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Effects of Glycogen Synthase Kinase-3 and IκB Kinase on Ligand-Dependent Activation of the Androgen Receptor

Dissertation

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1. Introduction

1.1 Prostate cancer

Prostate cancer (PCa) is one of the most frequently diagnosed neoplasms and the second leading cause of cancer death in elderly men of the Western World. When diagnosed at an early stage, patients suffering from PCa can be treated curatively by radical prostatectomy. However, in an advanced state of the disease, when metastases have spread to lymph nodes or bones, androgen ablation is the first line treatment. Androgen ablation takes advantage of the fact that prostate cancer cells, like healthy prostate cells, initially depend on continuous androgenic stimuli for growth and survival. As a consequence androgen-withdrawal inhibits the induction of androgen-dependent genes like the prostate specific antigen (PSA) and initiates apoptosis, thereby inhibiting PCa cell growth and progression.

Endocrine therapy involves androgen depletion by surgical castration, treatment with luteinising hormone releasing hormone (LHRH)-analogs as well as the blockade of the androgen receptor (AR) with anti-androgens. LHRH-analogs effectuate hormonal castration by inhibiting the pituitary gland, thereby blocking the secretion of testosterone from the testis. A combination of both LHRH-analogs and anti-androgens is termed “complete androgen ablation”. Unfortunately, the benefit from endocrine therapies is only transitory. After a period of around 2 years, nearly all prostate cancers progress to a state of the disease where they do no longer respond to endocrine therapies. These tumors are called “hormone refractory prostate cancers” (HRPCa).

While *in vitro* loss of the AR is the predominant mechanism for the failure of endocrine therapies, recent *in vivo* studies demonstrated that the AR is consistently expressed in the majority of HRPCa and their metastases. The mechanisms that facilitate survival and growth of PCa cells under castrate levels of androgens remain largely unknown. Amongst alterations in the AR signalling cascade, the deregulation of signalling pathways controlling cell proliferation, differentiation and survival like WNT- or NF-κB (described in Chapters 4.3 and 5.1), have been a matter of intensive research. Therefore, the following chapters will focus on the AR and some recently discovered mechanisms involved in the modulation of AR signalling.

1.2 Functional domains of the androgen receptor

The androgen receptor (AR) is a ligand-dependent transcription factor of the steroid receptor superfamily, other members being nuclear receptors like the estrogen receptor (ER), the glucocorticoid receptor (GR), the progesterone receptor (PR), the receptors for vitamin A (RXR) and vitamin D (VDR) as well as the most archaic member of the family, the ecdyson

receptor (EcR) of insects. Being located on the X chromosome on bands q11 and q12 (Lubahn et al., 1988b), the AR is organized in eight exons which are termed as exons A to H (see Figure 1). The AR-*gene* encodes a protein, which consists of 910 to 919 amino acids and a molecular weight of 110 to 114 kDa. The discrepancy in the number of amino acids or molecular weight respectively arises from the variable length of trinucleotide repeats (polyglutamine or polyglycine) at both ends of exon A (Lubahn et al., 1988a, Montgomery et al., 2001; Jenster et al., 1991, Wilson et al., 1992). As a member of the steroid receptor family, the AR has a characteristic structure consisting of four different functional domains: an amino-terminal transactivation domain (NTD) encoded by exon A, a central DNA-binding domain (DBD) encoded by exons B and C, a carboxy-terminal ligand-binding domain (LBD) encoded by the exons E to H, and a hinge region encoded by exon D which connects the DBD to the LBD.

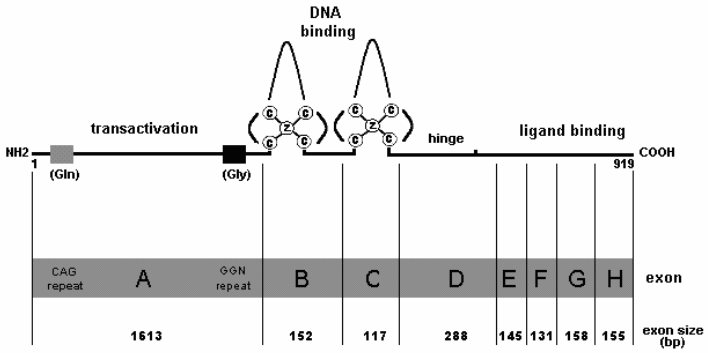


Figure 1: Structure of the androgen receptor (AR). The AR is organized in 8 exons (A-H) which encode the AR protein that comprises 919 amino acids. Structurally the AR consists of 4 different regions, the N-terminal transactivation domain (NTD), a central DNA-binding domain (DBD) with 2 zinc finger motives required for DNA-binding, a c-terminal located ligand binding domain (LBD) and a hinge region which serves as flexible linker between DBD and LBD. [Cronauer et al., Int J Oncol 23, 1095, 2003].

The most variable region of all steroid receptors is the NTD. In the AR, the **NTD** contains the transcription activation functions -1 (AF-1) and -5 (AF-5). AF-1 is required for hormone-dependent transactivation of the full-length AR, whereas AF-5 is necessary for the transactivation of a C-terminal deleted and therefore constitutively active AR (Jenster et al., 1995). AF-1 and AF-5 are both regulated by phosphorylation in a predominantly ligand-independent manner (Grisouard et al., 2009). The highly conserved central **DBD** consists of two cys_4-type zinc fingers composed of 4 cysteine residues surrounding a central zinc ion. These zinc fingers are necessary for DNA binding of the receptor. The C-terminal located **LBD** is involved in AR dimerization and contains the highly conserved ligand-dependent activation function-2 (AF-2) (Grisouard et al., 2009). The LBD consists of 12 α-helices and 4 β-sheets. In this respect, helix 12 plays a very important role, as the AF-2 is located within this helix (Danielian et al., 1992). Upon ligand binding to the LBD, there is a conformational change in helix 12 which facilitates co-activator binding to the LBD (Shiau et al., 1998; Moras

et al., 1998). Consequently, the AR is able to bind to androgen response elements (AREs) in the promoter region of different target genes, e.g. prostate specific antigen (PSA) or probasin (Claessens et al., 1996; He et al., 2002). The **hinge region** links the DBD to the LBD. However, this region is more than just a flexible linker between DBD and LBD as it contains one part of a nuclear localization signal (Zhou et al., 1994). Moreover, the existence of a nuclear export signal (NES) has also been discussed recently (Gioeli et al., 2002; 2006). As a result, the hinge region must be considered important in the nuclear translocation of steroid hormone receptors.

1.3 AR signalling in prostatic epithelial cells

The prostate, a prototype of an androgen-responsive organ, requires androgens for development, growth, and maintenance of its functional and structural integrity. The AR is the key regulator in the androgen signalling pathway. Two important androgens, testosterone and its metabolite dihydrotestosterone (DHT), mediate their effects through the AR. The male sex hormone testosterone (T) is mainly secreted by the testes, although an extremely small amount of T is also produced by the adrenal gland (Feldman and Feldman, 2001). T circulates in the blood predominantly bound to albumin and sex-hormone-binding globulin (SHBG). Only a small amount of T circulates freely in the serum. When T enters the prostate cell, it is almost completely converted to the more active dihydrotestosterone (DHT) by an enzyme called 5α-reductase (Bruchovsky et al., 1968). DHT has a fivefold higher affinity for the AR than T (Wilson et al., 1996). In the absence of hormonal stimuli, the AR is mainly located as monomer in the cytoplasm associated to heat-shock proteins (HSP) which stabilize the AR (Veldscholte et al., 1992a). Binding of androgens to the AR is thought to promote a conformational change of the AR, leading to a dissociation of the receptor-HSP complex, and a phosphorylation of the AR. The activated AR homodimerizes with another AR protein, thus enabling the homodimer to enter the nucleus. Once in the nucleus, AR dimers are able to bind to AREs in the promoter region of target genes (Kemppainen et al., 1992) where they recruit co-regulatory proteins (co-activators or co-repressors), the latter facilitating the interaction with the general transcription apparatus (GTA) (Chmelar et al., 2007).

