Docetaxel resistance in castration resistant prostate cancer: the role of c-Jun and the androgen receptor

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**Samenvatting**
Prostaatkanker is de op één na meest voorkomende maligniteit bij mannen en tot op heden de op één na meest voorkomende oorzaak van kankerge relatieve sterfte wereldwijd. Gemetastaseerde hormoon refractaire prostaatkanker (HRPC) is een lethale ziekte met een slechte prognose. Alhoewel er nieuwe, veelbelovende medicamenten op de markt zijn gekomen voor de behandeling van gemetastaseerd HRPC, neemt het chemotherapeuticum docetaxel wereldwijd nog steeds een belangrijke plaats in bij de behandeling van deze patiëntengroep. Echter reageert slechts de helft van de patiënten op therapie met docetaxel en ervaren de meeste patiënten progressie van de ziekte binnen het eerste behandelingssjaar, waardoor resistentie de belangrijkste beperkende factor is van de behandeling. Een beter inzicht in het effect van docetaxel op prostaatkankercellen kan in belangrijke mate bijdragen aan de ontwikkeling van mogelijkheden om het ontstaan van resistentie voor docetaxel te voorkomen of vertragen.

Eerdere studies toonden aan dat docetaxel tot een verhoogde expressie van de androgeenreceptor en het prostaat specifiek antigeen (PSA) leidde, maar hoe deze stimulatie geschiedt was niet bekend. Voor dit verslag werden PSA kwantificatie testen en Western blot analyses uitgevoerd om de rol van het proto-oncogen c-Jun en de androgeenreceptor bij docetaxelresistentie te bepalen. Er werd aangetoond dat docetaxel zorgde voor een verhoogde expressie van de androgeenreceptor, die onafhankelijk was van endogeen c-Jun, en dat de tijdsafhankelijk verhoging van het PSA gemedieerd wordt door de androgeenreceptor. Deze uitkomsten onderstrepen nogmaals het belang van verder onderzoek naar de regulatie van de androgeenreceptor, die betrokken is bij zowel het ontstaan als de progressie van de ziekte.

**Summary**
Prostate cancer is the second most common malignancy in men and has remained the second leading cause of cancer related death worldwide. Metastatic castration resistant prostate cancer (mCRPC) is a lethal disease with a poor prognosis. Although new promising drugs in mCRPC treatment have been approved, the chemotherapeutic agent docetaxel has remained a cornerstone of the standard of care in men worldwide. However, only half of patients will respond to docetaxel therapy and most men will experience progression of the disease within the first year of treatment, resulting in resistance being the main limitation of its efficacy. A better understanding of the impact of docetaxel on prostate cancer cells could provide opportunities for preventing or delaying the development of resistance to docetaxel.

Previous studies demonstrated that docetaxel increases the expression of androgen receptor and prostate specific antigen (PSA), but how these effects are mediated was yet unknown. In this report, PSA quantification tests and Western blot analyzes were performed to elucidate the role of proto-oncogene c-Jun and the androgen receptor in docetaxel resistance. It was shown that docetaxel treatment leads to an increased expression of the androgen receptor, which is independent of endogenous c-Jun levels and that the time dependent increase in PSA level is mediated by the androgen receptor. These results underline once more the importance of further research on the regulation of the androgen receptor, which plays an important role in both onset and progression of the disease.
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INTRODUCTION

Prostate cancer is the second most common malignancy in men.\textsuperscript{1} Although many men will have indolent disease that does not threaten their natural life span, many other men will have aggressive disease and death from prostate cancer, which remains the second leading cause of cancer related death worldwide. Albeit the vast majority of patients with localized disease is cured with surgery and/or radiation therapy, over 30\textendash40\% of patients will progress and develop advanced disease.\textsuperscript{2}

In most cases, previously untreated advanced prostate cancer is initially dependent upon androgens for its growth and patients typically respond to androgen deprivation therapy (ADT).\textsuperscript{3,4} However, resistance inevitably occurs after a median time of 18-24 months of ADT, leading to a state termed castrate-resistant prostate cancer.\textsuperscript{5}

Metastatic castration resistant prostate cancer (mCRPC) is a lethal disease. Patients diagnosed with mCRPC have a poor prognosis with an expected survival up to 18-19 months.\textsuperscript{6} It is now accepted that not only prostate cancer initiation, but also the progression to its castration resistant and metastatic form are dependent on androgens and androgen receptor signaling.\textsuperscript{7,8} These observations have led to the recent development of several new agents that target the androgen-androgen receptor pathway at the prereceptor, receptor or postreceptor ligand binding level, such as CYP17 inhibitor abiraterone and the novel androgen receptor antagonist enzalutamide.

Although since 2010 new promising therapies have been approved, the first-line chemotherapeutic agent docetaxel has remained a cornerstone of the standard of care in men with mCRPC worldwide. However, only half of patients will respond to docetaxel therapy and most men treated with docetaxel will experience progression of their disease within the first year from the start of treatment, resulting in \textit{de novo} and acquired resistance being the main limitation of its efficacy.\textsuperscript{9,10}

The rapid induction of docetaxel resistance suggests that at least some molecular mechanisms that contribute to the development of CRPC also play a role in the development of docetaxel resistance, but the exact mechanisms by which docetaxel mediates its effect on prostate cancer cells is not well known. A better understanding of the impact of docetaxel on prostate cancer cells could provide opportunities for preventing or delaying the development of resistance to docetaxel and, given the abundance of new drugs, help answer the clinically important question in what sequence these agents should be given. Previous studies have shown that docetaxel treatment increases expression of the androgen receptor and prostate specific antigen (PSA), which is an important tumor marker for prostate cancer.\textsuperscript{11,12} How docetaxel mediates these effects on androgen receptor and PSA level is yet unknown and will be the main focus of this report.

BACKGROUND

The prostate

The prostate gland is a secretory organ that is part of the male reproductive system. The human prostate is located just below the bladder, where it surrounds the proximal part of the urethra and has the size of a walnut.

