels from phase 1 studies [1,2].

Cell lines and xenografts

The PC346C patient-derived prostate cancer xenograft and cell line were developed and maintained as described previously [3,4]. The enzalutamide-resistant PC346Enza cell line was generated from parental PC346C by long-time culturing in the presence of enzalutamide (1 μM) [5]. Both PC346C and PC346Enza cells harbor wild-type androgen receptor (AR).

In vivo experiments

PC346Enza and parental PC346C cells were subcutaneously inoculated into immunodeficient male (NMRI) mice. Mice were castrated when tumors reached a volume of 150–200 mm^3. After castration, mice were randomized to treatment with a single intraperitoneal dose of docetaxel (33 mg/kg), cabazitaxel (33 mg/kg), or placebo when a tumor volume of 300 mm^3 was reached. Mice bearing the enzalutamide-resistant PC346Enza xenografts were kept under selection pressure with enzalutamide until they received their assigned treatment. To confirm enzalutamide resistance, castrate mice bearing PC346C and PC346Enza tumors were randomized to receive placebo or oral enzalutamide once daily (Axon Medchem, Groningen, The Netherlands) at a dose of 60 mg/kg, which is in line with the optimal biological dose of 30–100 mg/kg in mice as reported by Clegg et al [6]. All placebo-treated PC346C xenografts were pooled for analyses. Tumor volumes were measured twice a week, and blood samples were taken every 2 wk and analyzed for serum prostate-specific antigen (PSA) levels. Tumor volumes were analyzed after a follow-up of 77 d after the start of treatment. Mice were euthanized before day 77 if a tumor volume of >1500–2000 mm^3 was reached. Serum PSA levels measured at baseline (at least 2 wk after castration) were compared with PSA levels after approximately 77 d or at the end of the treatment, whichever came first. All animal experiments were approved by the Animal Experiments Committee under the Dutch Experiments on Animals Act. Experiments were analyzed using Graphpad 5.0. An unpaired *t*-test was used for statistical evaluation.

Pharmacokinetics

To determine whether enzalutamide affected the pharmacokinetics of docetaxel and cabazitaxel, a separate experiment was conducted in mice that were pretreated with enzalutamide (60 mg/kg) for at least 2 wk and subsequently received an intraperitoneal injection of docetaxel or cabazitaxel (both 33 mg/kg). Mice that were not pretreated with enzalutamide also received an injection with docetaxel or cabazitaxel. At 3 h after administration, blood samples were taken to determine the plasma concentration of both taxanes using a validated LC/MS/MS assay, as previously described [7,8].

RNA isolation and real-time PCR

Total RNA from the xenografts was isolated using RNA-Bee (Tel-Test, Friendwood, TX, USA). Real-time PCR was performed in duplicate using a 7500 Fast Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) as previously described [9]. Gene expression was normalized against the average of two housekeeping genes (*GAPDH* and PBGD) using the ΔC_t method.

Immunohistochemistry

Immunohistochemistry to determine AR nuclear localization and Ki67 expression of the xenografts was performed using formalin-fixed, paraffin-embedded tissue sections. AR nuclear localization was determined after incubation with an anti-AR antibody (SP107, Dako, Glostrup, Denmark), treatment with an anti-rabbit secondary antibody (UltraMap), and visualization with diaminobenzidine (DAB)/H_2O_2. Ki67 was used as a biotinylated anti-mouse complex, detected with streptavidin conjugated to horseradish peroxidase, and visualized using DAB/H_2O_2. AR nuclear localization scores comprised the sum of nuclear AR scores (0 for no stain, 1 for weak stain, and 2 for intense stain), each multiplied by the corresponding percentage of cells [10]. The Ki67 score was calculated by estimating the percentage of positive cells in the whole tumor section. Tissue sections that could not be evaluated because of necrosis or insufficient cancer cells were excluded from the analysis. Immunohistochemistry slides were scored by two readers (RJvS and CFK) blinded to the treatment group and tumor type. The final blinded score was assigned via consensus.

Supplementary figures

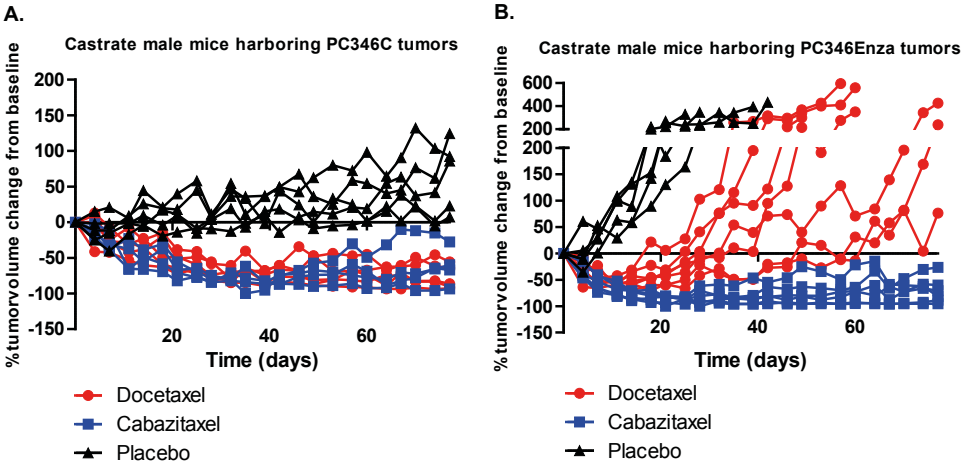


Figure S1. (A and B) Individual tumor growth curves over time in castrate mice bearing PC346C and PC346Enza tumors. Mice were treated with docetaxel (33 mg/kg), cabazitaxel (33 mg/kg) using a single intraperitoneal injection, or placebo.

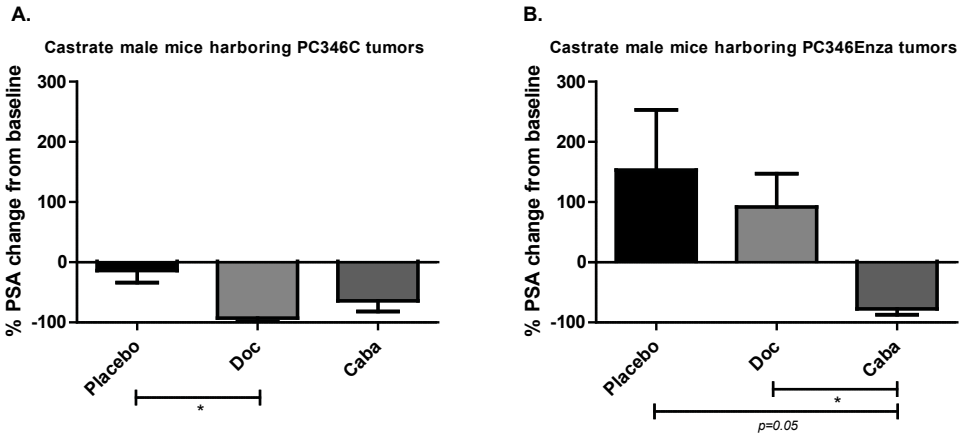


Figure S2. (A and B) Blood samples of mice harboring enzalutamide-resistant PC346Enza tumors and the parental PC346C tumors were taken every 2 weeks to determine serum PSA levels. Baseline serum PSA samples taken at least 2 weeks after castration were compared with PSA levels after approximately 77 days or end of treatment (whichever came first). The mean percentage of PSA change from baseline +/- SEM was plotted. Differences between groups were evaluated using an unpaired t-test. (*) represents $p<0.05$, (**) represents $p<0.01$. The exact p-values are quoted for comparisons with borderline significance ($0.05<p<0.10$), and the absence of a star indicates $p>0.10$.

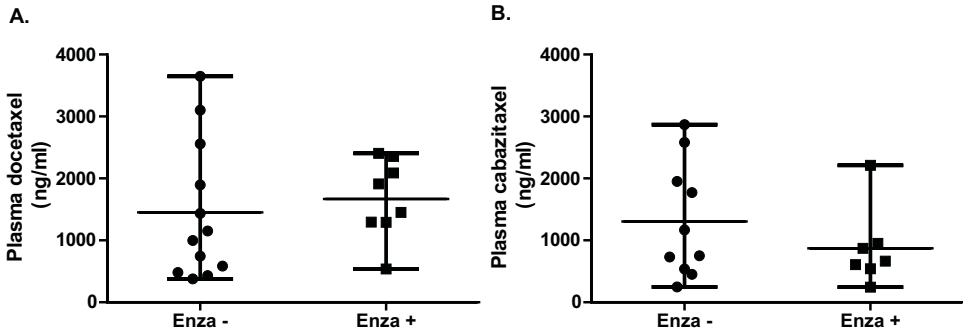
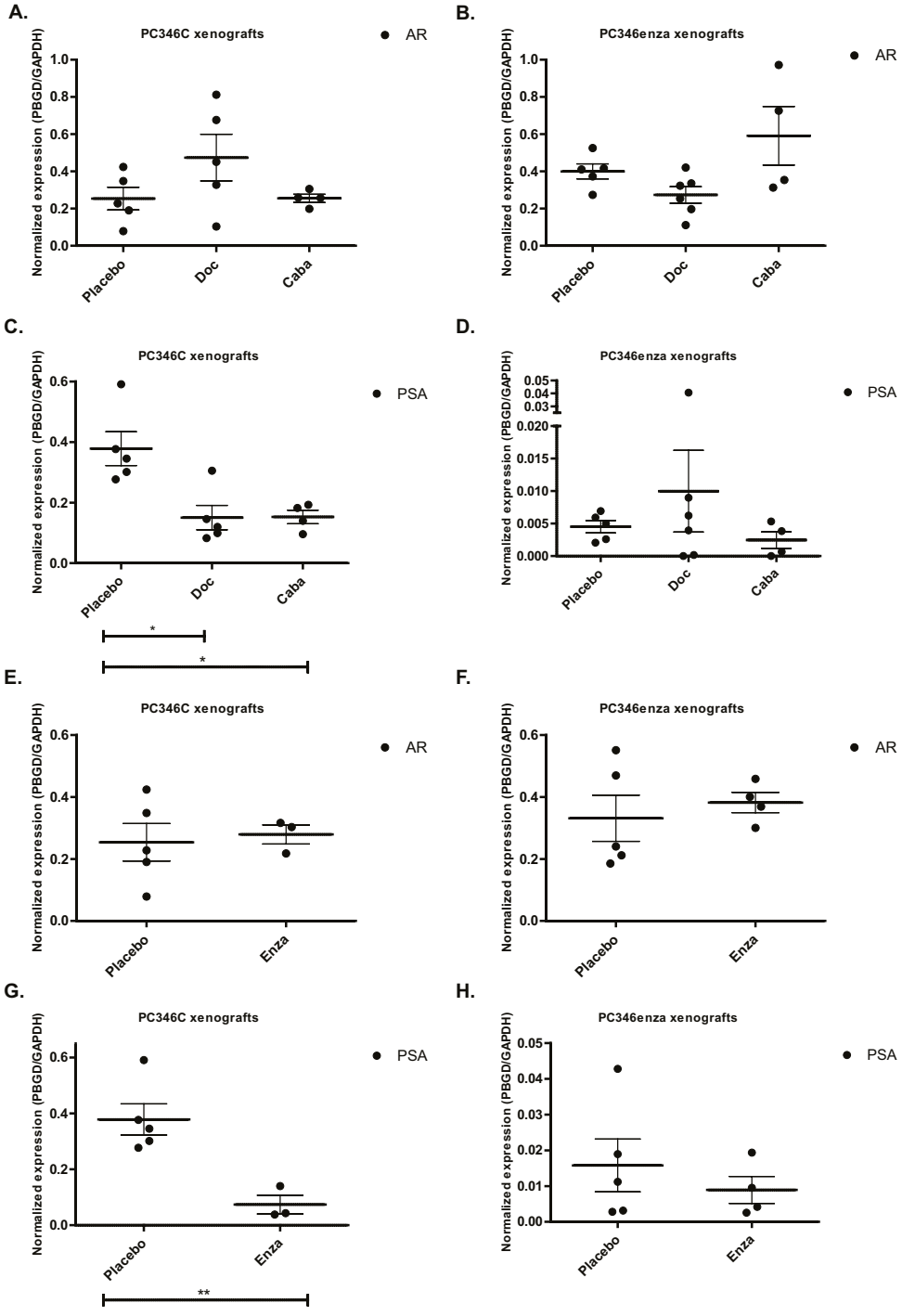


Figure S3. (**A and B**) Plasma concentrations 3 hours after intraperitoneal injection of docetaxel (33mg/kg) and cabazitaxel (33mg/kg) were measured in mice that were pre-treated with enzalutamide (60 mg/kg) and compared to mice that were non-pretreated. Plasma concentrations were measured using LC/MS/MS. Scatter plots including mean and range were used to represent the values. Differences between groups were evaluated using an unpaired t-test. Exact p-values are quoted in supplementary table 1. The absence of a star indicates that no statistical significant differences were observed.


A.
PC346C xenografts
AR
Normalized expression (PBGD/GAPDH)
1.0
0.8
0.6
0.4
0.2
0.0
Placebo
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Caba
B.
PC346enza xenografts
AR
Normalized expression (PBGD/GAPDH)
1.0
0.8
0.6
0.4
0.2
0.0
Placebo
Doc
Caba
C.
PC346C xenografts
PSA
Normalized expression (PBGD/GAPDH)
0.6
0.4
0.2
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Placebo
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PC346C xenografts
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Normalized expression (PBGD/GAPDH)
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PC346enza xenografts
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PC346enza xenografts
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Normalized expression (PBGD/GAPDH)
0.05
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Placebo
Enza

Figure S4. (**A and B**). Androgen receptor (AR) expression of enzalutamide-resistant PC346Enza tumors versus enzalutamide-naive PC346C tumors treated with docetaxel, cabazitaxel and placebo. (**C and D**) Expression of the downstream AR target gene PSA in PC346Enza versus PC346C tumors treated with docetaxel, cabazitaxel and placebo. (**E and F**) AR expression in PC346Enza versus PC346C tumors treated with enzalutamide and placebo. (**F and G**) Expression of the downstream AR target gene PSA in PC346Enza versus PC346C tumors treated with enzalutamide and placebo. RNA from the tumors was isolated and RT-PCR was performed as described in the supplementary methods. Gene expression was normalized against the average of two housekeeping genes (GAPDH and PBGD). Differences in gene expression were displayed using scatterplots including mean (+/- SEM) and tested using an unpaired t-test. (*) represents $p<0.05$, (**) represents $p<0.01$. The exact p-values are quoted for comparisons with borderline significance ($0.05<p<0.10$) and the absence of a star indicates $p>0.10$.

Supplementary references

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6

Understanding taxanes in prostate cancer; importance of intratumoral drug accumulation

Abstract

BACKGROUND: Resistance to docetaxel is common in metastatic castration-resistant prostate cancer (mCRPC) and may be caused by sub-therapeutic intratumoral drug concentrations. Cabazitaxel demonstrated survival benefit in docetaxel-pretreated and docetaxel-refractory patients. In this study we investigated whether the superior antitumor activity of cabazitaxel in mCRPC is explained by the higher intratumoral cabazitaxel levels. Since recent studies suggest a reduced efficacy of docetaxel following treatment with novel androgen receptor (AR) targeted agents, we also investigated taxane efficacy in an enzalutamide-resistant tumor model. **METHODS:** Intratumoral concentrations of docetaxel and cabazitaxel were correlated with antitumor activity in docetaxel-naïve, docetaxel-resistant and enzalutamide-resistant patient-derived xenografts (PDXs) of prostate cancer. **RESULTS:** Intratumoral drug levels were negatively related to intrinsic and acquired resistance to docetaxel. Also, the observed stronger antitumor activity of cabazitaxel was associated with increased cumulative exposure and higher intratumoral of cabazitaxel concentrations in all PDXs. **CONCLUSIONS:** The superior antitumor activity of cabazitaxel in docetaxel and enzalutamide-resistant tumors can be partly attributed to higher intratumoral drug concentrations. Especially for patients who are intrinsically resistant to docetaxel resulting from suboptimal intratumoral docetaxel concentrations, cabazitaxel may be the preferred chemotherapeutic agent.

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Introduction

Docetaxel is the standard first-line chemotherapy in metastatic castration-resistant prostate cancer (mCRPC). Unfortunately, there is a wide range in response, with some tumors being intrinsically resistant to docetaxel, while others progress during or within several months after cessation of docetaxel treatment(1-3). Cabazitaxel was developed for its ability to overcome docetaxel resistance. In preclinical studies it showed similar activity as docetaxel in a wide variety of docetaxel-sensitive tumor models and higher potency than docetaxel in tumor models with innate or acquired resistance to taxanes(4). Hence, cabazitaxel was approved for the treatment of mCRPC patients progressing during or after docetaxel(3). By inhibiting depolarization of the microtubules, both docetaxel and cabazitaxel block microtubule dynamics, which leads to cell trafficking disruption and cell cycle arrest in the G2/M phase and consequently apoptosis(5).