1.4 Putative mechanisms for the development of HRPCa

Like normal prostate development, primary PCas are largely dependent on androgens for growth and survival. The androgen dependency of prostatic epithelial cells is the reason why most PCas respond to androgen ablation. The so-called “androgen ablation therapy” effectively inhibits tumor cell growth for a variable period of time, but is then universally

followed by tumor regrowth despite castrate levels of androgens (= hormone refractory disease).

For a long time, it has been hypothesized that the development of hormone refractory prostate cancer (HRPCa) is due to a clonal selection of AR-negative cancer cells (Tang et al., 2007). This assumption was mainly based on a rat model (Dunning-rat) where the development of an androgen-insensitive state is linked to the loss of the AR in tumor cells during androgen withdrawal. Moreover, this theory was supported by the fact that human cell lines derived from advanced stage PCa, as well as primary cell cultures of PCa, rarely express AR *in vitro* (Peehl, 1994; Cronauer et al., 1997). However, *in vivo* studies showed that the AR is not only expressed but even up-regulated upon androgen withdrawal in the majority of HRPCa specimens (Hobisch et al., 1995; Visakorpi et al., 1995). Recent experimental studies have established a link between the clinical symptoms of HRPCa and the molecular biology of the AR. Based on these studies, Feldman and Feldman (2001) postulated five mechanisms enabling PCa cells to grow under sub-physiological levels of androgens. The mechanisms are summarized herein below (for review see Feldman and Feldman, 2001).

In order to survive the low levels of circulating androgens following androgen ablation therapy, PCa cells could lower their need for androgens via triggering the sensitivity of the AR signalling cascade. The mechanisms leading to the sensitizing of the AR signalling machinery are summarized in a model termed the **hypersensitive pathway.** As shown experimentally, one potential mechanism that increases the sensitivity of the AR signalling pathway is an increase in AR protein (Chen et al., 2004; Craft et al., 1999). The up-regulation of intracellular AR protein can be achieved either by amplification of the AR gene (Visakorpi et al., 1995) or by stabilisation of the AR protein (Gregory et al., 2001). Increased AR stability was paralleled by increased levels of nuclear AR (Gregory et al., 2001). Another putative mechanism leading to androgen-hypersensitive PCa cells is an increase in 5-α reductase activity, leading to increased local DHT production, thereby compensating the overall decline in circulating testosterone. A further possibility enhancing AR signalling is the alteration of AR coregulators. In this respect, the overexpression of coactivators, such as steroid receptor coactivator 1 (SRC1), cAMP response element-binding protein (CREB), AR-associated protein (ARA-70) or β-catenin, is another possible mechanism for the development of the hypersensitive properties (Yeh and Chang, 1996, Chmelar et al., 2007, Cronauer et al., 2005). Moreover, the decreased expression of corepressors, like nuclear receptor co-repressor (N-CoR), has been shown to produce similar effects, thus enhancing transcription of androgen-responsive genes (Lavinsky et al., 1998).

A further possibility for the development of HRPCa is the **promiscuous pathway**. This model focuses on the acquisition of genetic changes which lead to aberrant activation of the AR signalling cascade. In this respect, one possible mechanism leading to a promiscuous AR is the selection for gain of function mutants of in the AR. Mutations of the AR are mainly found in the LBD (Montgomery et al., 2001). Indeed several AR mutations have been shown to broaden the ligand specificity of the receptor (Culig et al., 1993; Shi et al., 2002), thereby conferring a growth advantage for the tumour cells that can now proliferate under the stimuli of other circulating steroids, like estrogen, glucocorticoids, progesterone, or even antiandrogens (Veldscholte et al., 1992b; Zhao et al., 2000).

The activation of the AR by ligand-independent mechanisms is termed the **outlaw pathway**. This pathway includes the activation of the AR by peptide growth factors, like insulin like growth factor 1 (IGF-1), keratinocyte growth factor (KGF), basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF), as well as overexpression of receptor tyrosine kinases (RTK), like the human epidermal growth factor receptor-2 (HER-2), as well as the activation of their downstream targets, the mitogen-activated protein kinases (MAPK) (Culig et al, 1994; Craft et al., 1999; Cronauer et al., 2000; Hobisch et al., 1998, Mellinghoff et al., 2004, Yeh et al., 1999). Cytokines, like the proinflammatory interleukin-6 (IL-6) or oncostatin M (OSM), are also able to activate AR signalling in a ligand-independent manner (Culig et al., 1994; Hobisch et al., 1998; Godoy-Tundidor et al., 2002). The exact mechanisms of the outlaw pathway are poorly understood. It is hypothesized that the activation of different elements of the RTK-pathways are able to phosphorylate the AR and/or AR cofactors, thereby modulating AR signalling. Indeed HER-2 has been shown to phosphorylate and activate the AR (Craft et al., 1999) through the activation of MAPK (Yeh et al., 1999).

Another pathway enabling PCa cells to circumvent an apoptosis signal normally generated following androgen ablation is referred to as the **bypass pathway**. A typical example of this pathway is the up-regulation of the anti-apoptotic BCL-2 protein or the down-regulation of the tumor suppressor protein PTEN (phosphatase and tensin homologue deleted on chromosome) in the majority of HRPCa cells (Berchem et al., 1995; McDonnell et al., 1992; Schmitz et al., 2007).

The fifth and final theory postulated by Feldman and Feldman in 2001 is the **lurker cell pathway**. This theory is based on the assumption that a subpopulation of androgen-independent multipotent epithelial stem cells is directing differentiation and proliferation of the epithelial compartment. According to this theory, transformed epithelial stem cells give rise to malignant epithelial cells, the PCa cells. Initially, in presence of physiological levels of

androgens, normal, as well as malignant epithelial stem cells, differentiate predominantly into androgen sensitive cells. Following androgen ablation, the androgen-dependent cells are eliminated, but the androgen-independent malignant epithelial stem cells, which have been lurking in the background all along, remain viable and continue to proliferate and differentiate into HRPCa cells (Tang et al., 2007).

An additional theory, leading to the development of a subset of HRPCa, is termed the **hyposensitive pathway** and was postulated by our group. This theory involves uncommon factors, like the tumor suppressor p53 or the free radical gas, nitric oxide (NO), in the down-regulation of AR activity (Cronauer et al., 2004, Cronauer et al., 2007).

The p53 tumor suppressor gene encodes a nuclear transcription factor, which is activated and which accumulates in cells in response to a variety of stresses inducing growth arrest or apoptosis. Loss of p53 function may compromise the ability of carcinoma cells to undergo apoptosis in response to genomic instability, thereby favoring uncontrolled cell growth. Although inhibition of p53 should promote PCa proliferation, recent studies revealed that the over-expression as well as the inhibition of p53 leads to a reduction in AR signalling (Shenk et al., 2001, Cronauer et al., 2004). Although the exact nature of this phenomenom remains poorly understood, there is experimental evidence that a balanced level between p53 and the AR is necessary to guarantee optimal AR transactivation (Cronauer et al. 2004).