Approximately 30\% of the prostate consists of fibromuscular stroma,\textsuperscript{13} comprising a.o. smooth muscle cells, fibroblasts and nerve fibres.\textsuperscript{14,15} The remaining 70\% consists of
glandular tissue: a collection of 30 to 50 tubuloacinar glands with ducts that together form branching secretory tubules and acini, embedded in the fibromuscular stroma.\textsuperscript{13,16} The tubuloacinar glands produce a fluid that contains various glycoproteins, enzymes and small molecules such as prostaglandins, that is stored until ejaculation when the smooth muscles in the stromal part contracts.\textsuperscript{16}

The glands are arranged in three glandular zones, which differ both histologically and biologically. The glandular tissue around the prostate urethra represents the \textit{transition zone}, comprising 5-10\% of the glandular prostate mass.\textsuperscript{17} The transition zone contains the mucosal glands and is the main site of origin of benign prostatic hyperplasia (BPH).\textsuperscript{16,17} The \textit{central zone} surrounds most of the transition zone, comprising 25\% of the gland and containing the submucosal glands. This region is relatively resistant to disease.\textsuperscript{18} The \textit{peripheral zone} is the outermost zone, comprising about 70\% of the prostate and containing the main glands. The peripheral zone is the most susceptible to inflammation and the main site of origin of carcinomas with approximately 88\% of prostate cancers occurring in this zone.\textsuperscript{18,19}

In all zones, the glandular epithelium include three major types of cells: secretory (luminal) cells lining the glandular ducts, basal cells adjacent to the basal membrane and neuroendocrine cells that are irregularly distributed throughout the acini.\textsuperscript{19} Secretory cells are the predominating cell type in glandular epithelium, whereas basal cells are the main proliferating cell type with less than 10\% of the basal cells believed to be stem cells for the prostate gland.\textsuperscript{20,21} Neuroendocrine (NE) cells play a regulatory role in normal prostate growth, differentiation and secretory processes in both an endocrine and paracrine manner.\textsuperscript{22,23} However, NE cells also have a role in the pathogenesis of prostate cancer and NE differentiation has been associated with progression of the disease towards an androgen-independent state.\textsuperscript{24}

\textbf{Prostate-specific antigen (PSA)}

Prostate-specific antigen (PSA), or human glandular kallikrein 3 (hK3), is a glycoprotein that is a member of the tissue kallikrein family. PSA is produced by both prostate epithelial cells and prostate cancer cells, although its expression in prostate cancer on a per cell basis is usually lower than in normal epithelial cells.\textsuperscript{25,26} The expression of PSA is strongly androgen-dependent and positively regulated by the androgen receptor (AR).\textsuperscript{27} PSA is primarily produced by the secretory cells of the prostate glands and functions as a serine protease cleaving semenogelin I and II to enhance sperm motility by liquefying the semen.\textsuperscript{28,29}

Normally, PSA is produced as the inactive precursor protein proPSA and secreted into the lumen,\textsuperscript{30} where it undergoes cleavage of the N-terminal seven amino acids to generate active PSA.\textsuperscript{31} The major activating enzyme is human kallikrein 2 (hK2), but PSA may also be activated by other prostate kallikreins, such as prostase (hK4).\textsuperscript{32} A fraction of the active PSA becomes inactive because of internal cleavages by proteases and enters the blood stream, where it circulates as free PSA.\textsuperscript{33} Alternatively, active PSA diffuses into the circulation, where it is rapidly bound by the protease inhibitors alpha-1-antichymotrypsin (ACT) and, to a lesser extent, alpha-2-macroglobulin and alpha-1-antichymotrypsin (bound PSA).\textsuperscript{34,35}

Compared to men who have a normal prostate or BPH, the percentage of bound PSA is higher in men with prostate cancer. In prostate cancer, there is a complete loss of the basement
membrane and disruption of the normal lumen architecture. Therefore proPSA has a decreased luminal exposure to proteases and direct access to the circulation, resulting in relative increases in bound PSA and proPSA in the serum.\textsuperscript{36-38} Decreased cleavage of proPSA by hK2 in prostate cancer also results in truncated forms of proPSA, which have extra amino acids compared to active mature PSA and are inactive. These truncated proPSA forms, including [-2]pPSA and [-5]pPSA, are elevated in prostate cancer and circulate as free PSA.\textsuperscript{39}

**Androgens and the androgen receptor**

Androgens are essential hormones in the male reproductive system, as they are indispensable for the development, differentiation and functioning of the prostate gland.\textsuperscript{40} However, it was already in 1941 that also the development of prostate cancer has been shown to be androgen dependent.\textsuperscript{41}

Free testosterone can enter the prostate gland, where the androgen is converted to its more active derivative 5α-dihydrotestosterone (DHT) by the enzyme 5α-reductase.\textsuperscript{42} Both testosterone and DHT mediate their effects through the AR, but the AR has a higher affinity for DHT and also a slower dissociation from this more potent derivative.\textsuperscript{43} Without ligand is the AR an inactive monomer located in the cytosol, associated with heat shock proteins (HSPs).\textsuperscript{44} Upon ligand binding, the AR undergoes a conformational change as described above, that results in the dissociation from HSPs and enables interactions between the AR and coregulators that facilitate nuclear transport and nuclear dimerisation.\textsuperscript{45}

Following dimerisation, the AR binds with coactivators and corepressors to specific genomic sequences in promoter and enhancer elements of DNA, called androgen response elements (AREs). Binding to these AREs can induce or inhibit the transcription of particular target genes, resulting in changes in the expression of genes and their encoded proteins to modulate androgenic effects.\textsuperscript{44}

**The androgen receptor (AR)**

The androgen receptor is part of the steroid receptor family and expressed in the reproductive organs, muscles, brain, kidney, spleen, heart, liver and salivary glands.\textsuperscript{46} In prostate epithelial cells AR acts as a ligand-dependent transcription factor that modulates both their growth and development.\textsuperscript{47}