Efficacy of chemotherapy is determined by actual concentrations of the drug that can be achieved and maintained in tumor tissue(6). However, data on taxane concentrations in solid tumors and its relation to antitumor activity are rare. It was recently reported that high tumor uptake of [C^{11}]docetaxel corresponded with improved tumor response in non-small lung cancer patients(7). For prostate cancer, the correlation between intratumoral concentrations and antitumor activity of taxanes has not been established. We hypothesized that chemotherapy- resistance is strongly related to the actual drug levels that can be achieved in the tumor.

Chemotherapy resistance as seen in human cancers can be either intrinsic or be acquired in time after or during chemotherapeutic treatment. The exact mechanisms underlying the resistance phenotype are not yet fully understood and most likely involve multiple molecular, cellular and/or systemic mechanisms, simultaneously(8). Generally, drug resistance mechanisms can be classified in two main categories; one characterized by an impaired delivery, uptake or retention of the drugs in the cancer cell, the other by genetic and/or epigenetic alterations within the cancer cells that affect drug sensitivity(9). In prostate cancer, the heterogeneity in the duration of clinical responses to docetaxel among patients suggests that both intrinsic and acquired resistance may play a role.

In this study we used a unique panel of clinically relevant docetaxel-naïve and docetaxel-resistant patient-derived xenograft (PDX) models for prostate cancer to test our hypothesis that intratumoral drug concentrations and taxane efficacy are positively related. As recent studies suggested reduced activity of docetaxel in patients progressing on treatment with novel androgen receptor (AR) targeted agents such as abiraterone and enzalutamide (10-14), we also included an enzalutamide-resistant tumor model. Our findings indicate that docetaxel resistance in our prostate cancer models is related to insufficient intratumoral drug concentrations, which may be reversed by improving intratumoral taxane concentrations with cabazitaxel.

Materials and Methods

Drugs

Docetaxel and cabazitaxel (Sanofi, Vitry-sur-Seine, France) were prepared in polysorbate-80 and ethanol (1:1, v/v) and further diluted in a 5% (w/v) glucose solution to a final concentration of 2.5 mg/ml. Enzalutamide (Axon, Medchem, Groningen, NL) was prepared in 1% carboxymethyl cellulose, 0.1% Tween-80 and 5% DMSO.

The animal Welfare and Ethical statement

All experiments were approved by the Animal Experiments Committee under the Dutch Experiments on Animals Act and adhered to the European Convention for Protection of Vertebrate Animals used for Experimental Purposes (Directive 2010/63/EU). This study complies with the recommendations of ARRIVE.

Patient-derived xenografts (PDX) of prostate cancer

Chemotherapy-naïve PDXs PC339, PC374 and PC346C, docetaxel-resistant PDXs PC339-DOC and PC346C-DOC, and enzalutamide-resistant PC346Enza were selected from our panel of PDX models **(supplementary table 1)** (15-17). Docetaxel-resistant PDXs were created by subjecting tumor-bearing mice to intraperitoneal (i.p.) injections of docetaxel (33 mg/kg) every 14 days. Regrowing tumors were serially passaged and recipient mice were treated as described. Enzalutamide-resistant PC346C cells were obtained by in vitro culturing in the presence of 1μM enzalutamide until resistance developed as indicated by a growth rate similar to the parental PC346C. The established enzalutamide-resistant PC346Enza cell line was subsequently inoculated in mice and kept under selection pressure with enzalutamide (60 mg/kg) (10). Tumor fragments of the selected PDXs were subcutaneously transplanted to the flanks of 8 weeks old male NMRI nude mice (NMRI-Foxn1^{nu}; Taconic, Ry, Denmark). Mice were kept on a 12h dark/light cycle. Food and water were provided ad libitum.

Taxane concentration measurements.

Mice bearing chemotherapy-naïve, docetaxel-resistant and enzalutamide-resistant PDXs were treated with a single dose of docetaxel or cabazitaxel intraperitoneally (i.p) at.the following doses: 8.3, 16.5, 33, or 50 mg/kg, at a tumor volume (TV) of 300 mm^3. TV was measured twice a week using calipers. Mice were sacrificed at various time points: day 2, day 7, and day 13 post-treatment. Antitumor activity of taxanes was defined by log (TV at day of treatment / TV at sacrifice). Upon sacrifice, part of the tumor was stored for immunohistochemical analysis, the other part was snap-frozen for intratumoral drug concentration measurements. Tumor tissue was homogenized in lithium-heparinized human plasma (1:5 w/v) and processed for further analysis by a validated LC/MS-MS assay to determine docetaxel and cabazitaxel concentrations (18,19). The lower limit of quantification was 1 ng/ml for docetaxel and 2 ng/

ml for cabazitaxel. Intratumoral drug concentrations and antitumor activity were correlated using a Spearman's Rank Correlation Coefficients test at the 7 day post-treatment time point. This 7-days time point was the earliest time point at which the antitumor effect on growth could be reliable evaluated and at which taxane levels were still measurable, as was previously described for gynaecological tumors (20). The cumulative exposure to taxanes of the tumor tissue was derived from the total exposure to taxanes between day 2 and day 13 post-treatment as calculated using WinNonlin version 6.3 (Pharsight Corp., Mountain View, CA).

Treatment efficacy of docetaxel-resistant PDXs

PC339-DOC and PC346C-DOC tumor-bearing mice received a single injection of docetaxel or cabazitaxel at a dose of 33 mg/kg, 50 mg/kg or placebo. Tumor response was monitored over time and TV was measured twice weekly. Mice were sacrificed when tumors reached a TV of 1500 mm^3. Antitumor activity of taxanes was established using log cell kill as described in (21).

Log cell kill was calculated using the formula: log cell kill = (tumor growth delay)/(3.32* tumor doubling time) after a single treatment with chemotherapy (21). According to the National Cancer Institute (NCI), log cell kill values are translated to antitumor activity as follows: <0.7= - (inactive); 0.7-1.2 = +; 1.3-1.9 = ++, 2.0-2.8= +++, >2.8= ++++. Resistance to taxanes was defined as log cell kill < 0.7.

Immunohistochemistry

Tumor tissues were fixed with 10% formalin and embedded in paraffin. Tissue sections (4 μm) were stained for the cell proliferation marker Ki-67 using a monoclonal mouse anti-Ki-67 antibody (1:100, MIB-1, Dako Cytomation, Denmark) according to a standard protocol as described previously(10). Percentage of Ki-67 positivity was scored independently by two investigators; 100 cells were counted per tumor.

Data analysis and statistical procedures

Data were analyzed using Graphpad Prism 5.0. (Graphpad software, La Jolla, CA). Data are presented as mean ± Standard error of the Mean (SEM) unless stated otherwise. Student's unpaired **t test was used to compare two groups.** P<0.05 was considered statistically significant.

Results

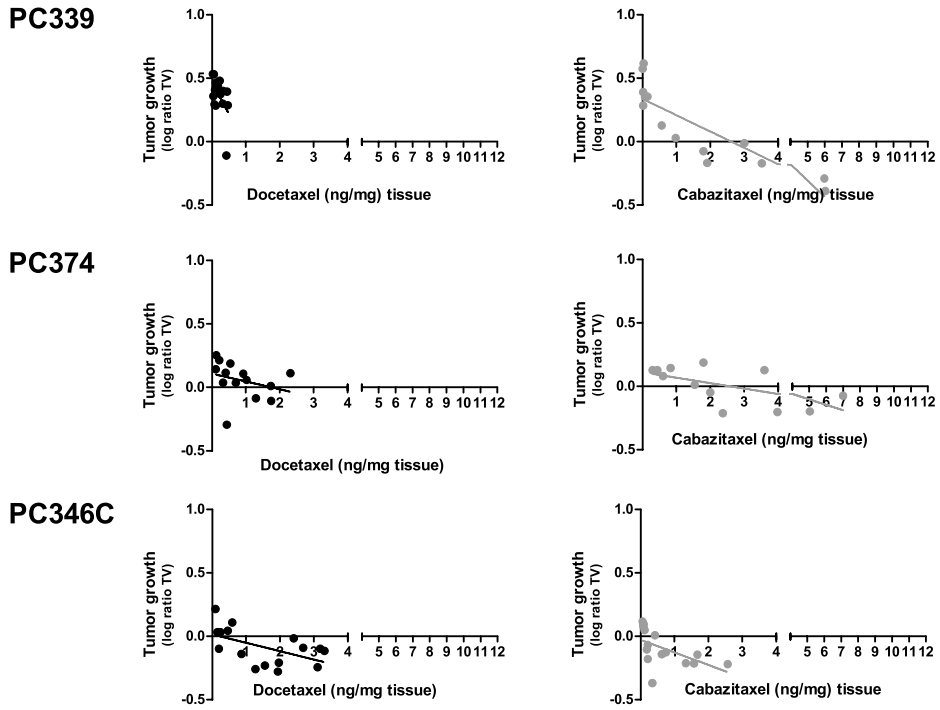
Tolerability and tissue distribution of docetaxel and cabazitaxel

Both taxanes showed comparable plasma pharmacokinetics **(supplementary fig s1)** and were tolerated well with no signs of toxicity or excessive body weight loss **(supplementary fig s2)**. Tissue distribution profiles of docetaxel and cabazitaxel at 7 days after treatment are

shown in **supplementary fig s3**. Interestingly, cabazitaxel accumulates in brain tissue, and both taxanes accumulate in mouse prostate.

Intratumoral taxane concentrations in chemo-naïve PDX correlate to efficacy

In chemotherapy-naive PDXs, PC339, PC374, and PC346C, a strong correlation was found between intratumoral concentrations and antitumor activity of docetaxel and cabazitaxel. In general, cabazitaxel levels were found to be higher as compared to docetaxel in docetaxel-naïve tumors **(fig 1)**.



Xenograft	Docetaxel				Cabazitaxel			
	Response	Spearman r	95% CI	P-value	Response	Spearman r	95% CI	P-value
PC339	-	-0.43	-0.78 / 0.12	0.11	+++	-0.93	-0.98 / -0.79	<0.0001
PC346C	+	-0.54	-0.83 / -0.05	0.03	+	-0.78	-0.93 / -0.43	0.0006
PC374	++	-0.57	-0.85 / - 0.04	0.03	++	-0.57	-0.87 / - 0.03	0.05

Figure 1: Antitumor activity correlates with intratumoral concentrations of taxanes. Increased intratumoral taxane concentrations associate to a stronger tumor growth inhibition in PC339, PC374 and PC346C. Individual xenograft bearing mice were given a single dose of docetaxel or cabazitaxel at the following concentrations: 8.3, 16.5 or 33 mg/kg. Mice (n=4-5 per dose cohort) were sacrificed 7 days after treatment. Tumors were analyzed for intratumoral taxane concentration measurements. Log (tumor volume at day of treatment / tumor volume at day 7 after treatment) was taken as a measure for antitumor activity. Log ratio > 0 indicates increased tumor growth, <0 indicates tumor growth inhibition. TV=tumor volume.

Cabazitaxel was retained longer in tumor tissue compared to docetaxel in PC339 and PC374 tumors ($p<0.001$ in both PDXs), corresponding to higher cumulative exposures of 57.3 ng*day/mg in PC339 and 47.3 ng*day/mg in PC374 (**fig 2)**.

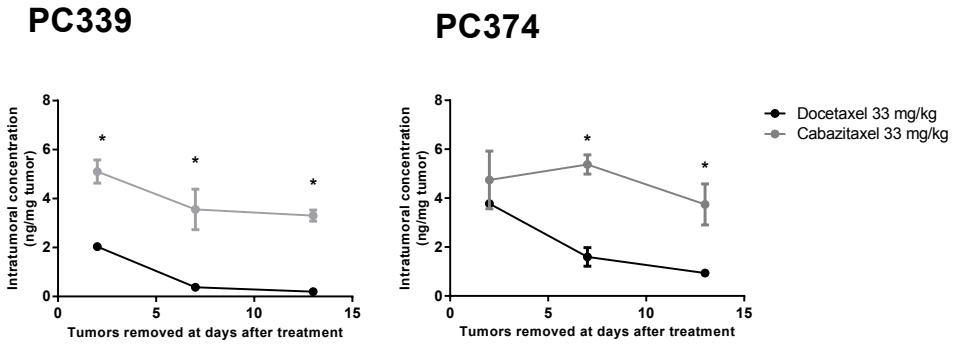


Figure 2: Cumulative exposure of taxanes in tumor tissue. Intratumoral taxane concentrations were measured after a single injection of either docetaxel 33 mg/kg or cabazitaxel 33 mg/kg. PC339 and PC374 tumors were removed at 2, 7 and 13 days after treatment. Each point indicates an average intratumoral concentration ± SEM of 3-7 tumors from independent mice (n=4-5). * $p<0.05$, intratumoral cabazitaxel concentrations differed significantly compared to the intratumoral docetaxel concentrations.

In contrast, docetaxel exposures were 9.8 ng*day/mg in PC339 and 24.8 ng*day/mg in PC374. The overall higher intratumoral accumulation of cabazitaxel was accompanied by an increased efficacy with stronger anti-proliferative effects as indicated by a reduction in Ki-67 expression **(fig 3)** and higher log cell kill values. Log cell kill values for cabazitaxel were 2.8 for PC339 and 1.8 for PC374, while log cell kill of docetaxel was 0.4 for in PC339 and 1.6 for PC374 **(supplementary table 1)**.

Acquired resistance to docetaxel is associated with decreased intratumoral concentrations

Based on the relative sensitivity of PC346C (log cell kill 1.0) for docetaxel and the extensive period of time of one year in which the tumor was passaged several times, to obtain the fully docetaxel resistant PC346C-DOC (log cell kill 0.2), we consider this PDX to represent an acquired resistance phenotype. When treated with the standard dose of docetaxel (33 mg/kg) the intratumoral concentrations of docetaxel were very low in PC346C-DOC compared to those in chemotherapy-naïve PC346C and very likely inadequate to induce tumor inhibition **(fig 4B)**.

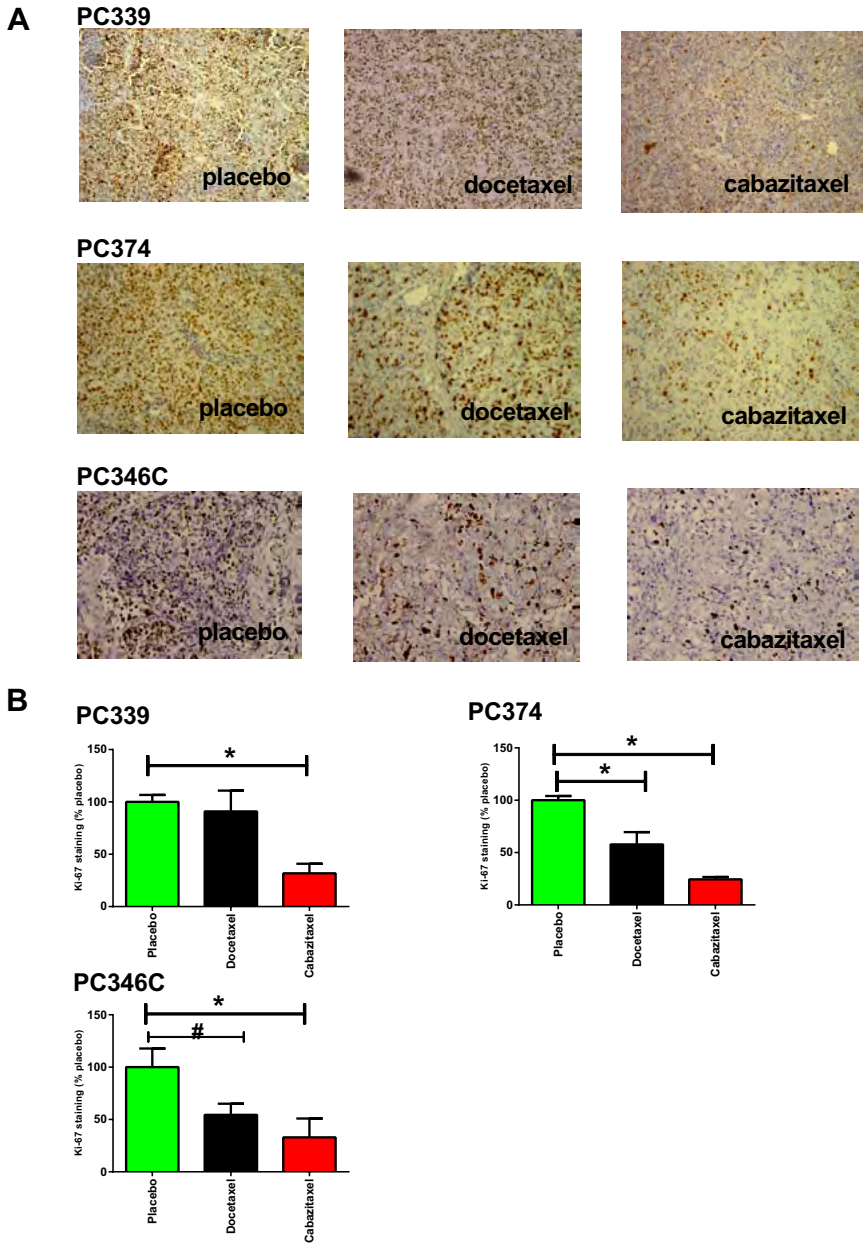


Figure 3: Ki-67 expression after treatment with taxanes. PC339-, PC374- and PC346C-bearing mice were treated with a single dose of placebo, 33 mg/kg docetaxel or 33 mg/kg cabazitaxel. **A)** Tumors were excised at day 7 after treatment, formalin fixed, paraffin embedded and stained for Ki-67 using immunohistochemistry. **B)** Percentage of Ki-67 positive cells quantified in 3-4 tumors derived from independent mice. Mean percentage of Ki-67 positive cells ± SEM is shown, * indicates p-value < 0.05, # trend p=0.06.