Various inflammatory processes have been shown to increase the frequency of cancer occurrence as well as cancer progression. One of these inflammatory stimuli is NO, a free radical gas known to be an important mediator of diverse physiological functions. Synthesis of high amounts of NO via inducible nitric oxide synthase (iNOS) has been demonstrated in a number of pathophysiological processes, such as inflammatory and autoimmune diseases and in tumorigenesis. Increased levels of iNOS and NO are detectable in more than 80% of PCa specimens, showing greatest expression in locally advanced and metastasized tumors. Interestingly, NO has been shown to inhibit AR-DNA-binding by nitrosating the zinc-finger structure of the receptor, thereby modulating AR transactivation in PCa cell lines (Cronauer, 2007). Due to the inhibitory effects of NO on AR signalling and cell viability, it is presumed that nitrosative stress leads to clonal selection of AR-negative or AR-insensitive PCa cells (Cronauer, 2007) resulting in the linking of inflammatory processes to PCa progression.

The so far discussed mechanisms enabling PCa cells to modulate AR signalling resulting in HRPCa cells are summarized in Table 1.

Table 1: Signalling pathways and mechanisms enabling hormone-refractory growth of PCa cells.

Signalling-pathway	Mechanisms	Effect(s)
AR-independent	loss of the AR	androgen-independent tumor growth
Hypersensitive AR	AR-amplification increased AR stability AR-mutations increased 5α-reductase activity	increased sensitivity to androgens enabling tumor cells to grow under subphysiological levels of circulating androgens
Prosmiscous AR	AR-mutations	increased survival/growth of PCa-cells due to broadened AR-ligand specificity
Outlaw-AR	AR-activation by growth factors or cytokines	increased survival/growth of PCa cells due to non-steroidal AR-activation
Bypass AR	anti-apoptotic pathways	decreased apoptosis in PCa cells
Lurker cells	malignant androgen-independent stem cells	androgen-independent stem cells control development and progression of PCa cells
Hyposensitive AR	various mechanisms	decreased AR-activity

In summary, the development of HRPCa is a multistep process that involves a variety of successive/simultaneous processes and pathways. The AR is an integrative nod for many pathways involved in PCa progression. Whereas traditional therapies are based on the depletion of androgens or competitive binding of anti-androgens to the AR, new experimental therapies tend to modulate AR functions. In order to develop such new strategies that will go far beyond the usual hormone ablation therapies, a more profound knowledge of AR posttranslational modifications is required.

1.5 Posttranslational modifications of the AR

In response to changes in environmental conditions, cells are able to rapidly modulate protein actions due to posttranslational modifications. Phosphorylation processes are the most important post-translational modifications of proteins (Meares and Jope, 2007); some others are acetylation, methylation, ubiquitinylation or sumoilation. Changes in the phosphorylation pattern of proteins are accompanied by alterations in the function of the target proteins like, for example, enzymatic activity**,** intracellular localization, or interaction with other molecules. The phosphorylation of proteins on serine/threonine or tyrosine residues is triggered by protein kinases. Comprising around 518 members, the protein kinases represent one of the largest superfamilies in the human genome (Johnson and Hunter, 2005), emphasizing once again the importance of phosphorylation processes in regulation of protein function (Meares and Jope, 2007).

Normal AR transcriptional activity is highly correlated with the phosphorylation status of the AR (Salas et al., 2004). Alterations in AR phosphorylation by protein kinases (like in the outlaw pathway) are believed to be key events in the deregulation of AR signalling that contribute to the development of HRPCa. Apart from serine residue 94 (S94), which is constitutively phosphorylated (Chen et al., 2006), many putative phoshorylation sites on the AR have been identified. However, the overwhelming majority of AR-phosphorylating kinases remains largely uncharacterised (Chen et al., 2006; Grisouard, 2007). Based on the Scansite program (www-scansite.mit.edu), Chen et al. (2006) predicted several kinases as targeting either serine or threonine sites on the AR (see Figure 2).

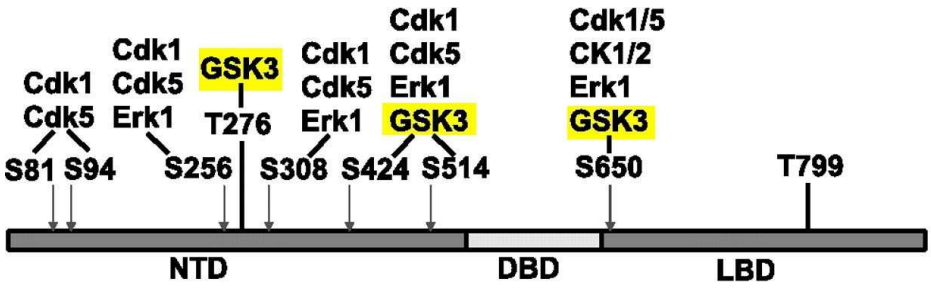


Figure 2: Predictions for kinases targeting Ser/Thr sites on the AR. (adapted from Chen et al., 2006).

2. Aims of the thesis

Normal transcriptional activity of the AR is highly correlated with its phosphorylation status. Although *in silico* analysis predicted various kinases targeting either serine or threonine sites on the AR the overwhelming majority of these putative AR-phosphorylating kinases remains largely uncharacterized. The aims of this thesis were to functionally characterize the role of two serine/threonine kinases namely the glycogen synthase kinase-3 (GSK-3) and the IkB-kinase (IKK) on AR signalling in human PCa cell lines.

3. Presentation of the results:

The results included in this thesis are presented in two parts. Part I referred to in Chapter 4 describes the role of GSK-3β in AR signalling and refers mainly to recently published manuscripts. Part II entitled Modulation of AR activity by IKK presented in Chapter 5 is based on yet unpublished results.

Part I: Role of GSK-3β in AR signalling (see Chapter 4)

This part/section refers to the following manuscripts outlined below.

Original articles

Rinnab L, Hessenauer A, Schütz SV, Schmid E, Küfer R, Finter F, Hautmann RE, Spindler K-D und Cronauer MV. Die Rolle des Androgenrezeptors im hormonrefraktären Prostatakarzinom – Molekulare Grundlagen und experimentelle Therapieansätze. Urologe A 47, 314-325, 2008.

Rinnab L*, Schütz SV*, Diesch J, Schmid E, Küfer R, Hautmann RE, Spindler K-D, Cronauer MV. Inhibiton of Glycogen-Synthase Kinase-3 (GSK) in Androgen Responsive Prostate Cancer Cell Lines - Are GSK-Inhibitors Therapeutically Useful? Neoplasia 10(6), 624-634, 2008. **(*these authors contributed equally to this work)**

Schütz SV, Cronauer MV, Rinnab L. Inhibition of glycogen synthase kinase-3β promotes nuclear export of the androgen receptor through a CRM1-dependent mechanism in prostate cancer cell lines. J Cell Biochem 2010 (DOI: 10.1002/jcb.22500).

Abstracts

Schütz SV, Cronauer MV, Schmid E, Spindler K-D, Hautmann RE, Rinnab L. Inhibition of glycogen synthase kinase-3β in androgen-responsive prostate cancer cell lines. Are GSK-3 inhibitors therapeutically useful? P 8.11, 60. Kongress der Deutschen Gesellschaft für Urologie 24.-27. September 2008, Stuttgart.

Schütz SV, Rinnab L, Diesch J, Cronauer MV. Inhibition of glycogen synthase kinase-3β modulates androgen receptor-signalling in prostate cancer cell lines. Abstract 33, pp.80 (abstract-band). Androgens 2008, 5th biennial meeting on androgen receptor function, October 1-3, 2008, Rotterdam, the Netherlands.