Human AR is encoded by a gene located on the short arm of chromosome X (Xq11-12), thereby giving karyotypically normal men one copy of this gene. The AR gene contains eight exons and shows a slightly varying open reading frame and therefore slightly varying protein length, due to variation in the length of the two codon repeat tracts CAG and GGN.\textsuperscript{48}

Resembling the other steroid hormone receptors, the AR consists of four functional domains:

1) **The N-terminal Transactivation Domain (NTD)**

The main role of the NTD is the recruitment of other proteins for AR’s transcriptional activity.\textsuperscript{49} Both Activation Function-1 (AF-1) and Activation Function-5 (AF-5) are responsible for the AR transactivating function, with AF-1 having the strongest transactivation potential in the ligand-activated receptor and AF-5 in the ligand-independent-activated receptor.\textsuperscript{50} The NTB also contains the polymorphic CAG and GGN-repeats that contribute to the proteins flexibility and may be an explanation for the differences seen in receptor activity.\textsuperscript{51}

2) **The DNA Binding Domain (DBD)**

The DBD consists of two zinc fingers that interact with DNA and a C-terminal extension.\textsuperscript{52} The first zinc finger contains the proximal-box (P-box): a sequence that coordinates specific interactions between the protein and the part of DNA where the
hormone responsive elements (HREs) are located. The second zinc finger contains the distal-box (D-box), that enables interaction with DNA by homo-dimerizing the ARs.

3) The hinge region
In this small region the nuclear localization signal (NLS) is found: it binds to the nuclear import factor importin-α, that mediates the transport of the AR through the nuclear pore complex into the cell nucleus. Mutations in this region can either reduce the binding affinity, that has been associated with the androgen insensitivity syndrome and impaired or lacking AR activity, or decrease AR activity by interrupting the NTD and LBD interactions.

4) The C-terminal Ligand Binding Domain (LBD)
Upon ligand binding, the LBD undergoes a conformational change: the ligand binding cavity closes and a hydrophobic cleft gets exposed, forming ligand-dependent activation function 2 (AF-2). In AR, this AF-2 motif binds mainly to the 25FQNLF27 motif in NTD.

There is a strong interaction between the LBD and NTD that has been shown to be necessary for complete AR activity. The precise function of this interaction is unknown, but it possibly facilitates AR activation by making protein-protein and protein-DNA interacting areas available at the surface to facilitate AR transactivation.

Castration resistant prostate cancer
It has been demonstrated that androgen-responsive genes continue to be expressed in men that were thought to be ‘androgen insensitive’ or ‘hormone refractory’ before, implying that the AR signaling pathway continues to drive prostate cancer growth in most patients. The means by which CRPC continues to grow, despite suppression of testicular androgen and blockade of the AR ligand-binding domain by anti-androgen drugs, is through a variety of mechanisms:

Persistent androgen receptor signaling
Persistent androgen suppression induces progression to castration resistance in prostate cancer cells, in which the AR signaling is maintained. In CRPC, the AR shows an increased expression, greater stability and nuclear localization, indicating hypersensitivity of the AR for low concentrations of androgens. Amplification of the AR gene is seen in 30% of the CRPCs from patients treated with androgen deprivation therapy (ADT), whereas no amplifications were found in untreated tumors. Patients with AR amplification also respond better to second-line maximal androgen blockade than patients without AR amplification, suggesting that those tumors may be androgen hypersensitive.

AR mutations are rarely found in untreated prostate cancers or tumors treated by castration alone, but have been detected in 20-25% of patients treated with anti-androgens, such as flutamide and bicalutamide. The most frequently found mutation in prostate cancer is the replacement of threonine 877 with alanine (T877A mutation), which corresponds to the mutation observed in LNCaP cells. It has been demonstrated that alterations in the ligand-binding domain of the AR in these cells allow AR activation by non-androgen ligands, such as estrogens, progesterone, adrenal steroids, receptor tyrosine kinases and, paradoxically, anti-androgens (associated with the anti-androgen withdrawal syndrome), which gives prostate cancer mutant receptors a survival advantage in an androgen-depleted environment.
Castration resistance can also be explained by the presence of androgen-receptor splice variants lacking the ligand-binding domain, but retaining the transactivating domain. These truncated proteins are constitutively active as transcription factors and promote activation of target genes, although these variants are unable to bind ligand.\(^67\)

**Ectopic androgen synthesis**

The major part of the circulating testosterone is produced by the testes. ADT primarily inhibits this gonadal androgen synthesis, resulting in a 95% decrease in serum testosterone levels. However, in CPRC the intraprostatic levels of testosterone and DHT may remain sufficient to stimulate the AR, as a result of local conversion of adrenal androgens to testosterone and *de novo* intratumoral synthesis of androgens through upregulation of steroidogenic enzymes, such as cytochrome P450 -17 (CYP17).\(^{68,69}\)

**Role of co-regulators of AR**

Coactivators and corepressors function as signaling intermediaries for AR-mediated transcription, promoting or inhibiting binding of AR to AREs. Alterations in genes encoding transcriptional coactivators (NCOA2), E1A binding protein p300, transcriptional corepressors (NCOR2), interacting transcription factors, and chromatin regulatory elements have been found,\(^70,71\) suggesting that both overexpression of coactivators and downregulation of corepressors are capable of facilitating AR-mediation transcription.