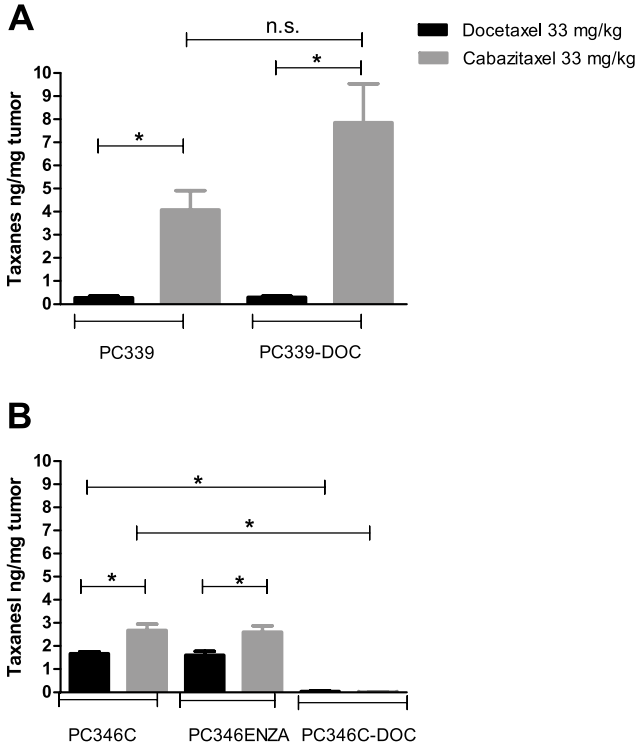


Figure 4: Intratumoral taxane concentrations in chemotherapy-naive versus docetaxel-resistant and enzalutamide-resistant PDXs. Intratumoral taxane concentrations were measured at day 7 after a single treatment with docetaxel or cabazitaxel at 33 mg/kg in A) PC339 vs PC339-DOC, B) PC346C vs PC346ENZA and PC346C-DOC. Each bar indicates an average intratumoral concentration ±SEM of 3-7 tumors derived from independent mice. * indicates P<0.05. n.s.: non-significant

Interestingly, this PDX also responded poorly to cabazitaxel as could be expected from the very low intratumoral cabazitaxel levels **(fig 5A, right panel). I**ncreasing the dose of docetaxel or cabazitaxel to 50 mg/kg did not improve neither intratumoral taxane levels nor antitumor activity **(fig 5B, right panel)**. Log cell kill values went from 0.2 to 0.4 for docetaxel, and from 0.2 to 0.6 for cabazitaxel, which in all cases is considered inactive indicating cross-resistance between both taxanes.

Intrinsic resistance to docetaxel can be overcome by enhancing intratumoral concentrations

In contrast to the acquired resistance of PC346C-DOC, the PC339 PDX (log cell kill 0.4) developed resistance to docetaxel within a couple of months, resulting in PC339-DOC representing an intrinsic resistance phenotype (log cell kill 0.2). Intratumoral docetaxel concentrations in PC339-DOC tumors reached after a standard dose of 33 mg/kg were

insufficient to induce an antitumor effect **(fig 4A)**. Interestingly, however, docetaxel resistance could be overcome by a higher docetaxel dose (50 mg/kg), significantly enhancing docetaxel response with log cell kill improving from 0.2 (inactive) to 0.9 (active) **(fig 5A, left panel)**. As expected, intratumoral docetaxel levels increased from 0.29 ± 0.06 ng/mg tissue after dosing with 33 mg/kg to 1.1 ± 0.11 ng/mg tissue (p=0.001) after treating the mice with 50 mg/kg.

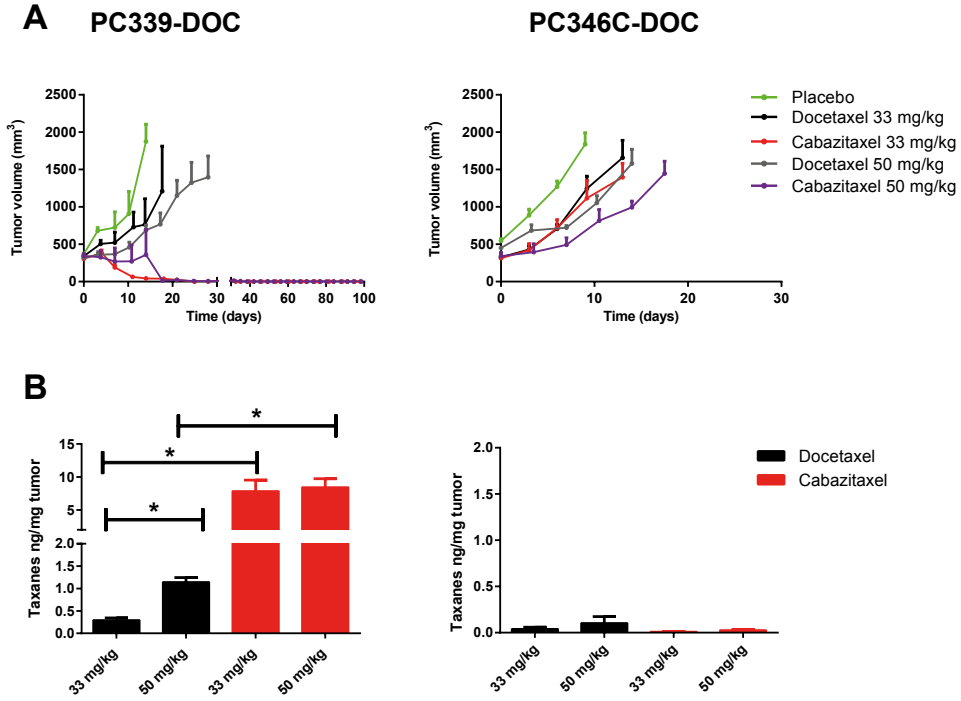


Figure 5: Response to taxanes of docetaxel-resistant PDX models. A) Average tumor growth curves are shown of PC339-DOC and PC346C-DOC after a single treatment with placebo, docetaxel 33 mg/kg, docetaxel 50 mg/kg, cabazitaxel 33 mg/kg or cabazitaxel 50 mg/kg. Average tumor volume ±SEM is shown for 4-5 mice per treatment cohort. **B)** Intratumoral taxane concentrations in PC339-DOC and PC346C-DOC after single treatment with either 33 mg/kg docetaxel/cabazitaxel or 50 mg/kg docetaxel/cabazitaxel. Average intratumoral taxane concentration ±SEM is shown of 4-5 mice per group. *p<0.05

Cabazitaxel yields higher intratumoral concentrations in docetaxel-resistant and enzalutamide-resistant tumors

Interestingly, in the intrinsically resistant PC339-DOC, cabazitaxel consistently yielded intratumoral concentrations that were on average 26.7 fold (p=0.01) higher: 7.9 ± 1.3 ng cabazitaxel /mg tumor tissue versus 0.29 ± 0.09 ng docetaxel /mg tumor tissue **(fig 4A),**

with corresponding superior antitumor activity (fig. 5A). Interestingly, in the enzalutamide-resistant PDX PC346Enza (10), on average 2.6 fold higher intratumoral levels of cabazitaxel could be achieved (2.61 ± 0.27 ng/mg) compared to docetaxel (1.00 ± 0.17 ng/mg) (p=0.01) **(fig 4B**), resulting in higher efficacy in this model as describes previously by our group(10).

Discussion

Intrinsic and acquired resistance mechanisms to docetaxel and potential cross-resistance with novel AR targeted agents affect the antitumor activity of docetaxel in mCPRC. Using clinically relevant PDX models for prostate cancer, we found that chemotherapy resistance is directly related to the capacity to accumulate intratumoral drug levels. Our observation that antitumor activity of taxanes depends on intratumoral accumulation is in line with previous findings in MCF-7 breast cancer cells, reporting faster uptake of cabazitaxel with longer retention longer compared to docetaxel (22). Our study further showed that intrinsic resistance can be overcome by enhancing intratumoral drug concentrations. Indeed, the superior antitumor activity of cabazitaxel in docetaxel-resistant as well as in enzalutamide-resistant tumors can be explained by its higher intratumoral drug concentrations.

Here we present two independent models of docetaxel-resistant prostate cancer representing intrinsic and acquired resistance to docetaxel. Both types of resistance were strongly related to intratumoral taxane concentrations. The finding that docetaxel-resistance could be reversed by increasing intratumoral concentrations in the intrinsically resistant PC339-DOC model suggests that patients with intrinsically resistant tumors may have insufficient intratumoral drug exposure and that such patients may benefit from increased docetaxel exposure. This may be achieved through increased dosing or prolonged docetaxel regimens, although this approach may be hampered by increased toxicity. Alternatively, cabazitaxel may be the preferred taxane, as our study indicated significantly higher intratumoral levels of cabazitaxel. To answer the subsequent question whether cabazitaxel should be the taxane of choice in the first line treatment of mCRPC, the Firstana (NCT01308567) randomized phase 3 trial was designed comparing docetaxel versus cabazitaxel as first line in mCRPC patients. The study has completed accrual and the results on overall survival and progression-free survival endpoints are awaited.
In addition to inefficient intratumoral concentrations, also other resistance mechanisms may contribute to the docetaxel resistant phenotype. For example, docetaxel resistance has been linked to increased expression of class III beta-tubulin, resulting not only in faster recurrence after radical prostatectomies but also in decreased docetaxel response and reduced survival (23). Such a stronger functional inhibition of microtubules by cabazitaxel may thus result in a stronger anti-proliferative and pro-cytotoxic effect and consequently higher antitumor activity as compared to docetaxel as was previously reported (22, 24).

The docetaxel resistance of the enzalutamide-resistant PDX PC346Enza as reported by our group(10) could not be readily explained by suboptimal intratumoral concentrations of docetaxel, as the observed levels measured in the tumor were not different from the levels detected in the naïve PC346C tumors. A possible explanation is the AR pathway mediated cross-resistance through interference with AR cellular trafficking via microtubules, which was previously reported by our group and others (10,25,26). However, the higher intratumoral concentrations of cabazitaxel in PC346Enza and hence higher antitumor activity, contrasts with the results with docetaxel and suggest the lack of AR pathway interference with cabazitaxel efficacy.

This study does not provide mechanistic data to explain the different intratumoral docetaxel and cabazitaxel levels. Due to its chemical properties, particularly its lipophilic nature, cabazitaxel uptake has shown to be faster and its retention in tissues longer than docetaxel and may partly explain the different intratumoral concentrations profiles (27, 28). In addition to these differences in properties, this study reveals that the expression of drug transporters with preference for one of the taxanes, may explain the different intratumoral concentration profiles of docetaxel and cabazitaxel. ABCB1 is an example of an efflux transporter for which the affinity of cabazitaxel is lower than for docetaxel (29). Although overexpression of ABCB1 has been shown to be involved in taxane resistance in some tumor types, its relevance for mCRPC is debatable; ABCB1 is expressed neither in our chemotherapy-naïve and resistant PDXs models nor in the majority of mCRPC (30, 31). Other drug transporters that may affect taxane uptake and could potentially play a role in docetaxel-resistance have been evaluated **(supplementary figure s4)**. Of these transporters the differential expression of ABCC10 and ABCC4 observed for PC339 and PC374 may be of interest for further study. To our knowledge, the affinity of cabazitaxel for these transporters has not been investigated yet. Clearly, the close relationship between intratumoral concentrations and antitumor activity of taxanes makes drug transporters an interesting target to improve taxane-based therapy.

To aid clinical decision making for mCRPC patients, knowledge of intratumoral taxane concentrations is a valuable and potentially predictive factor to select the most optimal taxane for individual patients or to adjust dosing. However, using intratumoral taxane levels as a predictive biomarker for personalized medicine requires invasive biopsy procedures to obtain tumor tissue shortly after - and at fixed time points - drug exposure. An interesting and novel non-invasive approach may be the estimation of [C^{11}]-docetaxel uptake using Positron Emission Tomography (PET) scanning, as was recently reported for non-small lung cancer(7). Introducing such a non/invasive uptake measurement of docetaxel and cabazitaxel, even in patients with nodal and-or visceral metastatic lesions, could enable clinicians to select the taxane with the greatest therapeutic potential.

This study provides an understanding that adequate intratumoral docetaxel levels are crucial for docetaxel responsiveness of prostate cancer. It also shows that resistance to docetaxel may be caused by different mechanisms as reflected by the PDX models developed in this

study. The recognition that in the PC339-DOC model, reflecting prostate cancer patients with intrinsic resistance, enhancing docetaxel dose, or the use of cabazitaxel, may revert the resistance phenotype is an important observation. On the other hand, the PC346C-DOC PDX model, reflecting acquired resistance, provide evidence for potential cross-resistance between docetaxel and cabazitaxel, underscoring the need to develop biomarkers to discriminate between these resistance phenotypes and to better select patients prior to both taxanes.

Conclusions

We demonstrated a strong association between intratumoral concentrations and taxane antitumor efficacy in prostate cancer using validated PDX models. The superior antitumor activity of cabazitaxel is related to higher achieved intratumoral concentrations and enhanced retention, possibly in combination with stronger antiproliferative and antimitotic effects. Especially for patients who are intrinsically resistant to docetaxel, as characterized by suboptimal intratumoral docetaxel concentrations, cabazitaxel may be the preferred chemotherapeutic agent. Clinical studies are underway to determine whether these patients will benefit from first line treatment with cabazitaxel.

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Supplementary methods

Dose finding in PDX models

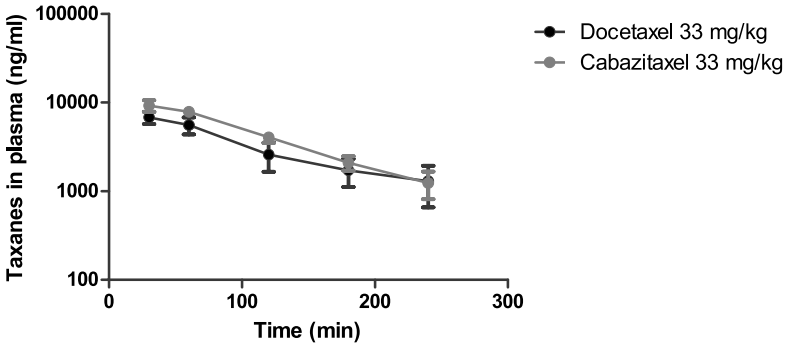
As a result of adverse toxicity observed in humans, the administered dose of cabazitaxel (25 mg/m^2) is three times lower than the dose of docetaxel (75mg/m^2)(1-3). As mice can display a different pharmacokinetic profile for drugs compared to humans, we performed a pharmacokinetic study comparing a standard dose of 33 mg/kg (4) for both docetaxel and cabazitaxel. Mice received a single i.p. injection of docetaxel (33 mg/kg) or cabazitaxel (33 mg/kg). Serial blood samples (150µl were collected at t=30 min, t=60 min (submandibular vein), t=120 and t=180 min (retro-orbital sinus). Final blood draw was obtained at t=240 by a cardiac punction. Plasma was isolated from whole blood samples by centrifugation at 1500xg for 5 minutes. Plasma levels of docetaxel and cabazitaxel were measured by a validated liquid chromatography/tandem mass spectrometry (LC/MS-MS) assay. Non-compartmental parameters were calculated using WinNonlin version 6.3 (Pharsight Corp., Mountain View, CA).