Schütz SV, Rinnab L, Cronauer MV. Modulation of androgen receptor-signalling by inhibition of glycogen synthase kinase-3β in prostate cancer cell lines. Abstract 18, pp.49 (abstract-band). 18th Meeting of the European Society for Urological Research (ESUR) October 16-18, 2008, Barcelona, Spain.

Rinnab L, Cronauer MV, Schütz SV. Inhibition of glycogen synthase kinase-3 promotes CRM1-dependent nuclear export of the androgen receptor. Abstract P10.11 (abstract cd-rom). 61. Kongress der Deutschen Gesellschaft für Urologie 16.-19. September 2009, Dresden.

Part II: Modulation of AR activity by IKK (see Chapter 5)

This part refers to yet unpublished results.

4. Role of GSK-3 in AR signalling

4.1 GSK-3, a key regulator of various physiological processes

The glycogen synthase kinase 3 (GSK-3) is a multifunctional enzyme expressed in a large number of different tissues. Initially, GSK-3 was identified as an important protein kinase in glycogen biosynthesis acting as “counterpart” of insulin. (Cross et al., 1995; Grimes and Jope, 2001). However, increasing knowledge has changed the image of GSK-3 to that of a key enzyme whose functions go far beyond mediating glycogen metabolism. Up to now, GSK-3 is known to play pivotal roles in a large number of cellular and physiological events, some of them being, WNT and Hedgehog signalling, insulin action, lipid synthesis, cell division, cell death and cell survival, transcription, patterning and axial orientation during development, differentiation, circadian rhythm as well as neuronal functions (Meijer et al., 2004).

GSK-3 is a ubiquitous serine/threonine kinase. In mammalian cells, two isoforms namely, GSK-3α and GSK-3β are expressed. Both GSK-3-isoforms share 84% overall sequence identity, whereas their catalytic domains share 98% identity. GSK-3α, unlike GSK-3β, contains a glycine-rich stretch on the N-terminus. This stretch results in the greater molecular weight (51 kDa) of GSK-3α in comparison to GSK-3β (47 kDa) (Meijer et al., 2004). Except for their discrepancy in structure, both GSK-3 isoforms share similar substrate specificity. However, GSK-3β seems to be the more essential isoform, as only a knockout of GSK-3β in mice results in an embryolethal phenotype (Hoeflich et al., 2000).

GSK-3β is a special kinase due to two major features. First, GSK-3β belongs to the few kinases that are catalytically active, i.e., phosphorylated on tyrosine 216 in resting cells (Jope and Johnson, 2004; Frame and Cohen, 2001) (see Figure 3). Phosphorylation of GSK-3β on the N-terminal Serine 9 otherwise inactivates GSK-3β, thereby blocking its catalytic centre for further substrate access (Meijer et al., 2004). ´

Figure 3: Structure of GSK-3β. GSK-3β is a serine/threonine kinase which’s activity is regulated by phosphorylation. The catalytical active form of GSK-3β is phosphoryltated on Tyrosin 216 (Y216), whereas phosphorylation on Serine 9 (S9) inactivates the kinase.

Second, GSK-3β has unique substrate specificity. The actions of GSK-3 are often regulated by the phosphorylation state of its substrates. In order to be phosphorylated by GSK-3β, most GSK-3β substrates need to be pre-phosphorylated on serine- or threonine-residues

four amino acids to the C-terminus of the actual GSK-3β phosphorylation site. This position at “n + 4” is also called the “priming phosphate”, resulting in the recognition motif –[S/T]-X-X-X-[S/T](P) for GSK-3 substrates, with S being serine and X being an arbitrary amino acid (Bhat and Budd, 2002; Jope and Johnson, 2004).

Examples of primed GSK-3-substrates are (1) glycogen synthase (GS), primed by casein kinase 2 (CK2), (2) the eukaryotic initiation factor-2B (eIF2B), primed by kinase DYRK (Dual specificity Y-phosphorylated and Regulated Kinase), (3) the transcription factor cyclic-AMP-response element binding protein (CREB), pre-phosphorylated by kinase A (PKA), and (4) the microtubule-associated protein tau, primed by various kinases in order to facilitate its phosphorylation by GSK-3 (Jope and Johnson, 2004). However, other proteins like axin and β-catenin, which are parts of the canonical WNT-pathway, have been identified as un-primed substrates of GSK-3 (Grisouard, 2007).

Other important proteins phosphorylated by GSK-3 are summarized in Table 2. Many of them are metabolic and signalling proteins, like GS, eIF2B, Cyclin D1, as well as structural proteins, like the microtubule binding protein tau. Moreover, GSK-3β has a key role as “gatekeeper” over a broad array of transcription factors, including β-catenin, T-cell factor (TCF), p53 and some steroid receptors including AR, ER and GR.

Table 2: Putative substrates of GSK-3. (Jope and Johnson, 2004; Grimes and Jope, 2001, Meijer et al., 2004)

Metabolic and signalling proteins	Structural proteins	Transcription factors
Adenomatous Polyposis Coli (APC) Amyloid Precursor Protein(APP) Axin Cyclin D1 eIF2B Glycogen Synthase (GS) Insulin receptor substrate-1 Presenilin-1	Dynamin-like protein Neural cell-adhesion protein neurofilaments tau	β-catenin p53 NF-κB CREB T-cell factors (TCF) steroid receptors (GR, ER, AR)

4.2 GSK-3β inhibitors in human malignancies

Multiple diseases are associated with the deregulation of GSK-3β activity. GSK-3β is implicated in disorders of the nervous system, in type II diabetes, and in various cancers (Meijer et al., 2004). Therefore, pharmacological inhibitors of GSK-3 have emerged as promising therapeutic agents for the treatment of a large variety of diseases (see Figure 4).

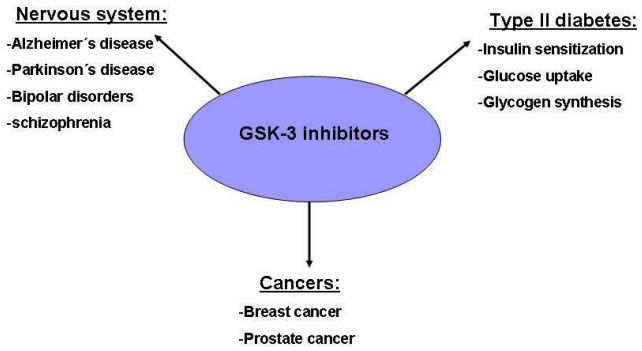


Figure 4: Therapeutic application of GSK-3 inhibitors in human malignancies

Promising results using GSK-3 inhibitors have already been achieved in the treatment of bipolar disorders and Alzheimer´s disease (AD), as well as Type II diabetes. Bipolar disorders, like manic depression, have been treated for years with lithium chloride, one of the first known GSK-3 inhibitors. GSK-3β is intimately linked to the two major pathological hallmarks of AD, the abnormal hyperphosphorylation of the microtubule-binding protein tau and the production of amyloid plaques, the latter being implicated in the degeneration of nerve cells and brain atrophy (Frame and Cohen, 2001; Bhat and Budd, 2002). Experimentally, inhibition of GSK-3β has been shown to protect neurons from β-amyloid-induced neurotoxicity. Moreover inhibition of tau phosphorylation renders GSK-3 inhibitors suitable as neuroprotective agent (Bhat and Budd, 2002). Inhibition of GSK-3 by lithium chloride (Hampel et al., 2009) and by thiadiazolidinone NP031112 (Luna-Medina et al., 2007) are now been tested clinically (see http://clinicaltrials.gov).