**Activation of compensatory AR-independent pathways**

In the presence of low or undetectable levels of androgens, signaling that is usually AR dependent can be triggered by activation of other receptor tyrosine kinases, such as insulin-like growth factor 1 (IGFR-1), epidermal growth factor-R (EGFR) and vascular endothelial growth factor-R (VEGR) and their signal transduction pathways PI3K/Akt and Ras/Raf/MAPK/ERK in CRPC.\(^72\)

Crosstalk has been demonstrated between the AR in the cytoplasm and tyrosine kinase HER2/neu, resulting in activation of the AR in the absence of androgens, and also HER3, the activation of tumor growth factor-βR (TGF-βR), Wnt/β-catenin, Src kinase and interleukin-6R (IL-6R) and their pathways have been implicated in crosstalk with the AR.\(^72\) Activation of antiapoptotic pathways may form another explanation for the development of castration resistance. Overexpression of the antiapoptotic protein Bcl-2 and the related factors Bcl-XL and survivin is frequently seen in CRPC, but not in hormone-responsive disease.\(^75\)

**Enzalutamide**

Historically, the treatment for metastatic prostate cancer has been focused on targeting the androgen-AR signaling. Secondary hormonal manipulations with addition of an AR antagonist such as bicalutamide, flutamide or nilatumide, have effect in 30% of patients progressing on ADT.\(^76\)

Enzalutamide is a novel AR antagonist with a five to eightfold higher affinity for AR compared to bicalutamide.\(^77\) In contrast with other AR antagonists, enzalutamide is a pure antagonist that does not become a ligand to AR in the setting of AR amplification.\(^78\)

**Taxanes**

In patients with castration-resistant prostate cancer, it was not until 2004 that a treatment was shown to be prolonging the median overall survival. Two landmark phase III trials, TAX-327 AND SWOG 99-16, demonstrated a survival benefit of 2 to 3 months for patients treated with docetaxel (75 mg/m\(^2\)) every 3 weeks. This led to the establishment of docetaxel as a first-line agent in patients with mCRPC.\(^9,10\)
Taxanes, such as docetaxel, paclitaxel and cabazitaxel, are chemotherapeutic agents that interfere with microtubules. Microtubules orchestrate an orderly separation of chromosomes during mitosis and are composed of α- and β-tubulin heterodimers. Taxanes bind to β-tubulin, leading to mitotic arrest of the cells in the G2/M phase by hyperstabilizing the microtubules and, ultimately, apoptosis of the cell. However, resistance is a major problem since approximately half of men treated with docetaxel do not respond to therapy and patients who initially do respond will develop resistance, most often already within the first year of treatment.

Mechanisms of docetaxel resistance in prostate cancer

The mechanisms responsible for the development of resistance to docetaxel can be divided into general mechanisms of drug resistance, as shared by other tumor types, and mechanisms specific for prostate cancer.

General mechanisms of drug resistance

Impaired drug distribution
Cancer cells release a variety of angiogenic growth factors and together with an interaction of the proliferating endothelial cells and the ECM glycoproteins, this results in vascularisation of the tumor with blood supplied from the surrounding tissue. Compared to normal tissue, tumor blood vessels are disorganized, more separated, have variable structure and chaotic flow. This leads to impaired delivery of nutrients and prostate cancers often contain regions of tumor hypoxia. These hypoxic conditions activate hypoxia-inducible transcription factors such as hypoxia-inducible factor 1α (HIF1α), inducing expression of genes that promote survival, treatment resistance and metastasis of prostate cancer cells. Hypoxia may therefore select for cancer cells with a more malignant phenotype. Coupled with an increased interstitial fluid pressure due to the disorganized and leaky vasculature and an impaired lymphatic drainage, the drug delivery into the tumor is impaired, since anticancer agents reach cancer cells via the blood circulation and the extracellular matrix. Also in situations where anticancer drugs are capable of gaining access to the tumor, the cancer cells most distant from the functional blood vessels tend to be slowly proliferating and resistant to cell-cycle-dependent drugs.

Heterogeneity of cancer cells

In prostate cancer, there are subpopulations of cancer cells with different proliferative capacities. Stem cells migrating to the tumor after the start of treatment has been associated with treatment failure. Additionally, only 0.1% of prostate cancer cells show the same marker profile that characterizes normal prostatic epithelium stem cells. Also neuroendocrine differentiation, as can be caused by ADT, is of major importance, since neuroendocrine cells become resistant to any treatment in a short amount of time.

Tumor microenvironment
Primary drug resistance can be induced by a paracrine amplification loop of stromal cytokines and growth factors, such as IL-6 and stromal cell-derived factor 1, and the adhesion of cancer cells to the ECM and stroma by their integrin receptors. This microenvironment promotes epithelial-mesenchymal transition and the acquisition of stem cell properties of the cancer cells, which have been linked to invasiveness, chemotherapeutic resistance and castration resistance.

Src family kinases (Src/SFK) are frequently overexpressed in both prostate cancer and bone cells. These kinases play an important role in cancer cell survival, proliferation, migration and...
angiogenesis, through modulating downstream signaling from membrane receptors, such as IGFR-1, EGFR, VEGFR, integrins and cytokine receptors. Chemokine (C-C motif) ligand 2 (CCL2) is one of the many growth factors produced by prostate cancer cells and various host cells. CCL2 is an important modulator of metastatic growth of prostate cancer in bone by stimulating cancer cells, osteoclasts and tumor-associated macrophages. Chemotherapy can induce secretion of this ligand, protecting prostate cancer cells from docetaxel cytotoxicity via activation of the PI3K/Akt pathway.

**Prostate cancer specific mechanisms of taxane resistance**

**Drug efflux pump**

Docetaxel has a high substrate affinity for P-glycoprotein (P-gp), the main drug efflux pump. P-glycoprotein is an ATP-dependent drug efflux pump bound to the membrane, encoded by the multi-drug resistance (MDR1) gene. Overexpression of this gene decreases the cellular drug accumulation and has been related to docetaxel and paclitaxel resistance. The drug efflux pumps ABCB4 and ABCC1 (encoded by the MDR2 gene and MRP1 gene) decrease the intracellular concentration of docetaxel as well.