Supplementary table 1: Characteristics and taxane sensitivity of patient-derived prostate cancer xenografts(4-6)

Tumor model	Origin	AR	PSA	Androgen sensitivity	Docetaxel sensitivity	Cabazitaxel sensitivity
PC339	TURP	-	-	-	-	+++
PC346C	TURP	+	+	+	+	+
PC374	SSM	+	+	+/-	++	++
PC339-DOC	PC339	-	-	-	-	+++
PC346C-DOC	PC346C	+	+/-	+/-	-	-
PC346Enza	PC346C	+	+	-	-	+

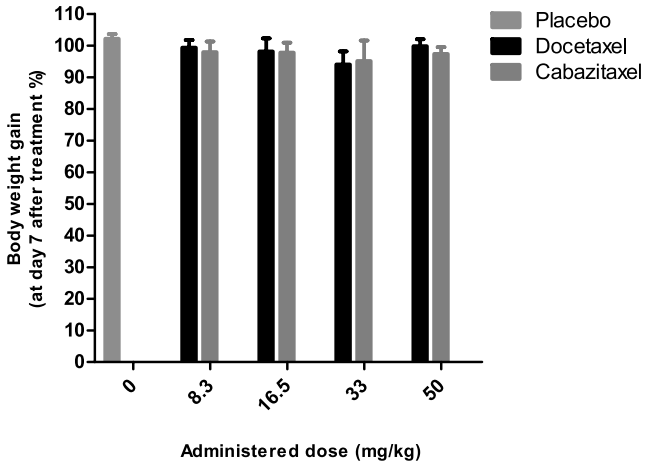
PC= primary prostate tumor, TURP= transuretheral resection of prostate, SSM= scrotal skin metastasis, AR= human androgen receptor, PSA=prostate specific antigen. Docetaxel and cabazitaxel sensitivity is experimentally determined using log cell kill to express antitumor activity. Log cell kill values are translated to antitumor activity as follows(7): <0.7= - (inactive); 0.7-1.2 = +; 1.3-1.9 = ++, 2.0-2.8= +++, >2.8= ++++. Resistance to taxanes was defined as log cell kill < 0.7. Androgen sensitivity was determined in vivo by castration experiments.

Supplementary results

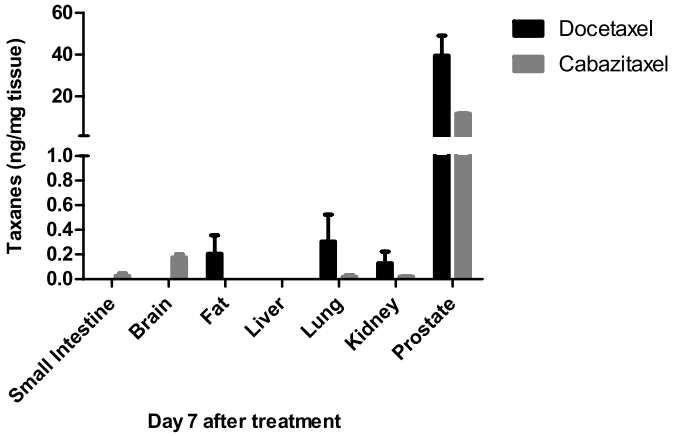


Drug	Clearance L/h/kg	AUC mg*h/L
Docetaxel	2.20 ± 0.64	15.89 ± 4.29
Cabazitaxel	1.70 ± 0.17	19.48 ± 1.90

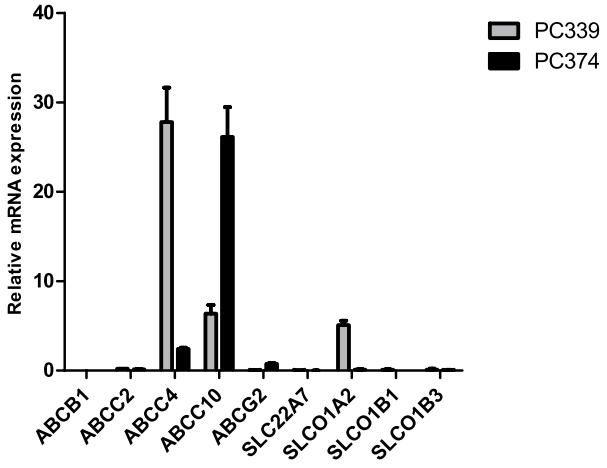
Supplementary figure s1: Plasma pharmacokinetics of docetaxel and cabazitaxel in NMRI nude mice. A single administration of docetaxel 33 mg/kg or cabazitaxel 33 mg/kg was given. Blood was drawn at different time points. The plasma concentration of taxane was analyzed using validated LC-MS/MS assays. The average plasma concentrations ± SEM (n=4-5) are shown. The clearance of docetaxel and cabazitaxel was comparable. There were no signs of toxicity after administration of a single i.p. injection of docetaxel 33 mg/kg or cabazitaxel 33 mg/kg, we therefore decided to use the same dose for docetaxel and cabazitaxel in our experiments.



Supplementary figure s2: Body weight gain after treatment with placebo, docetaxel or cabazitaxel. Mice received a single administration of placebo, docetaxel 33 mg/kg or 50 mg/kg, or cabazitaxel 33 mg/kg or 50 mg/kg at day 0 and were sacrificed at day 7. The mean body weight gain + SD is shown of 4 independent mice per group.



Supplementary figure s3: Tissue distribution and accumulation of docetaxel and cabazitaxel after a single administration. Mouse organs were harvested at day 7 after a single treatment with docetaxel 33 mg/kg or cabazitaxel 33 mg/kg. Concentrations of docetaxel and cabazitaxel were measured and corrected for the weight of the tissue. Mean + SD is shown of n=3 mice per treatment.



Transporter	Docetaxel is a substrate (8-14)
ABCB1	Yes
ABCC2	Yes
ABCC4	No
ABCC10	Yes
ABCG2	No
SLC22A7	Yes
SLCO1A2	Yes
SLCO1B1	Yes
SLCO1B3	Yes

Supplementary figure s4: Drug transporter expression profile of PC339 and PC374. We created a gene expression profile of drug transporters that are described in literature in relation with docetaxel transport. The mean +SD of the relative mRNA expression is shown of three independent tumors.

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71

17

Loss of SLCO1B3 drives taxane resistance in prostate cancer

BACKGROUND: Both taxanes, docetaxel and cabazitaxel are effective treatments for metastatic castration-resistant prostate cancer (mCRPC). However, resistance to taxanes is common. Our objective was to investigate mechanisms of taxane resistance in prostate cancer. **METHODS:** Two docetaxel-resistant patient-derived xenografts (PDXs) of CRPC were established (PC339DOC and PC346C-DOC) in male athymic nude mice by frequent intraperitoneal administrations of docetaxel. Next Generation Sequencing was performed on PDX tissue pre- and post-docetaxel resistance and gene expression profiles were compared. [^{14}C]-docetaxel and [^{14}C]-cabazitaxel uptake assays in vitro and cytotoxicity assays were performed to validate direct involvement of transporter genes in taxane sensitivity. **RESULTS:** Organic anion-transporting polypeptide (SLCO1B3), an influx transporter of docetaxel, was significantly downregulated in PC346C-DOC tumors. In accordance with this finding, intratumoral concentrations of docetaxel and cabazitaxel were significantly decreased in PC346C-DOC as compared to levels in chemotherapy-naïve PC346C tumors. Also, silencing of SLCO1B3 in chemo-naïve PC346C resulted in a 2-fold decrease in intracellular concentrations of both taxanes. Overexpression of SLCO1B3 showed higher sensitivity to docetaxel and cabazitaxel. **CONCLUSIONS:** SLCO1B3 determines intracellular concentrations of docetaxel and cabazitaxel and consequently influences taxane efficacy. Loss of the drug transporter SLCO1B3 may drive taxane resistance in prostate cancer.

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Background

To date, approximately 25% of patients diagnosed with prostate cancer will eventually progress to metastatic castration-resistant prostate cancer (mCRPC) (Rane *et al*, 2012). mCRPC patients are generally treated with docetaxel as standard first-line chemotherapy. Unfortunately, there is a large variability in response to docetaxel treatment among patients (de Bono *et al*, 2010; Petrylak *et al*, 2004; Tannock *et al*, 2004). While some patients show prolonged responses, others respond poorly and progress rapidly. Among new options for docetaxel-resistant patients, cabazitaxel is a strong alternative taxane with proven efficacy in docetaxel-resistant patients (de Bono *et al*, 2010). We previously showed that higher intratumoral concentrations of cabazitaxel in docetaxel-resistant tumors might lead to stronger antitumor activity (De Morree *et al*, 2016). Hence, it is highly relevant to determine tumor factors that may be linked to docetaxel resistance and cabazitaxel sensitivity. Such predictive biomarkers of taxane resistance will allow to define patients who are less likely to benefit from continued treatment with docetaxel, and thus might be candidates for treatment with cabazitaxel.

In order to mimic the development of docetaxel resistiance in patients, we set out to develop docetaxel resistance in a selection of patient-derived xenografts (PDX) of prostate cancer. PDX models have been demonstrated to largely resemble the complexity of prostate cancer including molecular diversity, cellular heterogeneity and histology (Kopetz *et al*, 2012). To investigate mechanisms of docetaxel resistance, we performed Next Generation Sequencing (NGS) and compared gene expression profiles of the created docetaxel-resistant tumors versus their parental tumors. We identified downregulation of SLCO1B3 as a potential mechanism of taxane resistance in CRPC. We show that silencing of SLCO1B3 resulted in decreased uptake of both docetaxel and cabazitaxel, while SLCO1B3 overexpression enhanced taxane sensitivity.

Methods

Drugs

Docetaxel and cabazitaxel (Sanofi, Vitry-sur-Seine, France) formulations were prepared in polysorbate-80-ethanol (1:1, v/v) and further diluted in 5% (w/v) glucose solution to a final concentration of 2.5 mg/ml for *in vivo* experiments. In uptake assays [^{14}C]-docetaxel and [^{14}C]-cabazitaxel (specific activity: 83–86 mCi/mmol/L; Sanofi-Aventis Deutschland GmbH, Germany) was used.

Development of docetaxel-resistant PDXs

PC339, PC346C and PC374 xenografts with respectively low, moderate and high sensitivity to docetaxel were selected from the Erasmus MC prostate cancer PDX panel (van Weerden *et al*, 2009). The 3 selected PDXs were derived from patients progressive under standard

androgen depletion therapy (ADT). Some molecular characteristics of the PDX models are listed in **Supplementary Table 1**. Docetaxel resistance was developed as described previously (De Morree *et al*, 2016). In short, tumor fragments of PDXs were subcutaneously transplanted on 8 weeks non-castrated male NMRI nude mice (NMRI-Foxn1^{nu}; Taconic, Hudson, NY) and when tumors were established, mice received one bolus injection of docetaxel (33 mg/kg) intraperitoneally (i.p.) every 14 days until tumors progressed. Mice were not castrated, as it was previously shown that androgen levels in male mice resemble the androgen levels of chemically castrated men, originating from adrenal androgens (Sedelaar et al 2013). Tumor fragments were consecutively passaged until growth rates of docetaxel-treated tumors were similar to those of the chemo-naïve original tumor. All experiments were approved by the Animal Experiments Committee under the Dutch Experiments on Animals Act in adherence of the European Convention for Protection of Vertebrate Animals used for Experimental Purposes (Directive 2010/63/EU).

Assessment of antitumor activity

Antitumor activity of taxanes in chemo-naïve and docetaxel-resistant PDXs was determined after a single intraperitoneal (i.p.) injection of docetaxel 33 mg/kg or cabazitaxel 33 mg/kg, or 0.9% (w/v) NaCl at a tumor volume (TV) of 300 mm^3 as described previously (de Morree et al 2016). In short, tumor volumes were measured twice a week by caliper. Antitumor activity of taxanes was monitored using the log cell kill formula (formula 1). This formula theoretically models the working mechanism of taxanes, assuming that a proportion of cells is killed after a single treatment while taking into account tumor doubling time.

$$Log\ cell\ kill = T\text{-}C\ (days) / (3.32 * Td)$$

Formula 1: Log cell kill closely mimics clinical end points, such as disease progression and takes into account how fast the tumor grows (tumor doubling time) in relation with the tumor growth delay induced by treatment according to the formula: T is the median time in days to reach a tumor volume of 1000 mm 3 in the treated mice. C is the median time in days to reach the same tumor volume in the control mice. Td is the tumor doubling time, derived from a log-linear growt h plot of the control tumors in exponential growth phase. Log cell kill values are translated to antitumor activity as follows (Corbett et al , 2003 ; Lloyd, 1975 ; Schabel Jr et al , 1977): 0.7-1.2 = +; 1.3-1.9 = ++, 2.0-2.8= +++, > 2.8= ++++. Resistance to taxanes was defined as log cell kill < 0.7

Next Generation Sequencing (NGS) of PDXs

Three independent tumors of each PDX: docetaxel-naïve PC339, PC346C and PC374, and of docetaxel-resistant PC339-DOC and PC346C-DOC, were analyzed by NGS/RNA seq. In brief: total RNA was extracted using RNA-Bee reagent (Tel-Test, Inc. Friendswood, TX) according to the manufacturer's instructions. RNA quantity and quality were analyzed using Agilent Laboratory-on-Chip analysis (Agilent Bioanalyzer 2100). RNA samples with RNA Integrity Number (RIN) ≥ 7 were included. Library and paired-end RNA sequencing were executed

by AROS (Applied Biotechnologies, Aarhus, Denmark) on a Illumina HiSeq 2000 with a sequencing depth of min. 35 mio reads.

mRNA expression validation using Real-Time PCR

Total RNA was isolated from PC346C and PC346C-DOC tumors to validate the expression levels of SLCO1B3. In addition, RNA from abiraterone and enzalutamide-resistant tumors PC346Enza and PC346Abi101 that were previously developed (Van Soest *et al*, 2015; van Soest *et al*, 2013), was isolated. SLCO1B3 mRNA (Taqman Assay On Demand, Hs00251986_m1, Applied Biosystems, Thermo Fisher Scientific, Waltham, MA) was measured using a 7500 Fast Real-Time PCR System (Applied Biosystems). Gene expression was normalized using the ΔC_t method against housekeeping genes hypoxanthineguanine phosphoribosyltransferase (HPRT, Taqman assay 4310890E) and Porphobilinogen Deaminase (PBGD, Taqman assay Hs00609297_m1).

Bioinformatics analysis

A detailed description of the bioinformatics approach and analysis is provided in the Supplementary methods. Gene expression profiles of chemotherapy-naïve and docetaxel-resistant tumors were compared. mRNA gene expression profiles were generated of which the expression was significantly altered in the docetaxel resistant tumors. A False Discovery Rate (FDR) of <0.05 was used.

Gene silencing of taxane-related drug transporters

To evaluate the involvement of drug transporters in taxane efficacy, the top 10 of transporters that correlated with docetaxel and the top 10 transporters that correlated with cabazitaxel sensitivity were selected (see supplementary methods for details on the selection process). Genes were silenced in parental PC346C cells using the Sigma Transporter Silencing Bank (Sigma Mission siRNA of Ion Channel and Transporter Panel S106100) focusing on adenosine triphosphate binding cassette (ABC) transporters and Solute Carrier (SLCs) families that were correlated with taxane sensitivity (for further details see supplementary methods). In short, PC346C cells were transfected with siRNA pools targeting various transporters. After 48h, a [^{14}C]-docetaxel and [^{14}C]-cabazitaxel uptake assay was performed. Retained [^{14}C]-cabazitaxel and [^{14}C]-docetaxel levels were counted with a scintillation counter and normalized to levels observed in control cells transfected with a non-targeting siRNA pool (Zimmerman *et al*, 2016).

Intratumoral taxane concentration

Mice bearing docetaxel-naïve or docetaxel-resistant PDXs received a single intraperitoneal injection of docetaxel 33 mg/kg or cabazitaxel 33 mg/kg at a tumor volume of 300 mm^3 and were sacrificed after 7 days. Tumor tissue was homogenized in lithium-heparinized human

plasma (1:5 w/v) and processed for further analysis by a validated liquid chromatography/tandem mass spectrometry (LC/MS-MS) assay to determine intratumoral docetaxel and cabazitaxel concentrations as previously described (de Bruijn *et al*, 2012; Engels *et al*, 2006, de Morree *et al, 2016*).

Cell proliferation assays

To substantiate the role of SLCO1B3 in taxane-sensitivity, SLCO1B3-negative PC339C and PC346C-DCC-G cells were stably transfected with a SLCO1B3 or GFP (control) expression construct (see supplementary methods). SLCO1B3-overexpressing cells and GFP-control cells were seeded in a 96-wells plate at a concentration of 2500 cells/well and incubated for 10 days with docetaxel or cabazitaxel (serial concentration range 0-10 nM). Proliferation was assessed using PrestoBlue Cell Viability reagent (Invitrogen). Data are expressed as mean ± SEM of three independent experiments with at least 6 replicates per condition. IC_{50} values were determined using GraphPad Prism 5.0. IC_{50} values were compared using the extra sum-of-squares F test with a boundary for significance of $p \leq 0.05$.