In its initial stage, Type II diabetes is characterized by resistance to insulin (Frame and Cohen, 2001; Jope and Johnson, 2004). Inhibitors of GSK-3, like the maleimide SB216763, have been described as potent drugs against diabetes by mimicking insulin signalling, thus lowering blood glucose levels, stimulating glucose transport, and facilitating the conversion of glucose to glycogen (Frame and Cohen, 2001; Jope and Johnson, 2004).

4.3 GSK-3β in the WNT/β-catenin pathway in PCa cells

As described in Chapter 1.4, AR signalling can be influenced by peptide growth factors and their corresponding pathways. One of the signalling pathways thought to be involved in progression of PCa is the canonical WNT signalling pathway (see Figure 5). Under normal conditions, the canonical WNT pathway is involved in the maintenance of stem cell populations, and the direction of embryological development and governing processes such as cell fate specification, proliferation, polarity, and migration (Yardy and Brewster, 2005).

A crucial step in the canonical WNT signalling pathway is the control of β-catenin degradation via GSK-3β. In the absence of a Wnt signal, a multiprotein complex, including GSK-3β, continuously phosphorylates free cytoplasmic β-catenin, thus marking it for proteasomal degradation. Activation of the canonical WNT pathway by binding of a WNT ligand to its membrane receptor leads to inactivation of GSK-3β. Subsequently β-catenin is no longer targeted for ubiquitination, and it accumulates in the cytoplasm and translocates into the nucleus. Nuclear β-catenin then associates with transcription factors of the T-cell factor/lymphoid enhancer factor family (TCF/LEF) and activates the transcription of WNT target genes, some of them being c-myc, c-jun, fra-1, and cyclin D1, which are important mediators in cell proliferation, the cell cycle, and cell migration (Yardy and Brewster 2005).

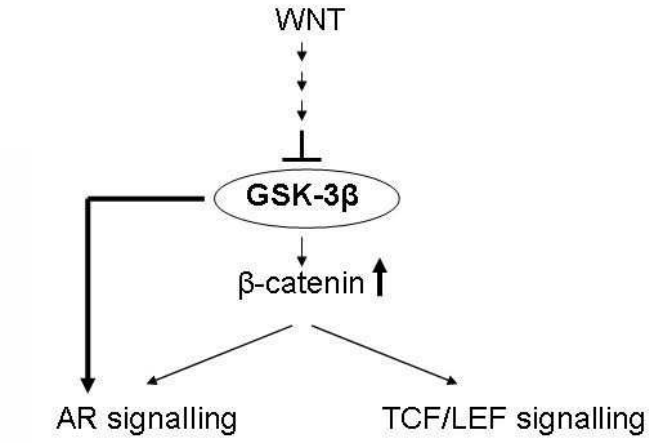


Figure 5: Schematic overview of the canonical WNT signalling pathway, including its involvement in the AR signalling cascade.

Interestingly β-catenin, an intracellular messenger of WNT signalling, is thought to act as a ligand-dependent co-activator of the AR (see Figure 5). Following transient over-expression, β-catenin has been shown to bind to the C-terminal AF-2 domain on the AR, thereby enhancing AR transactivation *in vitro* (Song et al., 2005). As a consequence, inhibition of GSK-3β in PCa cells should lead to an increase in intracellular β-catenin, the latter enhancing AR signalling. Surprisingly, inhibition of endogenous β-catenin by RNA interference in PCa cells increased, rather than decreased, AR activity (Mazor et al, 2004; Cronauer, 2007) suggesting that the regulation of AR transcriptional activity by endogenous β-catenin differs from what can be seen with ectopically overexpressed β-catenin. Interestingly, inhibition or down-regulation of GSK-3β in PCa cells lead to an inhibition of AR signalling (Mazor et al, 2004; Liao et al., 2004). However, these results were characterised as being controversial (Wang et al. 2004, Salas et al., 2004). In an attempt to clarify the controversy, the effects of a GSK-3 inhibition on AR signalling and function were analyzed (Rinnab et al., 2008, Schütz et al., 2010).

4.4 Role of GSK-3β in the direct regulation of AR signalling in human PCa cells

4.4.1 Cellular localization and formation of GSK-3β/AR complexes in PCa cells

Previous studies showing a co-localization of ER-α and GSK-3β in MCF-7 breast cancer cells (Medunjanin et al., 2005) prompted us to study the localization GSK-3β and AR in PCa cells. Using an Alexa Fluor 594 labeled antibody (red) (see Figure 6) directed against GSK-3β, the expression and co-localization of GSK-3β and AR was analyzed in PC3 cells transfected with a green AR-Eos fusion protein (AR-EosFP) (see Figure 6) using fluorescence-microscopy.

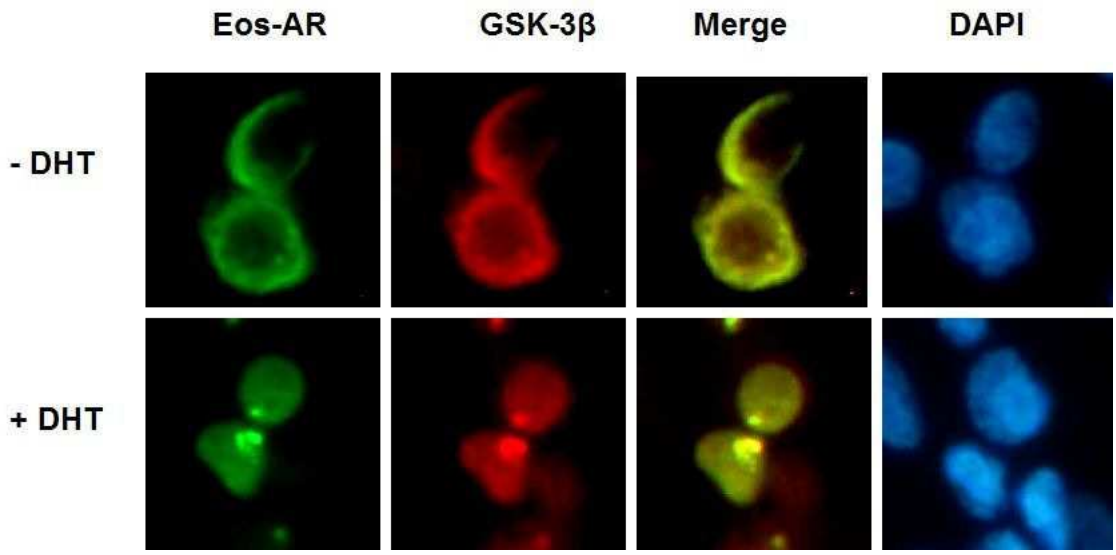


Figure 6: GSK-3β binds to the AR. Intracellular localization of GSK-3β and AR by fluorescence microscopy: PC3 cells were transfected with pAR-t1EosFP coding for the green fluorescent AR-fusion protein AREosFP. Following transfection (after 24h), cells were treated with/without DHT for another four hours. Cellular localization of GSK-3β was analyzed via red immunofluorecence (primary antibody: anti-GSK-3β antibody (27C10), secondary antibody: Alexa Fluor 594 goat anti-rabbit IgG). DAPI staining served as a control for nuclear localization.