**Alterations in microtubule structure and/or function**

Both structural and functional changes of the microtubules contribute to taxane resistance. Structural changes include mutations of β-tubulin affecting docetaxel binding, increased total cellular β-tubulin content, altered expression of β-tubulin isotypes such as βIII-tubulin overexpression, and post-translational β-tubulin modifications. Functional changes include alternative binding of docetaxel to β-tubulin, altered expression of microtubule-destabilizing phosphoproteins, promotion of microtubule-based microtentacles, altered interaction with other cytoskeletal components such as γ-actin, overexpression of microtubule-associated proteins (MAPs), elevated levels of kinesins and activation of the TXRI/thrombospondin pathway.

**Apoptotic defects and enhanced cell survival**

The antiapoptotic proteins Bcl-2 and secretory clusterin become upregulated during taxane therapy, impairing apoptosis. Also increased levels of lipid kinases, such as sphingosine kinase-1, and serine-threonine kinases, such as Pim-1 kinases, contribute to further prostate cancer cell proliferation. In addition, activation of a variety of signaling pathways that are linked with prostate cancer cell survival has also been associated to chemotherapy resistance. It has been shown that taxane sensitivity can be increased by blockade of these activated pathways, such as is the case in nuclear factor-κB/IL-6, EGFR/HER2/3, PI3K/mTor, Hedgehog and β-catenin pathways, Janus kinase and mitogen-activated protein kinase/extracellular signal-regulated kinase.

**The MAPK pathway**

The Mitogen Activated Protein Kinase (MAPK) pathway has been intensively studied for its role in human cancers. Following activation by extracellular stimuli, MAPK is capable of the regulation of cell growth, proliferation, apoptosis, differentiation and other sort like processes. The stress-activated MAPK, such as p38 MAPK and c-Jun N-terminal kinase (JNK), are important mediators in the cellular processes as triggered by chemotherapeutic agents, thereby determining both outcome and sensitivity to these agents.

P38 fulfils contra dictionary roles when it comes to cellular responses of chemotherapy. Many chemotherapeutics agents induce apoptosis by activating p38, but p38 MAPK can also
mediate resistance to apoptosis as was shown in prostate cancer: p38 phosphorylation significantly increased resistance to docetaxel-induced apoptosis in prostate cancer cells.\textsuperscript{123} In colorectal cancer was also seen that blockade of the p38 pathway led to cell cycle arrest and autophagic cell death.\textsuperscript{124}

The role of JNK in cell apoptosis is better elucidated: the inhibition of JNK is associated with resistance to genotoxic stimuli, as caused by chemotherapeutic agents.\textsuperscript{125,126} There are three JNK genes in mammal genome: JNK1, JNK2 and JNK3. Whereas JNK1 and JNK2 are ubiquitously expressed, JNK3 is mainly seen in the brain, cardiac smooth muscle and testes.\textsuperscript{127,128} Activated JNK regulates transcription factors as c-Jun, c-fos, ATF-2, activator protein 1 (AP-1), p53 and Elk, but also phosphorylates cytoskeletal and mitochondrial proteins as Bcl-2 and Bcl-xl.\textsuperscript{129,130}

There are two mechanisms by which JNK regulates apoptosis. The first one is the phosphorylation of c-Jun and ATF-2, what leads to the activation of AP-1 and the expression of Fas/FasL signaling pathway-related proteins.\textsuperscript{131} In docetaxel treatment, the oxidative stress-induced apoptosis is promoted by tocopherol-associated protein (TAP) via regulation of downstream signaling of JNK to the AP-1 complex and BH-3-only subfamily.\textsuperscript{132} The second mechanism consists of the mediation of the phosphorylation of the anti-apoptotic proteins Bcl-2 and Bcl-xl. Both mechanisms result in the activation of caspase 3, which induces apoptosis. In breast cancer cells has been shown that the inhibition of Bcl-2 and Bcl-xl via JNK pathway increased the sensitivity to paclitaxel.\textsuperscript{133} Activated JNK also modulates autophagy in two different ways: by promoting the Bcl-2/Bcl-xl phosphorylation and by upregulating the damage-regulated autophagy modulator (DRAM) and Sestrine 2.\textsuperscript{134,135} However, an answer to the question how JNK controls the balance of apoptosis and autophagy in response to genotoxic and oxidative stress has still not been found.

C-Jun

Cellular Jun (C-Jun) is a transcription factor that is member of the activator protein 1 (AP-1) family. The AP-1 family is constituted of the Jun, Fos, ATF and MAF subfamilies.\textsuperscript{136} AP-1 proteins have multiple functions and play a role in regulating stress response signals, cell growth and apoptosis.\textsuperscript{137} Under the control of the mitogen activated protein kinase kinase kinase 1 (MEKK1), the AP-1 proteins homo- or heterodimerize via the leucine zipper before binding to the promoter region of the target genes.\textsuperscript{138,139} The targeted gene for transcription is dependent on the AP-1 composition,\textsuperscript{140} and the composition of AP-1 dimers also modulates the transcriptional activity of AP-1. Heterodimers constituted of c-Jun and c-Fos increase transcriptional activity, whereas heterodimers constituted of c-Jun and JunB decrease transcriptional activity.\textsuperscript{141}

Whereas some AP-1 family members stimulate apoptosis, others activate cancer promoting genes.\textsuperscript{142} In order to be oncogenic C-Jun should be phosphorylated and phosphorylated c-Jun is frequently overexpressed in human cancers.\textsuperscript{143} Additionally, phosphorylated c-Jun has been linked to invasive properties of cells in some cancers, including prostate cancer.\textsuperscript{144} C-Jun is phosphorylated by c-Jun NH2-terminal Kinase (JNK) and Extracellular signaling Regulated Kinase (ERK), which are activated by the upstream MAPK-kinases and MAPKK-kinases. However, c-Jun activation by JNK can also be stimulated by other factors, such as migration inhibitory factor (MIF).\textsuperscript{145}

Regarding the human androgen receptor, c-Jun has two distinct non-transcriptional functions. As a transactivator, c-Jun has an antiproliferative function by interacting with the AR DNA
binding domain via the leucine zipper region. On the other hand, c-Jun has been shown to function as a coactivator with a proliferative function by mediating N-to-C interaction of the AR to enhance DNA binding.\textsuperscript{146}