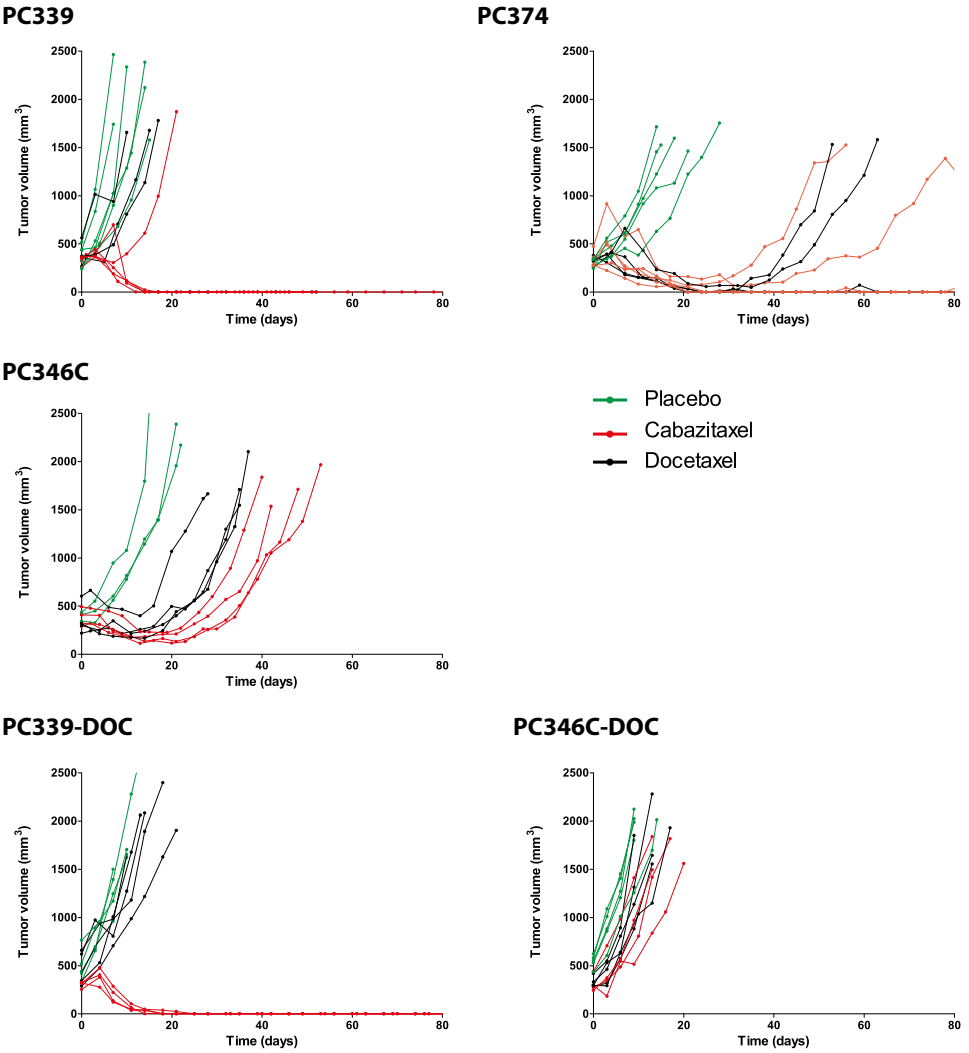
Results

Generation of docetaxel-resistant PDXs of prostate cancer

In order to develop docetaxel resistance in vivo, PC339, PC346C and PC374 tumors were serially passaged under docetaxel pressure. The chemotherapy-naïve PC339 PDX was characterized by a poor response to docetaxel with log cell kill values of 0.42 (log cell kill >0.7 is considered therapy responsive) **(Figure 1)**. Within 4 mouse passages, a docetaxel-resistant variant, PC339-DOC, was established translating in a log cell kill value of 0.18. In contrast, the chemotherapy-naïve PC346C was relatively sensitive to docetaxel treatment (log cell kill value of 1.0) and remained responsive for almost one year after the first docetaxel treatment before acquiring resistance (log cell kill 0.20). Finally, the chemotherapy-naïve PC374 was extremely sensitive to docetaxel treatment (log cell kill 1.6) and we were unable to create a resistant counterpart of this PDX. Cabazitaxel was at least as potent as docetaxel with corresponding log cell kill values of >2.8, 1.2, and 1.8 for the docetaxel-naïve PC339, PC346C and PC374, respectively. Interestingly, cabazitaxel exhibited high sensitivity in PC339-DOC (log cell kill >2.8) while showing cross-resistance with docetaxel in PC346C-DOC (log cell kill 0.23) **(Figure 1)**.

Significant downregulation of SLCO1B3 expression in docetaxel-resistant PDX

To unravel potential mechanisms of resistance, we performed NGS/RNA seq between parental chemo-naïve and docetaxel-resistant PDX tumors and identified differentially expressed genes based on a False Discovery Rate (FDR) of 0.05 (see **Table 1** for the top 15 gene list). The docetaxel uptake transporter SLCO1B3 was the most significantly down-regulated gene in PC346C-DOC.



	Docetaxel		Cabazitaxel	
PDX	**Log cell kill**	**Antitumor activity**	**Log cell kill**	**Antitumor activity**
Chemotherapy-naïve				
PC339	0.42	-	>2.8	++++
PC374	1.6	++	1.8	++
PC346C	1.0	+	1.2	+
Docetaxel-resistant				
PC339-DOC	0.18	--	>2.8	++++
PC346C-DOC	0.20	--	0.23	--

Figure 1: Taxane response in docetaxel-naïve and docetaxel-resistant PDXs. Tumor-bearing mice were treated with a single injection of either placebo (green), docetaxel 33 mg/kg (black) or cabazitaxel 33 mg/kg (red). Tumor volume was measured twice a week. Each line represents a single mouse. Log cell kill < 0.7 was considered as resistant. Table insert summarizes log cell kill values and antitumor activity of docetaxel and cabazitaxel in the various PDX models.

Table 1: Top 15 genes significantly[a] up or downregulated in the parental versus the docetaxel resistant PDX

PC346C vs PC346C-DOC	Function[b]	Log fold change	PC339 vs PC339-DOC	Function[b]	Log fold change
SLCO1B3	organic anion transmembrane transporter activity	-6.03	**SPATA21**	calcium ion binding	-2.90
RBM24	3'-UTR binding and nucleotide binding	-5.07	**MSMB**	member of the immunoglobulin binding factor family	2.69
HES7	protein dimerization activity and transcription factor binding.	4.02	**GRIP2**	undefined	-2.46
TNFRSF18	tumor necrosis factor-activated receptor activity	3.71	**EGFLAM**	glycosaminoglycan binding	2.38
VANGL2	regulation of planar cell polarity	3.70	**POTEM**	undefined	2.29
SLC30A4	zinc ion transmembrane transporter activity	-3.06	**AFAP1**	actin binding and phospholipid binding	-2.20
ATP2B4	calmodulin binding and sodium channel regulator activity	2.61	**USH2A**	collagen binding and myosin binding	2.08
FADS2	stearoyl-CoA 9-desaturase activity and iron ion binding	2.44	**TSHR**	thyroid-stimulating hormone receptor activity	1.93
MTRNR2L10	neuroprotective and antiapoptotic factor	2.36	**KRT6A**	structural constituent of cytoskeleton	1.83
GPR88	G-protein coupled receptor activity	-2.26	**ZNF407**	may be involved in transcriptional regulation	-1.82
MARCKS	cell motility, phagocytosis, membrane trafficking and mitogenesis	-1.97	**NEB**	structural constituent of muscle and actin binding	1.80
UTRN	protein kinase binding, calcium ion binding	-1.78	**HLA-A**	peptide antigen binding and receptor binding	1.72
COL7A1	serine-type endopeptidase inhibitor activity	1.75	**CTAG2**	undefined	-1.66
MT2A	binding of heavy metals	1.61	**TMEM176B**	undefined	1.59
IQGAP2	regulate cell morphology and motility	-1.59	**KRT17**	MHC class II protein binding and structural constituent of cytoskeleton	1.49

[a] False discovery rate was < 0.05 for all genes b Gene function was derived from www.genecards.org

SLCO1B3 was neither expressed in the docetaxel-naïve PC339 nor in the docetaxel-resistant PC339-DOC. Validation with qPCR confirmed the substantial downregulation of SLCO1B in PC346C-DOC **(Figure 2)**. To rule out that SLCO1B3 downregulation was caused by serial passaging of PC346C rather than by docetaxel treatment, we showed that SLCO1B3 expression was retained in parental PC346C tumors during propagation **(Supplementary figure 1)**.

Downregulation of SLCO1B3 in abiraterone and enzalutamide resistant cells

We have previously shown cross-resistance between AR-targeted therapies, such as abiraterone and enzalutamide, and docetaxel in vitro and in vivo (van Soest *et al*, 2014; van Soest *et al*, 2013). Therefore, SLCO1B3 expression was also evaluated in PC346C cell lines with acquired resistance to abiraterone (PC346abi101) and enzalutamide (PC346enza). Similar to PC346C-DOC, SLCO1B3 expression was low in both PC346abi101 and PC346enza cells **(Figure 2)**.

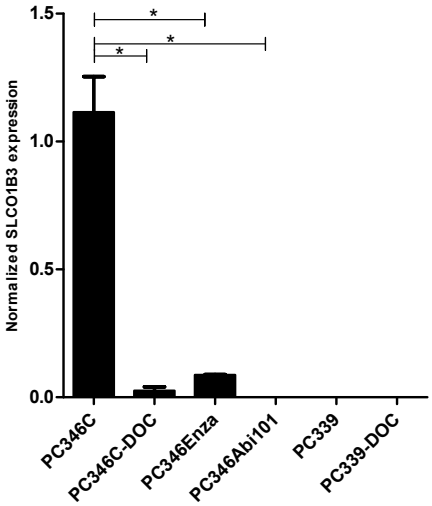


Figure 2: SLCO1B3 expression is downregulated in PC346C-DOC, PC346Enza and PC346CAbi101. Expression of SLCO1B3 was measured in PC346, PC346C-DOC, PC346Enza and PC346Abi101 tumors or cell lines using Real-time PCR. PC339 and PC339-DOC lack SLCO1B3 expression. SLCO1B3 mRNA expression was normalized to HPRT and PBGD. An average ± SEM of n=3-6 tumors is shown. *p<0.05

Decreased intratumoral taxane concentrations in docetaxel resistant PDXs

Since SLCO1B3 is a known transporter of docetaxel, downregulation of SLCO1B3 most likely result in reduced intratumoral taxane concentrations, inferring the observed resistant phenotype of PC346C-DOC. Indeed, intratumoral concentrations of docetaxel were significantly lower in PC346C-DOC as compared to chemo-naïve PC346C tumors (respectively P=0.003 and P=0.0006) **(Figure 3)**. As expected from the observed cross-resistance between docetaxel and cabazitaxel, intratumoral cabazitaxel levels were also reduced and not different from docetaxel in PC346C-DOC. In contrast, and in line with the efficacy of cabazitaxel in PC339 and PC339-DOC tumors, cabazitaxel levels were significantly higher than docetaxel levels in both PC339 (P=0.005) and PC339-DOC tumors (P=0.01) **(Figure 3).**The significance of SLCO1B3 in the regulation of effective intratumoral levels of taxanes is provided in figure 6.

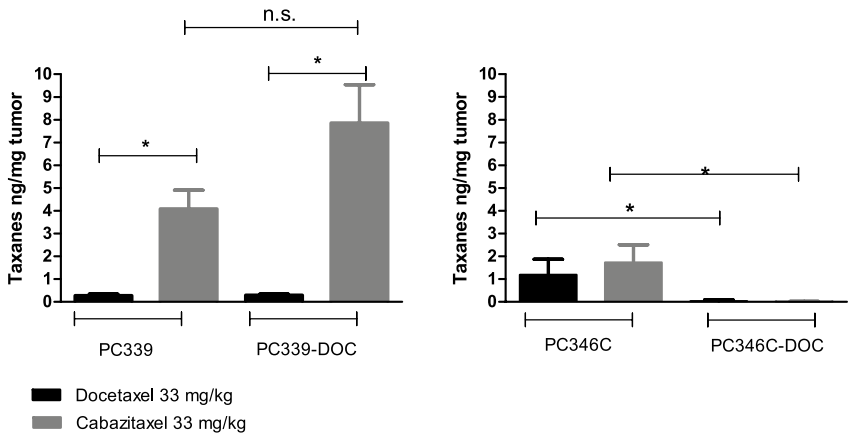


Figure 3: Intratumoral concentrations of docetaxel and cabazitaxel in parental versus docetaxel-resistant PDXs. Intratumoral concentrations were measured in docetaxel-naïve and docetaxel-resistant PDXs at seven days after a single dose with either docetaxel or cabazitaxel. Intratumoral concentrations of both docetaxel and cabazitaxel were significantly reduced in PC346C-DOC compared to PC346C. *p<0.05, n.s: non-significant.

Knockdown of SLCO1B3 significantly decreased cellular uptake of taxanes

To determine if other drug-transporters could have contributed to the decreased concentrations of docetaxel and cabazitaxel observed in PC346C-DOC tumors, an siRNA screen of putative taxane drug transporters was performed. The only significant (50%) reduction in uptake of docetaxel (p=0.01) and cabazitaxel (p=0.0003) was observed when PC346C cells were transfected with the SLCO1B3 siRNA pool**(Figure 4 and Supplementary figure 2)**.

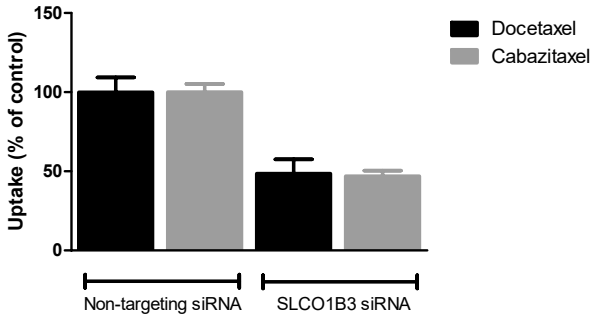
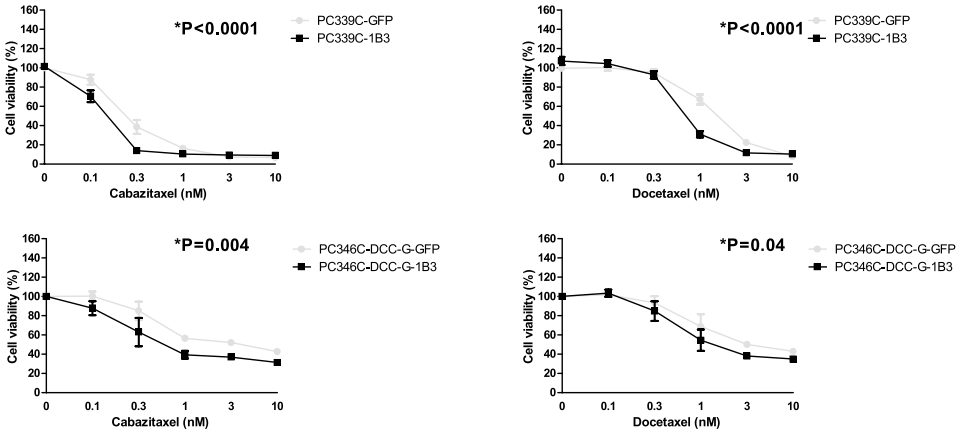


Figure 4: Silencing of SLCO1B3 leads to decreased uptake of docetaxel and cabazitaxel. Uptake and retention of [^{14}C]-docetaxel and [^{14}C]-cabazitaxel was measured in PC346C cells after silencing SLCO1B3. The levels of cabazitaxel and docetaxel taken up and retained in the cells was compared to the uptake of taxanes in cells transfected with CTRL siRNA. An average ± SD is shown of n=3 measurements. *p< 0.05 compared to the uptake in the control.

Higher sensitivity to taxanes in SLCO1B3 overexpressing cells

To further substantiate the role of SLCO1B3 in taxane-sensitivity, cell viability was measured after docetaxel and cabazitaxel exposure, in SLCO1B3 negative PC339C-GFP and SLCO1B3 overexpressing PC339C cells (see **Supplementary figure 3** for SLCO1B3-expression levels). SLCO1B3 overexpressing PC339 cells were more sensitive to taxane treatment than the control-transfected PC339C-GFP cells**.** A similar correlation between SLCO1B3 and taxane sensitivity was seen in SLCO1B3-transfected, originally SLCO1B3-negative, castration-resistant PC346C-DCC-G subline **(Figure 5)**.



Cells	Docetaxel IC50 value (nM) (95% CI)	Cabazitaxel IC50 value (nM) (95% CI)
PC339C-1B3	0.88 (0.69-1.1)	0.16 (0.13-0.21)
PC339C-GFP	1.6 (1.3-2.0)	0.30 (0.24-0.37)
PC346C-DCC-G-1B3	1.6 (0.98-2.7)	0.92 (0.54-1.6)
PC346C-DCC-G-GFP	3.0 (2.0-4.4)	3.2 (2.1-5.0)

Figure 5: Sensitivity to docetaxel and cabazitaxel is increased in SLCO1B3 overexpressing prostate cancer cells. Two independent prostate cancer cell lines were transfected with a lentiviral expression construct containing SLCO1B3 or turbo-GFP (GFP) as control. Cells were cultured for 10 days in the presence of 0 – 10 nM docetaxel or cabazitaxel. Average ± SEM is shown of n=3 independent experiments.