In the absence of androgens, AR and GSK-3β are predominantly localized in the cytoplasm. Only small amounts of GSK-3β are detectable in the nucleus (see Figure 6). After the addition of DHT, high levels of GSK-3β as well as AR are localized in the nucleus, suggesting a co-migration of AR-GSK-3β complex. The assumption that GSK-3β directly binds the AR is supported by previous findings (Salas et al., 2004; Schütz et al., 2010). In order to identify the functional domains of the AR that interact with GSK-3, an immunoprecipitation analysis on whole cell lysates from 22Rv1 cells was carried out (see Figure 7). 22Rv1 cells express two AR isoforms, a full length AR with a molecular weight of around 122 kDa and a C-terminally deleted 79 kDa-isoform (ARΔLBD) lacking the LBD of the AR. When analyzing the GSK-3 co-immunoprecipitates with an AR-antibody recognizing the C-terminal end of the AR, only the full-length AR was detectable. In contrast, an AR antibody directed against the N-terminal domain of the AR detected both AR isoforms. These observations suggest a binding site for GSK-3β at the N-terminal domain of the AR, ranging

from the transactivation domain to the hinge region (amino acids 1-672). Although additional interaction of GSK-3 with the C-terminal end of the AR could not be fully excluded, our finding is in agreement with that from a previous study analyzing the interactions of GSK-3 with ERα (Medunjanin et al., 2005).

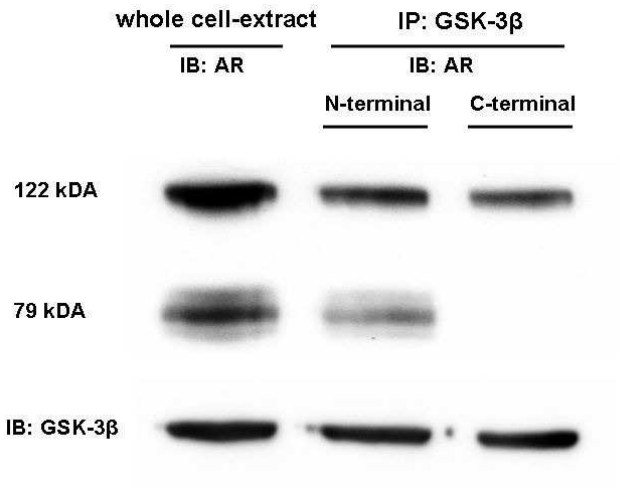


Figure 7: GSK-3β binds to the N-terminus of the AR. Immunoprecipitation analysis of whole cell lysates: 22Rv1 cells were grown for 24 hours under standard conditions. Whole cell lysates or GSK-3β immunoprecipitates were analyzed by immunoblotting (IB) with N-or C-terminally directed AR-antibodies (AR441: N-terminal or EP670Y: C-terminal) and GSK-3β antibody (anti-GSK-3β: 27C10) serving as loading control for IP.

In summary, there is experimental evidence that GSK-3β binds to the AR, most probably at the amino-terminal end of the receptor. Moreover, AR and GSK-3β are co-localized under the same physiological conditions.

4.4.2 GSK-3β phosphorylates the AR on serine and threonine residues

There is experimental evidence that the serine/threonine kinase GSK-3β is a key regulator of many transcription factors (see table 2). Indeed, GSK-3 has been repeatedly shown to phosphorylate steroid receptors like the estrogen receptor (ER) or the AR on serine residues (Medunjanin et al., 2005; Grisouard et al., 2007; Mazor et al., 2004; Schütz et al., 2010). Recently, our group was able to demonstrate for the first time that GSK-3β activity is involved in threonine phosphorylation of the AR *in vivo* (Schütz et al. 2010). Most interestingly, AR phosphorylation on threonine residues could be inhibited in 22Rv1 PCa cells by the maleimide SB216763, a commonly used inhibitor of GSK-3β (see Figure 8).

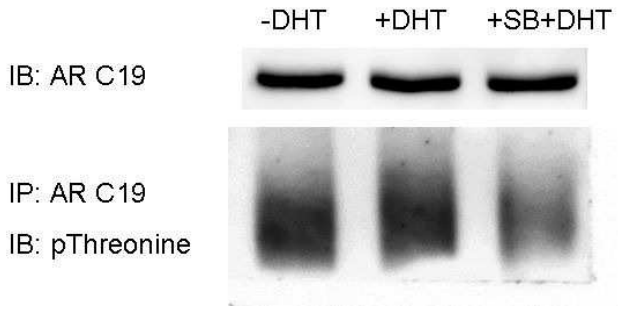


Figure 8: SB216763 prevents threonine phosphorylation of the AR. 22Rv1 cells were cultured in T25 flasks for 24 h under standard conditions (RPMI-1640 supplemented with 10% FBS (v/v) and antibiotics). Thereafter, medium was replaced and cells were grown for 24 h under steroid free conditions (RPMI-1640, 10% FBSdcc, antibiotics). Subsequently, cells were pre-incubated with/without 1 µM SB216763 for 30 min before addition of 10 nM DHT. Proteins were collected 1 h after addition of DHT. 250 µg of total protein extract was immunoprecipitated with 1 µg AR-C19 antibody. Immunoprecipitates were analyzed by a monoclonal antibody recognizing phosphothreonine residues.

4.4.3 Inhibition of GSK-3β modulates AR transactivation and AR stability

Inhibition of GSK-3 has been repeatedly shown to inhibit AR signalling (Mazor et al., 2004; Liao et al., 2004). However, these results have been considered controversial (Salas et al., 2004; Wang et al., 2004). Our group has shown that inhibition of GSK-3 leads indeed to a downregulation of AR signalling and intracellular PSA levels (Rinnab et al., 2008; Schütz et al., 2010) (see Figure 9).

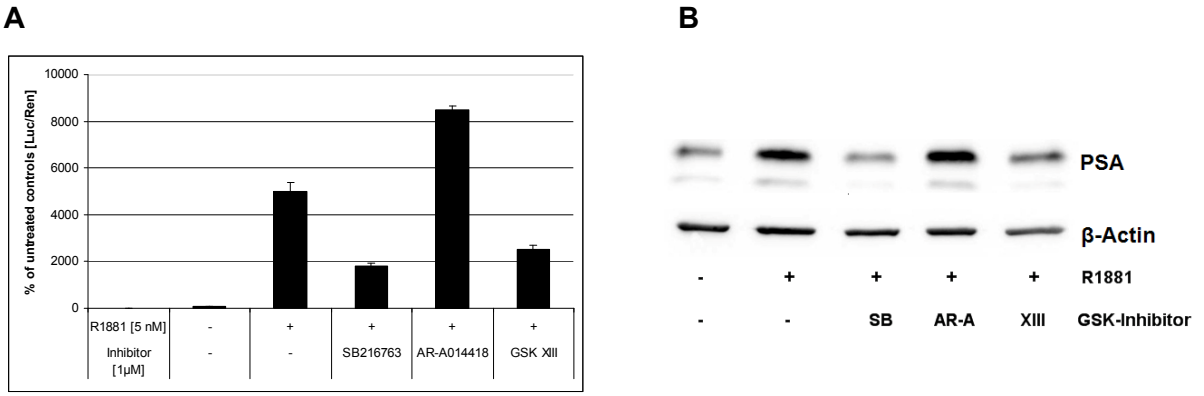


Figure 9: Modulation of AR transactivation and endogenous PSA-levels by pharmacological GSK-3 inhibitors. LNCaP cells were incubated with different pharmacological GSK-3 inhibitors in the presence/absence of 5 nM R1881 for 24 hours. (A) AR transcriptional activity was measured using an AR-reporter gene assay. Results are expressed as % of untreated controls. (B) Endogenous levels of PSA were detected by western blotting with Anti-PSA antibody (CHYH2, Santa Cruz Biotechnology) and anti β-Actin antibody as loading control (Schütz et al., 2010).