In docetaxel treatment in prostate cancer cells, c-Jun has been shown to be a potent antiapoptotic factor and a physical interaction of AR and c-Jun has been seen. Docetaxel treatment leads to increased expression of AR and PSA in LNCaP cells, whereas an inverse regulation of AR and PSA was seen in a castration resistant cell model.\textsuperscript{11} Taxane treatment enhances binding of c-Jun to the c-Jun gene and docetaxel leads to binding of the AR to the c-Jun promoter region. However, no recruitment of c-Jun to the PSA enhancer region was seen.\textsuperscript{12} Additionally, phosphorylation of c-Jun in taxane treatment occurs in a JNK pathway independent manner, suggesting that the phosphorylation of c-Jun may be a primary target of taxane therapy.\textsuperscript{11}

**RESEARCH QUESTIONS**

In this report two research questions will be addressed:

- Is the increased PSA level in docetaxel treatment due to a release from apoptotic cells or the result of AR activation?
- Is upregulation of the androgen receptor in docetaxel treatment mediated by c-Jun?

**MATERIALS AND METHODS**

**Cell lines and reagents**

The human prostate cancer cell line LNCaP was obtained from the American Type Culture Collection (ATCC, Manassass, VA). The LNSiJun cell line are LNCaP cells that are stably transfected with c-Jun plasmids, resulting in a small hairpin RNA knockdown for the c-Jun gene, and are a gift from Prof. Shemshedini L. (Department of Biological Sciences, University of Toledo, USA). The transfected cells were selected for experiments by using 0.8 mg/ml neomycine in the medium.

The cells were cultured in RPMI medium, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin-neomycin (PSN). The cells were directly exposed to 10 nM dihydrottestosterone (DHT, Steraloids Inc., Newport, RI), 10 nM docetaxel (Taxotere®, Sanofi Aventis) or 4 nM paclitaxel (Paclitaxel®, Actavis, Hafnarfjordeur, Iceland), or treated with 20 nM enzalutamide (Xtandi®, Astellas/Medivation) prior to start of the treatments. All Western blot reagents were purchased from Invitrogen (Carlsbad, CA, USA), except when indicated otherwise.

**Western Blot analysis**

Proteins were extracted from the cultured cells using radioimmunoprecipitation assay (RIPA) buffer (50 mmol/L Tris-HCl, 150 mM NaCl, 20 mM EDTA, 1% NP-40, 0.1% SDS, 1 mM Na$_3$VO$_4$, 1 mM NaF and 1 mM phenylmethylsulfonylfluoride), supplemented with the protease inhibitor cocktail Complete Mini (Roche, Mannheim, Germany). The protein concentrations were measured using BCA protein assay (Pierce, Rockford, IL). The protein samples (consisting of 20 μg protein, 4 μl sample buffer (10% β-mercaptoethanol) and MΩ to add up till 20 μl) were loaded on 12% SDS-polyacrylamide gels (MΩ,
Acrylamide-30%, Tris, 10% SDS, 10% APS and TEMED) for electrophoresis with running buffer at 100V. The gels were wet electrotransferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA) and checked with Ponceau Red for equal loading. After blocking with 5% milk-TBS-T, the membranes were probed overnight at 4°C with the following antibodies: anti-AR (N-20) 1:1000, anti-c-Jun (H-79) 1:1000, anti-p-c-Jun (ser 63/73) 1:500 (all from Santa Cruz Biotechnology, CA) or anti-β-catenin 1:1000 (Zymed, San Francisco, CA). Horseradish peroxidase-conjugated secondary antibodies anti-mouse IgG and anti-rabbit IgG (GE Healthcare) were used at 1:5000 dilution. Proteins were visualized using the Enhanced ChemiLuminescense (ECL) detection system (Pierce Biotechnology, Rockford, USA) and an Alphalmsger CCD system.

**PSA Quantification (ELISA assay)**

LNCaP and LNSi cells were maintained in RPMI medium supplemented with 10% FBS and 1% PSN. After more than 24 hours exposure to enzalutamide, the cells were treated with DHT, docetaxel or paclitaxel for 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 days. From each well, medium was collected and spun down to eliminate dead cells and debris. The concentration of PSA was measured with a PSA enzyme-linked immunosorbent (ELISA) assay according to the manufacturer’s instructions. Samples were analyzed on a DELFIA 1234 Plate fluorometer (Perkin Elmer Life Sciences, Turku, Finland). This commercial assay is based on a dual-label detection technique, which uses three carefully characterized, distinctly unique binding monoclonal antibodies to PSA. Cell free medium was used as reference standard.

**Real-time Quantitative PCR Analysis**

Total RNA was isolated using RNeasy Mini Kit (Qiagen, West Sussex, UK) following the manufacturers protocol and treated with RNase-free DNase (DNase I; Amersham Pharmacia Biotech, Sollentuna, Sweden) to eliminate potential genomic DNA contaminants. cDNA was synthesized by reverse transcription from 1 µg of total RNA using the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit and random hexamer primers (Thermo Scientific, Waltham, MA). Real-time PCR was performed with SYBR Green QPCR master mix (iScript from BioRad) in a MX 3000P detection system (Strategene) with an initiation step at 95°C for 10 minutes followed by 40 cycles at 95°C for 30 seconds, 56°C for 1 minute and 72°C for 1 minute. The following primers for each gene were used: PSA (F5’-AGGCCTTCCCTGTACACCAC-3’ and R5’-GTCTTGGCCTGCTATTTCC-3’), AR (F5’-GTACCTGTCAAGCCCTGAAC-3’ and R5’-GGAGAGCTGCTTTCCGTTAG-3’), GAPDH (F5’-CGGCCGCTTGGGTACGCCGTAG-3’ and R5’-GGAGAGCTGCTTTCCGTTAG-3’), and β-actin (F5’-CGAGGCTGCTTTCCGTTAG-3’ and R5’-GGAGAGCTGCTTTCCGTTAG-3’).