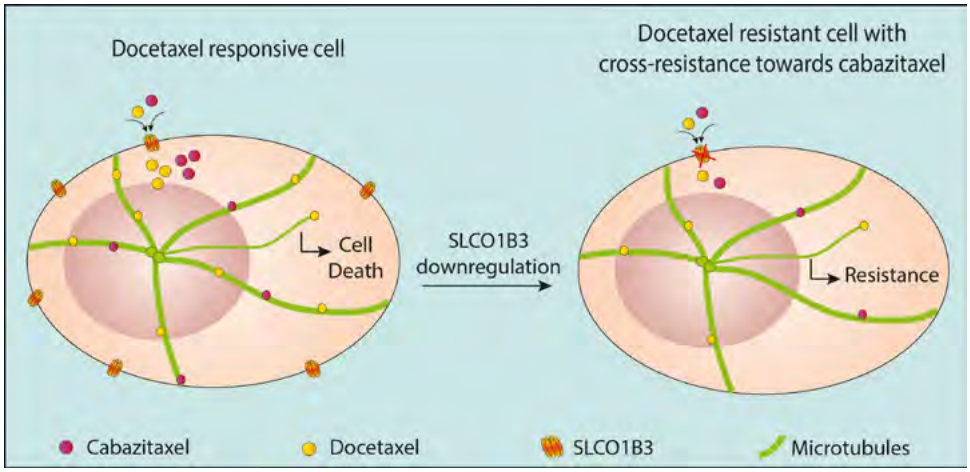


Figure 6: Mechanism of SLCO1B3-mediated resistance to docetaxel. Docetaxel-responsive cells express SLCO1B3. SLCO1B3 is a known influx transporter of docetaxel, and transports docetaxel into the cell. Cabazitaxel may also be a potential substrate of SLCO1B3, however this hypothesis needs further experimental validation. We previously showed that intratumoral concentrations of cabazitaxel were, generally, higher in docetaxel-naïve tumors compared to docetaxel. As docetaxel or cabazitaxel enter the cell, they inhibit microtubule dynamics, which leads to a cell cycle arrest in th G2/M phase and eventually to apoptosis. In docetaxel resistant cells, SLCO1B3 expression is downregulated. We found that intratumoral concentrations of both docetaxel and cabazitaxel were decreased in docetaxel-resistant PC346C-DOC xenograft tumors, compared to the parental PC346C xenograft tumors. Experiments in which SLCO1B3 was silenced in PC346C cells, showed decreased uptake of docetaxel and cabazitaxel , confirming that SLCO1B3 is at least partly involved in modulating intracellular concentrations of docetaxel and cabazitaxel. Decreased intratumoral concentrations leads to decreased response to therapy as was previously shown (De Morree et al. 2016)

Discussion

Predictive biomarkers for resistance to taxane chemotherapy are an unmet medical need in the management of mCRPC. Insights in mechanisms of taxane-resistance are crucial to identify and develop reliable predictive biomarkers and potential therapeutic targets. Here we show that downregulation of the influx transporter SLCO1B3 is associated with taxane-resistance in a PDX model of prostate cancer, pointing towards a role for SLCO1B3 in taxane sensitivity through regulation of drug uptake, determining intratumoral concentrations, and consequently efficacy. As such, SLCO1B3 may be a potential biomarker of docetaxel resistance that warrants further clinical validation.

The generation of two unique docetaxel resistant PDX models, PC346C-DOC and PC339-DOC, allowed to screen for somatic mutations underlying the resistant phenotype that could have been acquired during the development of docetaxel resistance. Interestingly, we did not find any treatment-induced somatic variants in the transcriptome of either PDX. This finding and the difference in time to development of docetaxel resistance of these

models, as well as their contrasting response to cabazitaxel, suggest that PC339-DOC and PC346C-DOC express different resistance mechanisms that are likely to be involved in chemo-resistance.

Differentially expressed genes that correlated to docetaxel resistance identified SLCO1B3 as an important gene associated with docetaxel resistance in PC346C-DOC. An extensive siRNA screen further revealed that the role of other ABC-transporters and transporters from the SLC families, that were previously linked to taxane sensitivity, was less pronounced compared to SLCO1B3 in this model. The lack of SLCO1B3 expression in naïve PC339 and resistant PC339-DOCmay explain the relative low sensitivity to docetaxel in this intrinsically resistant PDX, underscoring the relevance of SLCO1B3 in docetaxel sensitivity. This is further substantiated by the observation that overexpressing SLCO1B3 in PC339C cells indeed resulted in increased sensitivity to docetaxel. Interestingly, and in contrast to PC346C and PC346C-DOC, PC339 and PC339-DOC were highly sensitive for cabazitaxel, which was further augmented when overexpressing SLCO1B3. These data suggest that cabazitaxel influx and intratumoral levels also benefit from SLCO1B3 expression. This observation contrasts to previous reports indicating that cabazitaxel uptake may rely more on transmembrane diffusion than carrier mediated translocation across the plasma membrane due to its higher lipophilicity as compared to docetaxel, (Azarenko *et al*, 2014; Vrignaud *et al*, 2014). Clearly, further experiments are required to define if cabazitaxel is a substrate of SLCO1B3 and its importance for intracellular cabazitaxel levels.

SLCO1B3 has previously been shown to be expressed in at least 50% of prostate cancer specimens with increased expression in mCRPC compared to primary prostate cancer (Pressler *et al*, 2011; Wright *et al*, 2011). Furthermore, SLCO1B3 expression has been linked to the hormonal status of prostate cancer and to the response to androgen-deprivation therapies, as it is also an influx transporter of testosterone (Hamada *et al*, 2008). Of note, in our enzalutamide and abiraterone- resistant cell lines(van Soest *et al*, 2015; van Soest *et al*, 2013) that represent cross-resistance with docetaxel, SLCO1B3 expression was lost, suggesting that pretreatment with hormonal agents, like enzalutamide and abiraterone, may affect taxane sensitivity via reduction of SLCO1B3 expression. Therefore, SLCO1B3 may not only mediate the cellular uptake of docetaxel, but may also alter the androgen-responsiveness of the cell. This potential mechanism of cross-resistance may have implications for potential treatment sequences in the management of prostate cancer. Such possible relationship between SLCO1B3 and androgen status of the patient, inflicted either by conventional androgen deprivation therapy (ADT) or by the use of novel AR-targeted agents, may be particularly relevant in light of recent data, showing a robustly greater survival benefit by docetaxel when given in addition to ADT in patients with metastatic hormone-sensitive prostate cancer (mHSPC) as compared when given at the time of castration-resistance (Sweeney *et al*, 2015, James et al, 2015). The value of SLCO1B3 expression as a potential candidate biomarker for taxane response in mCRPC and mHSPC patients requires a

prospective clinical study in which tumor lesions are biopsied pre- and post- taxane treatment. As an alternative, circulating tumor cells may be considered as a liquid biopsy to evaluate SLCO1B3 expression over time and determine its predictive value.

Conclusions

We have shown that SLCO1B3 expression is associated with taxane resistance. Additionally, SLCO1B3 may also have a role in cross-resistance with hormonal agents like enzalutamide and abiraterone. Clinical studies are needed to further investigate the potential role of SLCO1B3 as biomarker in patients with mCRPC treated with taxane chemotherapy.

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Supplementary methods

Bioinformatics analysis of NGS data

Sequencing reads were aligned to a pre-indexed human reference genome (hg19, available via the bowtie2 homepage including UCSC based annotation) using TopHat2 (version 2.0.4){Kim, 2013 #62} (Kim *et al*, 2013). To increase accuracy, reads were aligned against the indexed transcriptome prior to alignment to the genome via setting "transcriptome-index". RNA expression levels were quantified via featureCounts (version 1.3.5-p5) using the UCSC hg19 annotation provided by the TopHat2 developers (Liao *et al*, 2014) . Subsequently, edgeR R-package (version 3.0.4) was used to investigate differentially expressed genes between different treatment conditions (Robinson et al 2010). Variants were called using samtools mpileup (version 0.1.19) and VarScan (version 2.3.6) in somatic mode with default settings between docetaxel resistant and naïve tumors (Koboldt *et al*, 2012; Li *et al*, 2009) . All TopHat2 alignments were performed on the surfSARA High Performance Computing Cloud (https://www.surfsara.nl/systems/hpc-cloud). Downstream analysis was run on a Dell Precision with two Intel Xeon E5507 Quadcores and 24 GB RAM running Ubuntu Linux 12.10.

Selection of drug transporters related to taxane response

Putative drug transporters related to taxane uptake and sensitivity were identified by correlating the expression levels of ABC-transporters and SLCs (determined by next generation sequencing) with log cell kill using Spearman's Rank Correlation Coefficients. For this analysis docetaxel-naïve (PC346C, PC339, PC374) and docetaxel-resistant (PC339-DOC and PC346C-DOC) PDXs were listed from low sensitivity (low log cell kill values) to high sensitivity (high log cell kill values) for docetaxel and cabazitaxel separately. Drug transporter genes with an absolute correlation ≤ 0.85 for either docetaxel or cabazitaxel sensitivity, and high overall expression (log_2CountsPerMillion / CPM > 3) were selected. A top 10 list of drug transporter genes with the strongest correlation for docetaxel and cabazitaxel sensitivity was generated for further validation studies.

siRNA screening of drug transporters

To identify transporters involved in the uptake/efflux of taxanes in prostate cancer cells, we silenced the expression of selected transporters in PC346C cells using the Sigma transporter silencing bank (Sigma Mission siRNA of Ion Channel and Transporter panel SI06100). PC346C cells were plated at a concentration of 30.000 cells/well in a 96-wells plate (96 well white, clear bottom ViewPlates from PerkinElmer #6005181, pre-coated with poly-D-lysine (Sigma, P6407). At a confluency of 60-70% cells were transfected with 60 nM siRNA (pool of three different siRNAs targeting the same transporter) using Lipofectamine RNAiMAX (Life Technologies)

as a transfection agent. A non-targeting siRNA pool (#D-001810-01-05, Dharmacon) was used as a transfection control. After 48 hours the transfectants were exposed for 5 minutes to 10 µM [^{14}C]-docetaxel or [^{14}C]-cabazitaxel (Sanofi, Vitry-sur-Seine, France) and washed 3 times with PBS. Cells were dissolved in 20 µl MicroScint (PerkinElmer#6013621) scintillation cocktail and radioactivity retained in the cells was quantified using a microplate scintillation counter (TopCount #9904; a 2 Detector Radioisotopic and Luminescent Variable Plate reader). Intracellular cabazitaxel and docetaxel levels were normalized to levels observed in control wells treated with non-targeting siRNAs.

Development of SLCO1B3 overexpressing cell lines

PC339C cells were derived from the PC339 prostate cancer PDX by digesting tumor tissue using a collagenase A solution in DMEM/F12 (250 U/mg) for 30 minutes at 37^{0}C and 5% CO_2. Cells were centrifuged at 500g for 5 minutes. A single cell suspension was plated in culture flasks and grown as 3D cell culture in Prostate Growth Medium (PGM, as previously described (Marques *et al*, 2011)). PC346C-DCC-G cells were created by long-term culturing of the parental PC346C cells in steroid depleted PGM. PC339C and PC346C-DCC-G cells were transfected with a lentiviral construct of SLCO1B3 (h-SLCO1B3 plenti-GIII-CMV-GFP-2A-PURO, constructed by ABM Inc, Canada) or with TurboGFP as control (pCDH-CMV-MCS-EF1-Puro-Turbo GFP, constructed by ABM Inc, Canada). Transfected cells were grown under continuous selection with 2.1 µg/ml puromycin (Life Technologies) in PGM.

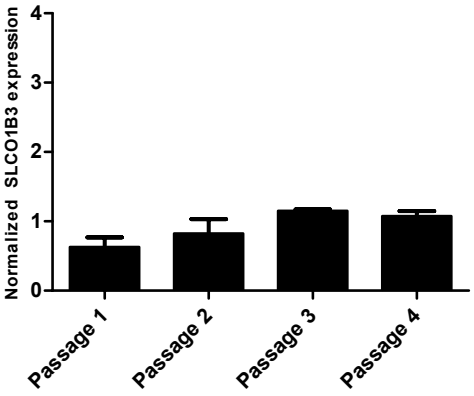
Supplementary data

Supplementary table 1: Characteristics of patient-derived xenografts of prostate cancer.

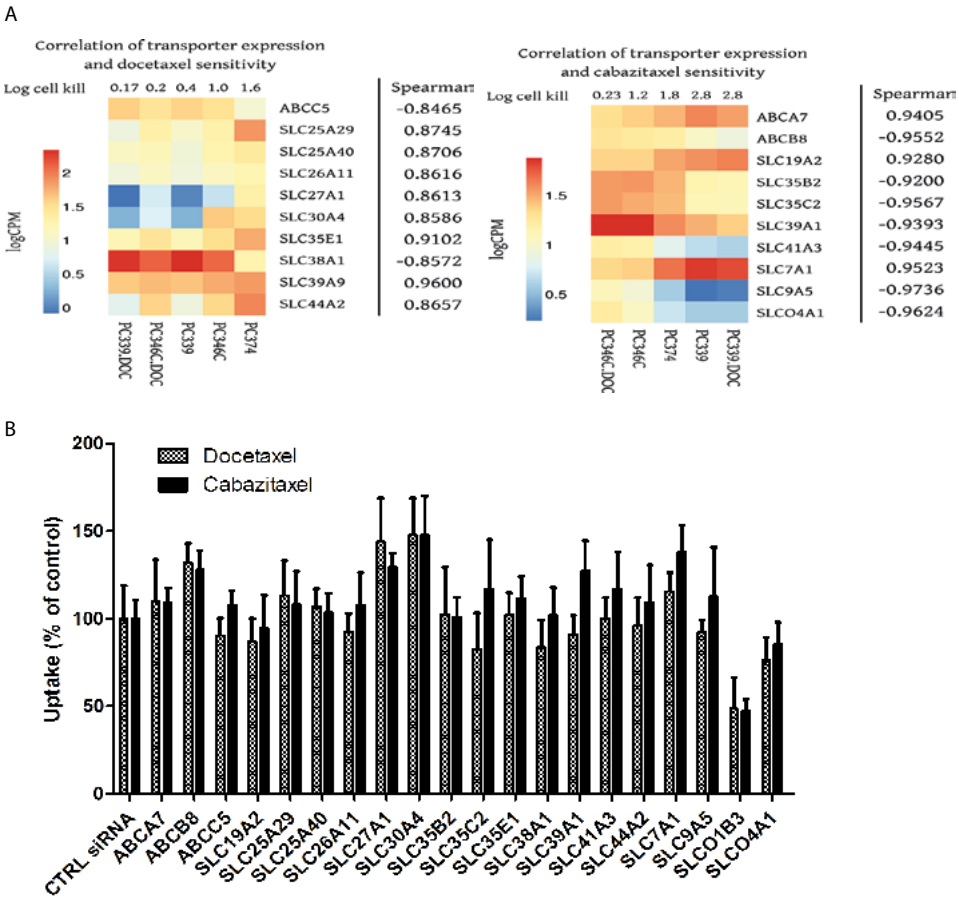
Tumor model	Origin	[d]AR	[e]PSA	p53	PTEN	TMPRSS2 fusion[h]
PC[a]-339	TURP[b]	-	-	Wt[f]	Wt[f]	FLI1
PC[a]-346	TURP[b]	+	+	Wt[f]	Mt[g]	-
PC[a]-374	SSM[c]	+	+	Wt[f]	Mt[g]	ETV-1

[a]PC = primary prostate tumor; [b]TURP = transurethral resection of the prostate; [c]SSM = scrotal skin metastasis, [d]AR = human androgen receptor expression, [e]PSA = prostate specific antigen production, [f]wt = wild- type, [g]mt = mutant, [h] fusion between TMPRSS2 and various ETS-genes

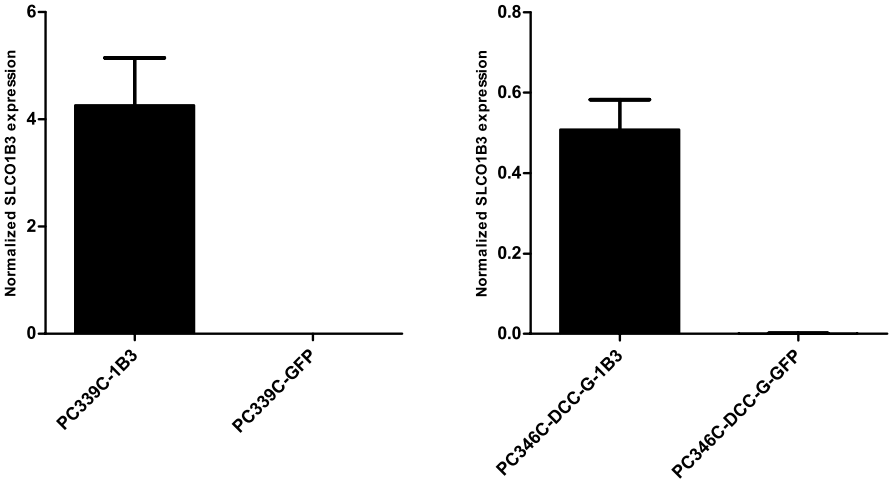
Table is adapted from previous publications (van Weerden et al, 2009; van Weerden et al, 1996) All tumor models were derived from chemotherapy-naïve, abiraterone-naïve and enzalutamide-naïve patients.