The discrepancy between our findings and the findings of Salas (2004) and Wang (2004) could partly be explained by the observation that some commonly used GSK-3 inhibitors, like thiazole-urea AR-A014418 have androgenic properties (Rinnab et al., 2008) (see Figure 10).

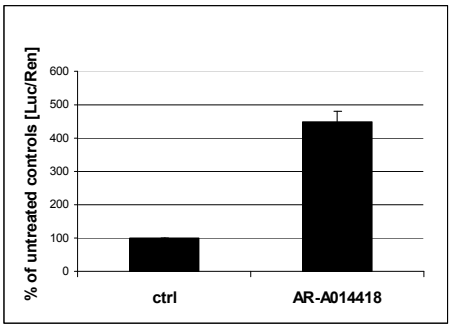


Figure 10: Androgenic properties of the GSK-3 inhibitor AR-A014418. 22Rv1 cells were treated for 24 hours with AR-A014418 in the absence of androgens (ctrl). AR-activity was determined using an AR-reporter gene assay (According to Rinnab et al., 2008).

As described by Mazor et. al. (2004), inhibition of AR signalling, after long-term inhibition of GSK-3 with the pharmacological inhibitors SB216763 and SB415286 (maleimides), was paralleled by a down-regulation of AR protein. The mechanism for this phenomenon, however, has remained unknown. Prolonged inhibition of GSK-3 (96 hours) nearly results in loss of the AR protein (see Figure 11). In this respect, both depletion of GSK-3 by short hairpin RNA (shRNA), as well as pharmacological inhibition of GSK-3 function, lead to a down-regulation of AR protein in 22Rv1 and LNCaP cells, thereby confirming the data of Mazor et al (2004) (see Figure 11).

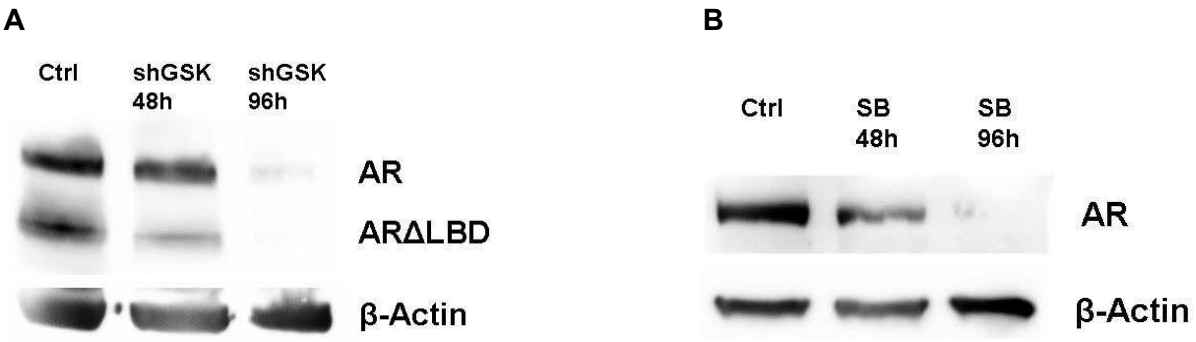


Figure 11: Prolonged inhibition of GSK-3 in PCa cells leads to tremendous decrease of AR protein. (A) 22Rv1 cells were transiently transfected with shRNA directed against GSK-3. (B) LNCaP cells were incubated with 1 µM of the pharmacological GSK-3 inhibitor SB216763. Protein extracts were collected after 48 hours or 96 hours, respectively, and analyzed by western blot analysis using AR antibody (AR441) and β-actin antibody as loading control (Schütz SV, unpublished).

Interestingly, diminished intracellular AR protein levels following GSK-3 inhibition could be rescued by pre-incubation with the proteasome inhibitor MG-132, suggesting that the inhibition of GSK-3 leads to proteasomal degradation of AR protein (see Figure 12).

Figure 12: Decrease in AR protein levels by GSK-3 inhibition is due to proteasomal degradation. LNCaP (A) or 22Rv1 (B) cells were grown in the presence/absence of the proteasome inhibitor MG-132 (5 µM) one hour before addition of GSK-3 inhibitor SB216763 (1 µM). Another 60 minutes later R1881 (5 nM) or DHT (10 nM) respectively was added to the medium. Whole cell extracts were collected 6 hours later and subsequently analyzed be western blotting using AR-antibody (AR441) or β-Actin antibody as loading control (Schütz SV, unpublished).

The observation that inhibition of GSK-3 leads to a proteasomal degradation of the AR is consistent with a similar observation in breast cancer cells (Grisouard et al., 2007). This furthermore supports the assumption that GSK-3 activity plays a crucial role in the stabilization of the AR protein.

4.4.4 GSK-3β inhibitors enhance AR dimerization

In order to investigate if the proteasomal degradation of AR protein following GSK-3 inhibition is due to reduced AR dimerization, a mammalian two hybrid (M2H)-assay was carried out. This system, based on the yeast two-hybrid system, relies upon co-transfection of three plasmids in mammalian cells: (1) The ACT-vector containing a VP16 transcriptional activation domain (TAD), (2) the BIND-vector containing the yeast GAL4 DBD and *Renilla reniformis* luciferase, which allows normalization for differences in transfection efficiency; and (3) the GAL4 reporter vector, which contains five GAL4 binding sites upstream of a firefly luciferase reporter gene. Interaction of the proteins ACT and BIND consequently leads to the association of the Gal4 DBD with the TAD resulting in their binding to the promoter region of the firefly luciferase gene. Thus transcriptional activity of the luciferase is activated. In order to investigate if pharmacological GSK-3 inhibitors alter AR dimerization, an AR-wild type construct was cloned into both the ACT- and the BIND-vectors. Therefore, dimerization of two full-length AR-molecules results in the activation of firefly luciferase-activity.

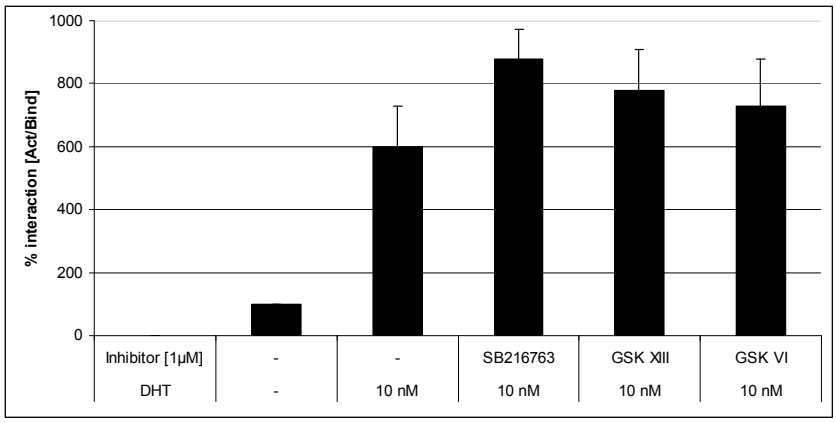


Figure 14: GSK-3 inhibitors slightly enhance AR-dimerization. AR-negative PC3 cells were transfected with AR-ACT, AR-BIND and Gal4 reporter and subsequently incubated for 24 hours with 1 µM of the GSK-3 inhibitors, SB216763, GSK-3 inhibitor XIII, and GSK-3 inhibitor VI in the presence of 10nM DHT. Luciferase activity was measured using the dual luciferase system (Promega). Results are means of three separate experiments and are expressed as % interaction AR-ACT/BIND (Schütz SV, unpublished).