The mRNA amount was determined by using the comparative C_T method. Samples were analyzed in triplicates and the expression of mRNA in non-treated controls set as a reference value. (Unfortunately, due to unforeseen circumstances, results could not be generated yet and it remains unclear when the problem will be solved. Therefore, the results of qPCR are not included in this report.)

**Statistical Analysis**

Results were obtained from at least two experiments performed and are expressed as the mean ± SD. Statistical comparisons between treatments groups were performed using Student’s unpaired t-test. All statistical tests were two-sided, and values of P < 0.05 were considered to be statistically significant.
RESULTS

Effects of taxane treatment on PSA level

Previous experiments showed a time dependent increase in PSA level in the medium of LNCaP cells treated with docetaxel, whereas in LNCaP cells treated with paclitaxel the opposite was seen after an initial flare. This was prompting to further elucidate the effect of docetaxel on the level of PSA. LNCaP cells and LNCaP cells treated with enzalutamide were exposed to DHT, docetaxel or paclitaxel as described, where after the medium was collected and PSA measurements were carried out. To determine whether downregulation of c-Jun effects the production of PSA in LNCaP cells, the same experiment was carried out in LNSi cells.

Docetaxel treatment increases PSA level over time

Exposure to DHT led to an increase in PSA level, with significant differences seen in LNCaP cells treated with enzalutamide and LNSi cells (p<0.001 and p=0.008, DHT vs 0) (Fig 1B, 1C) and treatment with enzalutamide significantly decreased PSA levels in LNCaP and LNSi cells for both treatments and controls (p<0.001) (Fig 1B, 1D). Docetaxel treatment lowered PSA level in all groups (p<0.001, Doc vs control). However, docetaxel treatment led to a significantly higher PSA level compared to paclitaxel (all p<0.001, except for LNSi cells treated with enzalutamide p=0.32) (Fig 1D). In docetaxel treatment a time dependent increase in PSA level is seen in LNCaP and LNSi cells, whereas cells exposed to paclitaxel or enzalutamide show a more constant level of PSA (Fig 2).

Downregulation of c-Jun decreases PSA production

In all treatments and controls, downregulation of c-Jun significantly reduced the PSA level (p<0.001), suggesting that c-Jun plays an important role in the production of PSA and that this role is not treatment specific (Fig 1).

Interestingly, downregulation of c-Jun also results in a markedly increase in the percentage of bound PSA. The free to total PSA ratio in LNSi cells is significantly lower than in LNCaP cells ($\mu = 36.5\%$ vs. $\mu = 91.9\%$, p<0.001) (Fig 2).

Figure 1. Evaluation of the production of PSA in taxane treatment for 10 days. Cell were either treated with DHT, docetaxel or paclitaxel. 0 served as a control. The median PSA level (horizontal lines) with error bars represent the range within which PSA levels were obtained. The boxes represent PSA values between the first and third quartile. A) Results from LNCaP cells. B) PSA levels in LNCaP cells that have been co-treated with the androgen receptor antagonist enzalutamide. C) LNSi cells (LNCaP cells silenced for c-Jun) show significant lower PSA levels for all treatments and control. D) Results from LNSi cells that have been co-treated with enzalutamide. The only group in which no significant difference in PSA level between docetaxel and paclitaxel was seen.
Figure 2. Assessment of PSA production in taxane treatment over time. Cells were treated for 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 days with DHT, docetaxel or paclitaxel or served as a control (0). The medium was collected and the concentration of PSA was measured. The blue bars represent the concentration of free PSA in the medium, the purple bars the concentration of total PSA. A) Production of PSA in LNCaP cells over time. B) LNCaP cells that are co-treated with enzalutamide show significant lower PSA levels compared to non co-treated cells. C) LNSi cells have significant lower PSA levels than LNCaP cells and show a significant rise in the concentration of bound PSA. D) Results for LNSi-cells co-treated with enzalutamide.

Effects of taxane treatment on protein levels of AR, c-Jun and p-c-Jun

Docetaxel treatment has been demonstrated to increase the expression of AR and PSA in LNCaP cells. To further examine the upregulation of AR in taxane treatment and whether the expression of AR is regulated by c-Jun, Western blot analysis was carried out to measure the expression of AR, c-Jun and p-c-Jun on protein level.

Downregulation of c-Jun promotes AR expression in 0 and DHT treated cells

In LNCaP cells an upregulation of AR is seen upon docetaxel treatment, whereas paclitaxel leads to a decreased expression of AR. As to be expected, the use of androgen receptor antagonist enzalutamide results in a reduced AR expression. However, this effect quickly diminishes and after 72 hours the enzalutamide treated cells show an AR upregulation for the DHT treated cells and controls that is not seen in LNCaP cells (Fig 3A). LNSi cells have stronger AR expressions in DHT treated cells and controls than LNCaP cells, suggesting that c-Jun normally downregulates the AR in LNCaP cells. For docetaxel and paclitaxel no differences were seen between both cell lines (Fig 3B).

Figure 3. Evaluation of the effect of taxane treatment on AR. Cells were treated with DHT, docetaxel or paclitaxel for 24, 48 or 72 hours or served as a control (0). Western blotting analysis was carried out to show the expression of AR on protein level, β-catenin was used as a loading control. A) Results for LNCaP cells and LNCaP cells treated with enzalutamide. B) Results for LNSi cells and LNSi cells treated with enzalutamide.
**Taxane treatment increases c-Jun and p-c-Jun levels**

Taxane treatment leads to an increase of c-Jun expression on protein level, compared to controls and DHT treated cells. The expression of c-Jun expression is not AR dependent, as there are no differences seen when these results are compared to cells that are co-treated with enzalutamide (Fig. 4A, 4C).