Supplementary figure 1: SLCO1B3 expression in the chemotherapy-naïve PDX PC346C. Tumor fragments of PC346C were consecutively passaged. SLCO1B3 mRNA expression was measured in the tumors and normalized against HPRT and PBGD. SLCO1B3 expression remains stable over passaging. Average ± SEM is shown of three independent tumors for each passage.



Supplementary figure 2A) Top 10 listing transporters that correlated with taxane sensitivity. Antitumor activity of taxanes was experimentally determined in docetaxel-naïve and docetaxel resistant PDXs after a single treatment with either docetaxel 33 mg/kg, cabazitaxel 33 mg/kg or placebo. mRNA expression levels of ABC-transporters and SLCs as determined by Next Generation Sequencing were correlated to log cell kill of taxanes using a Spearman's Rank Correlation Coefficients test. Log CPM stands for counts per million and indicates expression. **B)** Uptake and retention of [14C]-docetaxel and [14C]-cabazitaxel was measured in PC346C cells after siRNA mediated silencing of selected drug transporters. PC346C cells were transfected with siRNA pools directed against the indicated transporters. After 48h the transfectants were exposed to [14C]-docetaxel and [14C14]-cabazitaxel for 5 min and subsequently extensively washed. The levels of cabazitaxel and docetaxel taken up and retained in the cells was compared to the uptake of taxanes in cells transfected with CTRL siRNA (arbitrarily set at 100%). An average ± SD is shown of n=3 measurements.



Supplementary figure 3: SLCO1B3 expression in overexpressing cell lines. PC339C and PC346C-DCC-G cells were transfected with a lentiviral expression construct containing SLCO1B3 or GFP as a control. Gene expression was measured using RT-PCR. Average ± SEM is depicted (n=3).

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8

General discussion

Clinical aspects of docetaxel responsiveness

Docetaxel responsiveness may depend on both the pharmacokinetics (PK) and the pharmacodynamics (PD) in patients. Interactions with the PK, may alter the exposure to docetaxel and thus interfere with the antitumor activity. We described in our review that drug-drug interactions on the level of CYP3A4 metabolism interfere with the PK of docetaxel. Furthermore we described that hormonal status such as castration might influence the clearance of docetaxel. Regarding the PD, we found that expression of EG5 in prostate cancer tissue was predictive for docetaxel response. An interaction between docetaxel activity and EG5 is likely, since their working mechanism is both directed towards microtubules. Taxanes inhibit microtubule dynamics, and EG5 functions by crosslinking anti-parallel microtubules in the mitotic spindle. As EG5 is mainly expressed in mitotic cells, it may be a biomarker similar to Ki-67 reflecting cell proliferation. Therefore, both EG5 and Ki67 may reflect docetaxel responsiveness through the assessment of proliferating cells that are generally more sensitive to chemotherapy as compared to senescent cells. To further confirm its use as a predictive marker of docetaxel response, EG5 expression needs to be validated in prospective clinical trials.

Considering the availability of new treatment options for mCRPC patients, it is necessary to evaluate the optimal treatment strategy. This could be obtained not only with the use of predictive biomarkers, but also by treating patients with the optimal total number of docetaxel cycles. Previously, the optimal total numbers of docetaxel treatment has not been investigated in mCRPC patients. We are the first to show in a posthoc analysis of Mainsail, one of the largest phase 3 clinical trials reported, that the total number of docetaxel cycles had independent prognostic significance for survival. This is an important finding, which may influence clinical practice. The differences in median overall survival in relationship with the total numbers of cycles of docetaxel is remarkable. Patients who received >10 cycles had a median OS of 33.0 months compared to 26.9 months in patients treated with 8-10 cycles and 22.8 months for patients treated with 5-7 cycles of docetaxel ($P<0.0001$) in the Mainsail study. These data indicate that patients who appear to have clinical, radiological or biochemical benefit by docetaxel and tolerate treament well should continue beyond 6 cycles. A prospective clinical trial to investigate the impact of the total number of cycles is warranted. Since this would no longer seem feasible in the context of mCRPC as was studies in Mainsail a study in the setting of hormone naïve disease where 6 cycles was recently established appear more appropriate. Two pivotal studies showed survival benefit by the early use of docetaxel with 6 cycles in these patients (1, 2). A prospective study in metastatic hormone sensitive prostate cancer (mHSPC) patients in which 6 vs 10 cycles would be compared would be needed to further investigate the optimal number of docetaxel cycles.

Preclinical aspects of taxane response

Need for docetaxel-resistant models

In order to investigate mechanisms of docetaxel resistance and to identify biomarkers of response, a collection of patient samples prior and post therapy treatment is needed. Such a collection of samples would allow gene expression profiles to be linked to therapy responses, resulting in a potential responder and non-responder profile as putative biomarkers of response. However, it is often rather complicated to collect sufficient tumor material from mCRPC patients. As an alternative, prostate cancer cell lines have often been used to study mechanisms of resistance and sensitivity to docetaxel. However, such resistant cell lines are frequently created in the constant presence of docetaxel, which is not in line with the patient situation. These limitations of cell lines may account for the frequent inconsistencies observed between in vitro derived data and clinical data. This has been particularly shown in the case of overexpression of the ATP-binding efflux transporter ABCB1, which is a commonly found in vitro mechanism of resistance (3-5), while its expression in prostate cancer samples is highly ambiguous (6, 7).

The development of docetaxel-resistant patient-derived xenografts of prostate cancer

Using appropriate preclinical models may help to narrow the search for potential candidate biomarkers to be validated in patients. A valuable alternative to cell lines is the use of preclinical patient-derived xenografts (PDX) models. Such models have been established also for prostate cancer capturing clinically relevant stages of disease and allowing for exploration of mechanisms of tumor growth regulation and treatment response and resistance. PDX models are well-accepted model systems to reflect clinical disease, as they more accurately resemble the complexity of a tumor regarding molecular diversity, cellular heterogeneity and histology as seen in patient samples (8, 9). The PDX panel used in this thesis represents general genetic characteristics of prostate cancer and has been successfully validated as a discovery protein platform for serum biomarkers for prostate cancer (10). It has been effectively used to test new targeted therapies, such as PI3-kinase inhibitors and to optimize prostate cancer targeted imaging (11-13). In this thesis we described the in vivo development of docetaxel-resistant PDX models. Over the years, the panel has been extended with castration resistant-(14) as well as abiraterone-resistant and enzalutamide-resistant models (15, 16) to represent the current new treatment modalities available for prostate cancer patients. In addition, novel PDXs are being generated directly from docetaxel and/or cabazitaxel progressing patients in order to extent the value of our prostate cancer PDX panel. Together, this robust contemporary panel will allow for candidate biomarkers to be validated for taxane-response and other therapies in mCRPC.

Identification and functional validation of SLCO1B3 as a candidate biomarker for taxane response

The current understanding of docetaxel-resistance is still rather poor and so far potential predictive biomarkers, such as EG5 and KI67, have been linked predominantly to the proliferation status of tumors. We have shown a direct correlation between intratumoral concentrations and taxane efficacy and therefore further investigated the role of drug transporters in response to taxanes. Through Next Generation Sequencing (NGS) of chemotherapy-naïve and docetaxel-resistant PDX tumors, we selected ABC transporters and solute carriers that showed a correlation between intratumoral accumulation and taxane responsivenesse in our PDX panel. We identified a direct relationship between SLCO1B3 tumor expression, intratumoral taxane levels, and response to docetaxel and cabazitaxel. Interestingly, SLCO1B3 is known to be a transporter of endogenous compounds such as testosterone, which might imply a dual role of SLCO1B3 in hormone dependent cancer. Also, in light of the reported cross-resistance with androgen receptor-targeted agents, SLCO1B3 may play a role. As abiraterone has structural resemblance with testosterone, abiraterone might be a substrate of SLCO1B3, which would provide a mechanism for the cross-resistance that we observed in the abiraterone-resistant cell line model. Others have indeed reported that expression of SLCO1B3 was linked to intratumoral abiraterone concentrations (17). The relevance of SLCO1B3 as a putative tissue biomarker for taxane responsiveness clearly requires subsequent thorough validation in appropriate patient samples.

Clinical validation of SLCO1B3 as predictive marker of response

As a first step, a retrospective study of SLCO1B3 expression in archival tumor samples from patients who were treated with docetaxel needs to be performed. This may be done by assessment of SLCO1B3 expression determined by immunohistochemical staining and related to surrogate markers of response such as biochemical response (PSA) and response evaluation according to RECIST criteria (18). The major hurdle here is the scarce availability of relevant tumor samples and the variability in time between sampling and docetaxel therapy between patients. The latter is particularly crucial as it was found that SLCO1B3 expression may change during disease progression being increased in late stage mCRPC patients as compared to chemo-naïve patients (22). Therefore, prospective study designs allowing for tumor biopsies pre- and post- taxane treatment are essential to obtain the relevant tumor tissue to validate SLCO1B3 expression in relation to docetaxel treatment.

To use such tissue-related biomarkers, it would be highly relevant to develop analysis methods that would allow such biomarkers to be identified in tumor cells that require less invasive methods. Liquid biopsies from blood or urine may serve as a more readily available alternative to obtain tumor-derived materials that may be more easily applicable. and enhance implementation in a clinical setting. Further studies are required to assess if SLCO1B3 expression identified on circulating tumor cells (CTCs) or cell-free RNA reflect taxane responsiveness.

Cabazitaxel, the taxane of choice?

In five out of six PDX models of prostate cancer studied here, cabazitaxel was at least as effective as docetaxel. Cabazitaxel has been reported to induce higher antiproliferative and procytoxic effects and is a stronger inhibitor of the microtubule dynamics (19, 20). In contrast to docetaxel showing cross-resistance with AR targeted agents like enzalutamide, we did not observed this for cabazitaxel, indicating that cabazitaxel efficacy might be less affected by AR-targeted agents. Furthermore, we found that cabazitaxel intratumoral concentrations were significantly higher in all of our prostate cancer PDX models as compared to docetaxel, which is in line with faster uptake in cells as reported by others (17).

In order to select patients who would be intrinsically resistant to docetaxel and may benefit from cabazitaxel as a first line of therapy, molecular markers would strongly aid the choice for the most optimal treatment for each individual patient. Because of the distinct intratumoral concentration profiles of docetaxel and cabazitaxel, a drug transporter profile as biomarker for taxane responsiveness is evident. In addition to molecular biomarkers, direct assessment of intratumoral concentrations of docetaxel and cabazitaxel may be an interesting alternative, if feasible, as was done for docetaxel in non-small lung cancer patients showing a clear link to response (21).

All together, these findings indicate that cabazitaxel may be generally more potent than docetaxel and may be the preferred taxane in an unselected mCRPC patient population. However, the question if cabazitaxel should be the taxane of choice and become the first line of treatment for mCRPC can only be answered in a randomized phase 3 trial. The FIRSTANA (NCT01308567) trial is the head to head comparison of docetaxel versus cabazitaxel. This trial has completed accrual and results on overall survival and progression free survival endpoints are eagerly awaited

Conclusions

Response to taxanes in mCRPC patients is dependent on several factors, both relating to pharmacokinetic and pharmacodynamic parameters.

The total number of docetaxel cycles has independent prognostic significance for Overall Survival. These data indicate that patients who appear to have clinical, radiological or biochemical benefit by docetaxel should continue beyond 6 cycles, as long as they tolerate their treatment well.

We showed that cabazitaxel had superior antitumor activity in chemotherapy-naïve and docetaxel-resistant PDX models of prostate cancer, which was linked to higher intratumoral concentrations and enhanced accumulation of cabazitaxel. Furthermore, cabazitaxel activity seemed less dependent on AR-pathway activity as compared to docetaxel.

These findings suggest that an optimal treatment sequence may exist for each patient with potential preference for one of the two taxanes. Predictive biomarkers that could distinguish patients who are intrinsically resistant to docetaxel, but may respond to cabazitaxel, is highly relevant to select patients who will benefit from first line treatment with cabazitaxel. We provided evidence that SLCO1B3 may have a potential role as a predictive biomarker of taxane response.

SLCO1B3 is also highly interesting for its putative dual role as it is not only a transporter of taxane, but also of testosterone. Hence it may also contribute to the observed cross-resistance with AR pathway targeted agents like enzalutamide and abiraterone. To further validate SLCO1B3 as a predictive biomarker of taxane response prospective clinical studies are strongly recommended.

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91

Summary / Samenvatting

Summary

Intrinsic or acquired resistance to docetaxel is common in mCPRC patients and the response to docetaxel is highly variable. In view of the ongoing debate about the best treatment sequence strategy for mCRPC and the novel approach of using docetaxel in hormone naïve patients, insight into factors that influence interpatient variability and mechanisms of taxane resistance is crucial. In this thesis an overview is provided of currently known pharmacokinetics factors that influence the observed inter-patient variability in docetaxel response **(chapter 2**). Patient factors such as gender and, hormonal status, but also therapies that inhibit docetaxel metabolism, such as drugs targeting CYP3A4, contribute to individual difference in docetaxel pharmacokinetics. We further investigated whether the number of docetaxel cycles was an independent factor of overall survival and thus docetaxel response in the Mainsail study **(chapter 3).** The total number of docetaxel cycles administered was independently associated with overall survival, which can have important implications for treatment decisions on the duration of docetaxel chemotherapy. On a molecular level of the tumor, we investigated Eg5 expression on prostate cancer samples of patients who were treated with docetaxel. Eg5 was shown as a marker of more aggressive prostate cancers that showed better response to docetaxel **(chapter 4)**. To identify potential novel biomarkers of docetaxel response we studied molecular mechanisms of resistance in docetaxel-resistant, abiraterone-resistant and enzalutamide-resistant patient-derived xenografts of prostate cancer. In **chapter 5** cross-resistance between novel AR targeted agents and taxanes was investigated. Antitumor activity of docetaxel, but not cabazitaxel, was decreased in enzalutamide-resistant tumors, confirming that indeed a treatment sequence may exist. In **chapter 6** a positive relation between intratumoral concentrations of taxanes and antitumor activity in chemotherapy-naïve and docetaxel resistant tumors was found. Resistance to docetaxel was linked to inadequate intratumoral concentrations. Intratumoral concentrations of cabazitaxel were higher compared to docetaxel in our models and were linked to superior antitumor activity of cabazitaxel in most of our models. We further screened for drug transporters that influenced intratumoral concentrations as potential predictive biomarkers of docetaxel-resistant in our models. In **chapter 7** we described a next generation sequencing analysis to identify potential drug transporters as biomarkers of docetaxel response in docetaxel-resistant and chemotherapy-naïve PDXs. SLCO1B3, a known influx transporter of docetaxel, was significantly downregulated in docetaxel-resistant tumors. We functionally validated this transporter and showed that silencing of SLCO1B3 decreased uptake of docetaxel and cabazitaxel. Moreover, SLCO1B3 overexpressing-cells were more sensitive to taxanes treatment. These findings suggest that SLCO1B3 is a potential candidate biomarker of taxane response and possibly also for cross-resistance with abiraterone and enzalutamide. In **chapter 8** we discussed our research on clinical and preclinical aspects of taxane response, with a special focus on our approach to develop a preclinical candidate biomarker. Furthermore, interpretation is given with focus

on the translation of our findings towards a potential biomarker such as SLCO1B3 to predict taxane sensitivity in patients.