Interestingly, paclitaxel strongly upregulates c-Jun under all conditions, whereas docetaxel only upregulates c-Jun in LNCaP cells (Fig 4A, 4C). The c-Jun and p-c-Jun results are matching in both LNCaP and LNSi cells, implying that all c-Jun is phosphorylated (Fig 4B, 4D).

**DISCUSSION**

The results for the level of PSA in LNCaP cells are as to be expected. The expression of PSA is strongly androgen-dependent and positively regulated by the androgen receptor (AR). AR has a high affinity for DHT and together AR and DHT form a very stable, slowly dissociating complex. Cells treated with DHT therefore have a higher PSA level compared to controls. The sudden rise in PSA level on day 6 of DHT treatment in LNSi cells treated with enzalutamide is most likely due to inaccurate handling of the medium and can therefore not be validated.

The finding that docetaxel treatment leads to a time dependent increase in PSA level that is not seen in paclitaxel treatment, is in line with previous experiments. Since no time dependent increase is seen in cells that have also been treated with the androgen receptor antagonist enzalutamide, it is likely that the docetaxel induced rise in PSA level is mediated...
by AR. In paclitaxel treatment, there are rises in PSA level on day 2 and 3 in LNSi cells treated with enzalutamide. Paclitaxel-induced rapid genetic instability might be the cause this initial PSA flair, but the experiment should be repeated in order to rule out inaccurate handling.

In all treatments and controls, downregulation of c-Jun significantly reduced the PSA level, suggesting that c-Jun plays an important role in the production of PSA and that this role is not taxane specific. This is in line with previous findings that no recruitment of c-Jun to the PSA enhancer region was observed. In LNSi cells, enzalutamide lowers the level of PSA and DHT treatment induced a lower PSA level than in LNCaP cells, implying that the effect of c-Jun is not AR-dependent.

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In the human body, the major activating enzyme of PSA is human kallikrein 2 (hK2), but other prostate kallikreins, such as prostase (hK4), may also be capable of activation. Active PSA is rapidly bound by the protease inhibitors alpha-1-antichymotrypsin (ACT) and, to a lesser extent, alpha-2-macroglobulin and alpha-1-antichymotrypsin (bound PSA). Interestingly, downregulation of c-Jun results in a substantial increase in the percentage of bound PSA. This is unexpected, since none of the protease inhibitors is a defined component of RPMI or FBS. How can downregulation of c-Jun result in an increase in bound PSA? Are there other secretory proteins that bind PSA or is the pro-PSA produced affected by the c-Jun downregulation? Or is it just artifact? It warrants further investigation.

In human AR, c-Jun can function as both a transactivator and a coactivator. As transactivator, c-Jun has an antiproliferative function by interacting with the AR DNA binding domain via the leucine zipper region. As coactivator, c-Jun has a proliferative function by mediating N-to-C interaction of the AR to enhance DNA binding. In docetaxel treatment, c-Jun has been shown to be a potent antiapoptotic factor. In line with previous experiments, an upregulation of AR was seen upon docetaxel treatment and a downregulation of AR in paclitaxel treatment. Downregulation of c-Jun resulted in increased expressions of AR in controls and cells treated with DHT, suggesting that c-Jun functions as a transactivator in LNCaP cells and that this function is impaired in docetaxel treatment. Since downregulated c-Jun did not affect the expression of AR in docetaxel and paclitaxel, it is unlikely that in taxane treatment AR is regulated by endogenous c-Jun.

The stress-activated MAPK, such as p38 MAPK and c-Jun N-terminal kinase (JNK), are important mediators in the cellular processes as triggered by chemotherapeutic agents, thereby determining both outcome and sensitivity to these agents. Many chemotherapeutic agents induce apoptosis by activating p38, but p38 MAPK can also mediate resistance to apoptosis in prostate cancer: p38 phosphorylation significantly increased resistance to docetaxel-induced apoptosis in prostate cancer cells. Activated JNK regulates transcription factors as c-Jun and inhibition of JNK is associated with resistance to genotoxic stimuli, as caused by chemotherapeutic agents. There are two mechanisms by which JNK regulates apoptosis. The first one is the phosphorylation of c-Jun and ATF-2, what leads to the activation of AP-1 and the expression of Fas/FasL signaling pathway-related proteins. The second mechanism consists of the mediation of the phosphorylation of the anti-apoptotic proteins Bcl-2 and Bcl-xL. The antiapoptotic proteins Bcl-2 and secretory clusterin become upregulated during taxane therapy and in breast cancer cells has been shown that the inhibition of Bcl-2 and Bcl-xL via the JNK pathway increased the sensitivity to paclitaxel. As expected, it was demonstrated that taxane treatment increased the levels of c-Jun and p-c-Jun. Furthermore, it was shown that paclitaxel strongly upregulated c-Jun under all
conditions, whereas docetaxel only upregulated c-Jun in LNCaP cells and not in LNSi cells. This implies that the expression of c-Jun in docetaxel is the result of direct transcription of the c-Jun gene, which is supported by findings from a previous study that regulation of c-Jun in docetaxel treatment occurs in a JNK pathway independent manner.\textsuperscript{11} It was described that docetaxel led to binding of the AR to the c-Jun promoter region.\textsuperscript{12} In these experiments no differences in the level of c-Jun were seen in cells with and without treatment with enzalutamide, suggesting that binding of AR to the c-Jun promoter region is independent of the ligand binding domain.

**CONCLUSIONS**

Docetaxel treatment leads to higher expression levels of the androgen receptor in LNCaP cells and causes a time dependent increase in PSA level, that is mediated by the androgen receptor. Although downregulation of c-Jun results in a substantial decrease in PSA level, the upregulation of the androgen receptor in docetaxel treatment is independent of c-Jun. These results underline once more the importance of further research on the regulation of the androgen receptor, which plays an important role in both onset and progression of the disease.

For the future, it would be interesting to repeat the experiments in a castration resistant setting to gain a more profound understanding of the mechanism of docetaxel resistance.

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