Samenvatting

Docetaxel en cabazitaxel behoren tot de geneesmiddelengroep van de taxanen en zijn respectievelijk de eerste- en tweedelijnschemotherapie voor gemetastaseerde castratie resistente prostaatkanker patiënten. Intrinsieke of verkregen resistentie voor docetaxel is veelvoorkomend in mCRPC patiënten en de response voor de therapie is variabel. Inzicht in factoren die docetaxel response kunnen beïnvloeden, is noodzakelijk om de optimale behandeling van mCRPC patiënten vast te stellen. Dit is ook van belang voor hormoon gevoelige prostaatkanker patiënten die recentelijk een goede response op eerstelijns chemotherapie behandeling met docetaxel hebben laten zien. In dit proefschrift is een overzicht gegeven in de vorm van een review van onderzochte factoren die van invloed zijn op de pharmacokinetiek van docetaxel en de variatie in patiënten kunnen voorspellen **(hoofdstuk 2)**. Factoren zoals geslacht en castratiestatus zijn van invloed op de kinetiek van docetaxel. Ook andere geneesmiddelen die via hetzelfde CYP3A4 enzym gemetaboliseerd worden, kunnen de afbraak van docetaxel beïnvloeden en daardoor van invloed zijn op de pharmacokinetiek van docetaxel. Daarnaast hebben we onderzocht of het aantal kuren docetaxel een onafhankelijke factor op de overleving van mCRPC patienten in de Mainsail studie is. Uit deze analyse bleek dat het totaal aantal kuren docetaxel dat toegediend is, onafhankelijk gerelateerd was aan de overleving. Dit kan een belangrijke impact hebben op de behandeling van mCRPC patiënten en de duur van de docetaxel chemotherapie **(hoofdstuk 3).** Op moleculair niveau is Eg5, wat een eiwit is dat tot expressie komt tijdens de celdeling, getest in prostaatkanker tumoren afkomstig van patiënten die met docetaxel zijn behandeld. We vonden dat tumoren waar Eg5 tot expressie kwam agressievere prostaatkankers waren en dat patiënten beter op docetaxel reageerden **(hoofdstuk 4)**. Om mogelijke andere potentiele markers te identificeren en mechanismen van docetaxel resistentie en response op cabazitaxel verder te onderzoeken, hebben we docetaxel-resistentie humane xenograft modellen ontwikkeld. Omdat mCRPC patiënten naast docetaxel behandeling ook met nieuwe androgeen depriverende middelen zoals abiraterone en enzalutamide behandeld kunnen worden en er mogelijk kruisresistentie bestaat met taxanen, hebben we ook enzalutamide en abiraterone resistente humane xenograft modellen ontwikkeld. In **hoofdstuk 5** hebben we kruisresistentie tussen docetaxel/cabazitaxel en enzalutamide onderzocht. De antitumor activiteit van docetaxel, maar niet van cabazitaxel, was verminderd in enzalutamide resistente humane xenograft modellen. Deze bevinding suggereert dat er inderdaad een optimale behandelingsvolgorde voor mCRPC patiënten bestaat. In **hoofdstuk 6** beschrijven we de belangrijke bevinding dat intratumorale concentraties van docetaxel en cabazitaxel gerelateerd zijn aan de response. Hoge concentraties van beide chemotherapeutica leidt tot een betere respons. Resistentie voor docetaxel was gerelateerd aan inadequate concentraties in de tumor. Cabazitaxel concentraties in de tumor waren hoger dan voor docetaxel, wat voor een deel kan verklaren

waarom cabazitaxel effectiever is in zowel chemotherapie-naïve als docetaxel-resistente tumoren. Omdat intratumorale concentraties van docetaxel voorspellend waren voor de response, is een screening gedaan op een selectie van drug transporters die mogelijk de intratumorale concentraties van docetaxel en/of cabazitaxel zouden kunnen beïnvloeden. Deze selectie is tot stand gekomen door met behulp van *Next Generation Sequencing* een profiel te maken van transporters die correleren aan gevoeligheid met docetaxel of met cabazitaxel in een panel humane xenografts (**hoofdstuk 7)**. In ditzelfde hoofdstuk werden de RNA expressie profielen van chemotherapie naive en docetaxel-resistente tumoren vergelijken. We vonden dat de expressie van SLCO1B3, een bekende opname transporter van docetaxel, significant naar beneden was gegaan in docetaxel resistente tumoren. Met behulp van functionele validatie experimenten werd vastgesteld, dat de opname van zowel docetaxel als cabazitaxel naar beneden ging als de expressie van SLCO1B3 uitgezet werd. Ook waren cellen die SLCO1B3 hoog tot expressie brachten gevoeliger voor behandeling met docetaxel, en cabazitaxel. Deze resultaten laten zien dat SLCO1B3 een interessante kandidaat marker is van taxane response in mCRPC. Dit moet echter nog gevalideerd worden in prospectieve klinische studies waarin de expressie van SLCO1B3 gerelateerd kan worden aan de response op de chemotherapie en mogelijk ook kruisresistentie met abiraterone en enzalutamide. In **hoofdstuk 8** bediscussiëren we de preklinische en klinische aspecten van taxaan-gevoeligheid in castratie resistentie prostaatkanker en exploreren we de mogelijkheid voor SLCO1B3 als marker voor taxaan response.

IA

Dankwoord

Curriculum Vitae

List of publications

PhD Portfolio

Dankwoord

Een tijdje geleden, hier ver vandaan in het land van professoren en doctoren, liep een student op een dag het Erasmus Medical Center (EMC) binnen. Ze ontmoette dr. Wytske Van Weerden, professor Ronald de Wit, dr. Erik Wiemer en dr. Herman Burger. "Goh", zei deze student, "Heeft u niet een baan voor mij? Ik ben heel ambitieus en ik houd van hard werken. "Zo, ambitieuze OIO-to-be, weet jij wel wat promoveren inhoudt," werd er gevraagd. "Nou, ik denk het wel," zei ze. "Ik heb het weekend nog een vriendin-OIO huilend aan de telefoon gehad. Het is vallen en vooral opstaan". Tevreden knikten de doctoren en de professor en praatten vervolgens de rest van de tijd uitsluitend over zichzelf. "Je mag hier komen werken voor 4 jaar op het project over docetaxel resistentie, we verwachten dat je je in zult zetten en van je onderzoek zal gaan houden". En zo geschiedde. Professor Ronald de Wit werd de promotor van de OIO. Hij zou haar gedurende haar promotie altijd goed in de gaten houden en haar helpen met het stellen van kritische vragen om tot de kern te komen en vooral de link met de kliniek te behouden. En dr. Van Weerden werd haar co-promoter. Met dr. Van Weerden zou ze inhoudelijk en goede gesprekken voeren, en ook gezellige soy-chai lattes drinken. De OIO was heel dankbaar dat ze deze kans gekregen heeft van dr. Van Weerden en Professor de Wit en ging met goede moed aan de slag.

Het bureau van de OIO kwam te staan in een kamer met het nummer BE-331. Hier werkte ze zij aan zij met medeonderzoekers Yin, Robert, Matthijs, Lisanne aan mooie publicaties. Ze deelden koffie, migraine-pijnstillers, lief en leed en niet te vergeten: tosti ham-kaas. Maar ook buiten de gezellige eigen kamer was het goed toeven in het EMC. Bijvoorbeeld met haar andere OIO-vriend(inn)en van medische oncologie: Annemieke, Lisette en Anne-Joy, Jacqueline en Sander. De OIO voelde zich altijd gesteund om de, toch wel zware, taak van het promoveren te volbrengen.

Daarnaast kwam ze ook andere lieve mensen tegen op weg naar het lab: Wilma, Sigrun, Mirella, Natasja, Joke, Diana, Peter, Inge en Mei. Met de pipet in de hand, waren ze heer en meester in het urologie-en-medische-oncologie-land. Met overal een protocolletje voor, kookten de dames en heren eiwit-soepjes, chemo-concentraten en andere lekkere blotjes. Ook de andere doctoren van het lab: dr. Hanneke, dr, Rute, dr. Petra en dr. Elena, hadden altijd een goede oplossing, voor zowel persoonlijke als inhoudelijke problemen. De OIO is heel blij dat zij ook in het land van professoren en doctoren rondliepen. Iedereen stond altijd klaar om de OIO te helpen en de fijne kneepjes van het vak te leren (onder het mom van 'pipetteren kun je leren').

Na een beetje ingewerkt te zijn, was de OIO klaar om prostaatkankercellen te pesten door chemotherapeutica op ze te gooien. Diverse prostaatkankercellen legde het grote lood, maar niet allemaal. "Wat zou er toch met die cellen aan de hand zijn?," vroeg de OIO zich

af. Corrina, Sander (zelfbenoemde prins charming), Agnes en Debra maakten samen met de OIO klinisch relevante docetaxel-resistente prostaatkanker xenograft modellen om docetaxel-resistentie te onderzoeken. Wat een geweldig werk! De OIO was zeer verheugd toen ze meerdere nieuwe modellen hadden ontwikkeld!

Toen de modellen er waren, ging de OIO (met een take-away coffee in de hand,) vervolgens op pad om te ontrafelen wat er nu anders was in de docetaxel-resistente cellen. "Denk eraan," zei professor Guido Jenster, "als je het pathway niet meer kan vinden, het gaat om restrictie en preferentie." Maar aan deze algemene en toch wel abstracte woorden had de OIO natuurlijk niet zoveel, ze raakte al snel verdwaald in het land-van -20.000-genen-en-RNA expressie-profielen. Tot op een dag OIO-René daar was om haar te redden uit de kluwen data. Met zijn computer en programmeer-talent toverde hij hele genexpressie profielen van docetaxel-resistente tumoren naar voren. Er was echter één gen, dat het meest betoverend was van alle genen! SLCO1B3, een drugtransporter was helemaal verdwenen in de docetaxel-resistente tumoren. Deze transporter werd uiteraard het lievelingsgen van de OIO en ze nam het mee, op weg naar het land van validatie en publicatie!

Zo nu en dan, op weg naar dit veelbelovende land, kreeg de OIO goede raad van de professoren. "Denk eraan," zei professor Bangma: "jouw onderzoek is niks zonder patiëntenmateriaal". Dat hoefde professor Bangma maar één keer te zeggen om indruk te maken op deze OIO. En ze besloot aan de slag te gaan. Met behulp van geweldige enthousiaste dokters: dr. Egbert Boeve, dr. Paul Hamberg, dr. Joost Boormans, dr. Martijn Busstra, dr. Arno van Leenders en dr. Harm van Mellick en de belangeloze medewerking van patiënten juist op kwetsbare momenten in hun leven, lukte het om keer op keer weefsel te verzamelen.

Ook andere professoren en doctoren zoals professor Ron Mathijssen en dr. Herman Burger wisten de OIO op het rechte pad te houden. Daarnaast waren de maandelijkse video-meetings tussen de OIO en alle doctoren en professoren betrokken bij het project onontbeerlijk, al werden de meest interessante dingen pas besproken na afloop van de meetings als men niet in de gaten had dat de verbinding nog live was.

Als de OIO het even niet meer zag zitten, wisten zowel dr. Erik Wiemer als dr. Ellen Schenk altijd wel een goede spreuk om de motivatie in de OIO aan te wakkeren. Uiteraard hielp koekjes eten ook altijd om emotionele tegenspoed veroorzaakt door deprimerende JNI-meetings te laten verdwijnen. Hetgeen waar de OIO het meest van genoot waren congressen. De OIO deed niks liever dan praten over haar onderzoek en uren voor haar poster staan, of een Oral presentation geven op het Prostate Cancer UK forum in Baltimore, of winkelen in Boston met dr. Van Weerden.

Het was een stormachtige dag toen de OIO eenmaal aangekomen was in het land van validatie en publicatie. Ashraf kwam toen aan haar zijde. Deze analist werkte hard, erg hard. Zo hard had niemand ooit gewerkt. Altijd maar kweken van die vervelende cellijnen: cellijnen met en zonder SLCO1B3. Die lieve Ashraf had ze allemaal. En na een tijdje was het daar: de cellen met SLCO1B3 bleken gevoeliger voor chemotherapie te zijn. Ashraf en de OIO maakten een dansje van plezier. Dat was voer voor een publicatie en misschien wel voor twee!

Op een nacht droomde de OIO van een ander lab ergens in het grote Amerika. Namelijk bij professor Alex Sparreboom en professor Sharyn Baker in Memphis. Haar droom duurde wel 3 maanden en in het St Jude Children's Research Hospital leerde Alice haar *ins and outs* van opname experimenten. De OIO kreeg een nieuwe boost om verder te gaan met haar PhD, dankzij de erg inspirerende gesprekken. Dr. Jolieke maakt de OIO wegwijs in Memphis, leerde haar waar de gevaarlijke supermarkt was en hoe door Memphis te scheuren met haar huurauto. De OIO wist het erg te waarderen dat dr. Wytske van Weerden haar was komen opzoeken in Memphis! De OIO vond het alleen wel onverstandig van dr. Van Weerden om in de ghetto te willen hardlopen en was blij dat ze haar kon weerhouden van dergelijk gevaarlijke activiteiten.

Eenmaal wakker geworden uit haar droom en terug uit Memphis, was het einde alweer in zicht. Nog maar één jaar te gaan in het land van professoren en doctoren. Omdat het nu eenmaal traditie was om in het laatste jaar van je PhD zwanger te worden op het uro-lab, kon de OIO haar geluk niet op dat ze ook aan deze traditie kon deelnemen. Sommige professoren maakten zich zorgen of de hersenen van de OIO nog wel hetzelfde zouden zijn tijdens en na de zwangerschap, maar gelukkig bleef de grijze massa van deze OIO prima intact (zegt ze zelf).

De paranimfen van de OIO, Frank en Natalie, weken gedurende haar promotie-traject geen minuut van haar zij. Intensieve sessies met haar broer Frank waarin de OIO het wel en wee van het promotietraject deelde, eindigden standaard met "Laat je niet gek maken hoor, keep on going" en vriendin Natalie had in tijden van tegenspoed altijd het goede advies te gaan shoppen voor schoenen en jurken. En dat hield de OIO op de been. Uiteraard waren de familie en vrienden buiten het professoren en doctoren land ook erg belangrijk voor de OIO om vol te houden en niet het bijltje erbij neer te leggen. En niet te vergeten, Francis.
"Caro Francis, thank you for always being there for me and preparing the best coffee in the world. Ti amo."

"Hoe snel de tijd kan gaan, die vier jaar is zo weer voorbij!", verzuchtte de OIO in haar laatste maanden. De OIO heeft besloten het land van professoren en doctoren te verlaten en de wijde wereld in te trekken. Met warme gevoelens denkt ze vaak terug aan deze geweldige tijd, waar ze iedereen hartelijk voor wil bedanken die bij heeft gedragen aan dit mooie verhaal. Uiteraard leefde ze nog lang en gelukkig met haar man Mathijs en hun dochtertje Saar.

The End

Curriculum Vitae

Eleonora Susanne Terwindt-de Morrée werd geboren op 27 december 1987 te Gorinchem. In 2006 behaalde zij haar diploma aan het Gymnasium Camphusianum te Gorinchem. Aansluitend startte zij met de studie Farmacie aan de Universiteit Utrecht. Daarnaast heeft zij diverse commissies gedaan. Ze organiseerde het NK debatteren in 2008 en was secretaris van de multidisciplinaire studievereniging Hygieia in 2008 en 2009. Na haar bachelor Farmacie behaald te hebben, besloot zij zich meer te willen focussen op onderzoek en heeft vervolgens de master Toxicology and Environmental Health cum laude afgerond in 2011. Gedurende haar bachelor en master heeft zij tevens succesvol deelgenomen aan het honoursprogramma Farmacie. In 2011 startte zij met promotieonderzoek op de afdelingen Urologie en Medische Oncologie in het Erasmus Medisch Centrum te Rotterdam, onder begeleiding van professor Ronald de Wit en dr. Wytske van Weerden. Tijdens haar PhD heeft ze drie maanden in het St. Jude Children's Research Hospital (Memphis, Verengide Staten) een deel van haar onderzoek uitgevoerd, onder begeleiding van professor Alex Sparreboom. Sinds januari 2016 werkt Ellen als Medical Advisor Oncology bij MSD. Ellen woont samen met haar man Mathijs en dochtertje Saar in Den Haag.

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*both authors contributed equally

Portfolio

Name PhD Student: E.S. Terwindt-De Morrée
PhD period: 2011-2015
Erasmus MC Department: Urology / Medical Oncology
Promotor: Prof. Ronald de Wit
Research School: Molecular Medicine
Supervisor: Dr. Wytske van Weerden

	Year	ECTS
General Courses		
Biomedical English Writing and Communication	2012-2013	4
Course on molecular diagnostics	2012	1
Introduction in GraphPad Prism	2015	1
Analysis of microarray and RNA seq expression data	2013	1
Frame course	2012	1
Basic data analysis on gene expression arrays	2011	1
Seminars and Workshops		
Journal Club (monthly)	2012-2015	1
JNI meetings (weekly)	2011-2015	1
Lab meeting Urology	2011--2015	1
Lab meeting Medical Oncology	2011-2014	1
Get out of your lab days	2013	2.5
Presentations national		
Dutch uro-oncologic study group (DUOS)	2012, 2015	1.5
IKNL bijscholingsavond	2012	0.5
Tour d'Europe Rotterdam	2014	0.5
Presentations international		
ESUR 2012, 2013, 2014	2012-2014	1.0
PCTRE 2013	2013	1.0
AACR 2013	2013	1.5
EORTC-NCI-AACR 2012	2012	1.5
AACR-NCI-EORTC 2013	2013	1.5
AACR-NCI-EORTC 2015	2015	1.5
Prostate Cancer UK forum 2014	2014	1.0
Lecturing		
Supervision of Junior Med School	2013	1.5
Supervison of profielwerkstuk high school students	2013	1.5
Supervision of HBO and Master students	2012-2014	4.0