As shown in Figure 14, AR dimerization is largely enhanced (600 ± 128% interaction) upon stimulation with DHT in comparison to untreated controls (100% interaction). The use of different pharmacological GSK-3 inhibitors did not prevent, but rather slightly enhanced AR dimerization. GSK-3 inhibitor SB216763 caused an increased interaction by 877 ± 98%. GSK-3 inhibitors XIII (aminopyrazole) and VI (chloromethyl-thienyl-ketone) increased the interaction by 779 ± 130% and 728 ± 148%, respectively. Consequently, proteasomal degradation of the AR following inhibition of GSK-3 is not due to an inhibition of AR dimerization.

4.4.5 Effects of GSK-3β inhibitors on nuclear translocation of the AR

4.4.5.1 Cellular localization of the AR in HRPCa cells

In advanced PCa cell lines, an increased sensitivity to androgens enabling the cells to proliferate under sub-physiological androgen levels has been associated with increased AR stability and/or nuclear localization, even in the absence of a ligand (Gregory et al., 1998; 2001). This observation agrees with previous findings from our laboratory comparing the hormone sensitive LNCaP with the hormone refractory subline LNCaP-SSR (see Figure 15). In the absence of androgens, only a small amount of AR is located in the nucleus of the androgen-sensitive LNCaP cells. A strong increase in nuclear AR, however, could be observed upon stimulation with the androgen DHT. In contrast to these findings, the AR of the hormone-refractory LNCaP-SSR cells is predominantly located in the nucleus, even in the absence of androgens.

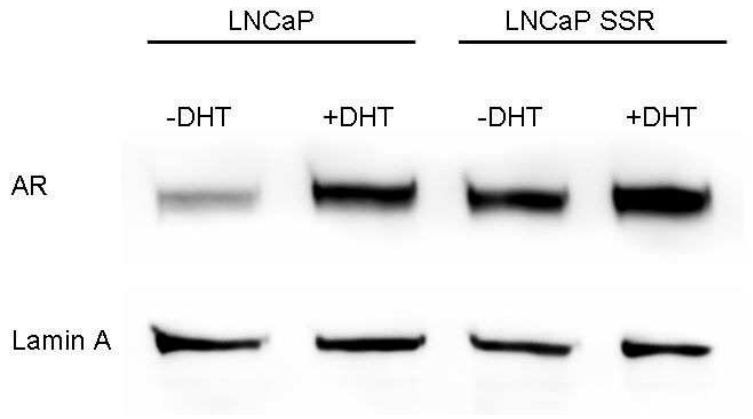


Figure 15: Localization of AR in normal and hormone refractory LNCaP cell lines. LNCaP cells and LNCaP SSR cells were grown in FBSdcc for 24 hours. Subsequently, cells were treated with/without 10 nM DHT for another 24 hours followed by an extraction of cytoplasmic and nuclear fractions. Nuclear AR was detected by western blotting using AR441 and Lamin A antibodies (Schütz SV, unpublished).

Besides its effects on steroid receptor stability, GSK-3 has also been shown to modulate the nuclear translocation of various proteins and transcription factors (Grimes and Jope, 2001; Jope and Johnson, 2004). However these effects have not been shown in members of the steroid receptor superfamily. As a decrease in nuclear AR protein could not only inhibit AR signalling but also favour its degradation in the cytoplasm, we investigated the effects of GSK-3 inhibitors on nuclear translocation of the AR.

4.4.5.2 Pharmacological inhibition of GSK-3β modulates AR localization

The ability of GSK-3 inhibitors to modulate the nuclear translocation of the AR was tested in PC3 cells transfected with the geen fluorescent fusion protein Eos-AR (see Figure 16). In the absence of the androgen DHT, the AR fusion protein was located in the cytoplasm, where it translocates into the nucleus upon androgenic stimulation. When the cells were incubated with the GSK-3-inhibitior SB216763, prior to androgen treatment, a dramatic decrease in nuclear AR was detected (see Figure 16). This phenomenon suggests that there is a reduction in AR import into the nucleus, although in cannot be excluded that there may be an enhancement of AR nuclear export is enhanced following GSK-3 inhibition.

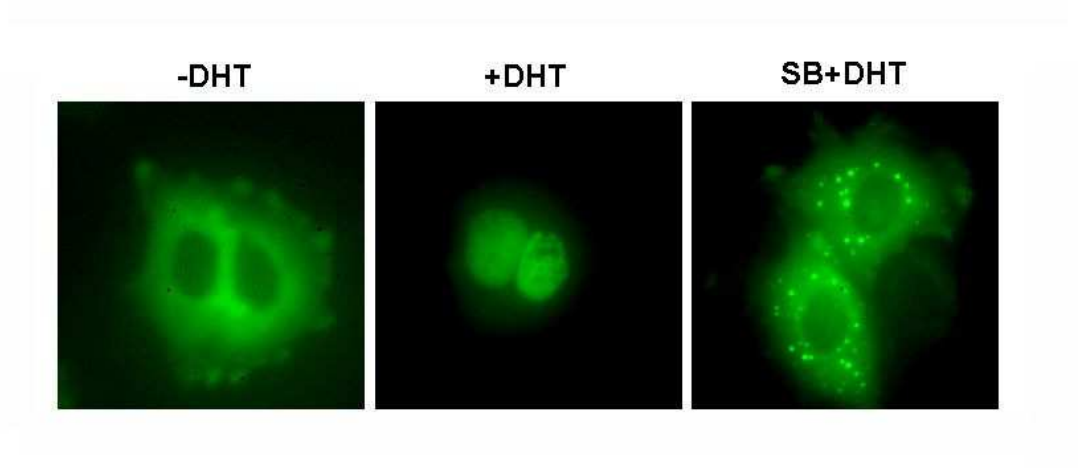


Figure 16: GSK-3 inhibition prevents AR nuclear import. AR-negative PC3 cells were transfected with pAR-t1EosFP. 24 hours later cells were incubated with 1 µM of the GSK-3 inhibitor SB216763 30 minutes prior to the addition of 10 nM DHT. AR-EosFP localization was measured four hours later by fluorescent microscopy. (Adapted from Schütz et al., 2010)

In cells pre-treated with androgens, SB216763 was able to induce a nuclear export of Eos-AR. The GSK-3 inhibitor-triggered nuclear export of Eos-AR is a very fast acting mechanism, as nuclear localization of Eos-AR was reduced by 70% in less than 30 min (see Figure 17).

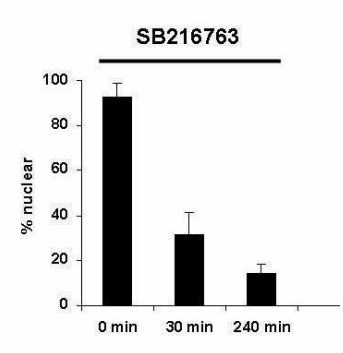


Figure 17: Inhibition of GSK-3 by SB216763 induces a rapid export of Eos-AR from the nucleus. PC3 cells were transfected with pAR-t1EosFP. Subsequently, cells were treated with 5 nM R1881 for 30 minutes resulting in >90% nuclear localization of EosAR. Subsequently, cells were treated with/without 1 µM SB216763 for 30 and 240 minutes, respectively. Fluorescent cells were counted using fluorescence microscopy (30 cells/ well). Results are expressed in % of green fluorescent nuclei ± SD (Rinnab et al., 2008).

The effects of the GSK-3 inhibitor SB216763 on nuclear export of the AR was not restricted to the AR-fusion protein Eos-AR but is also true for the endogenously expressed AR in 22Rv1 cells (see Figure 18).

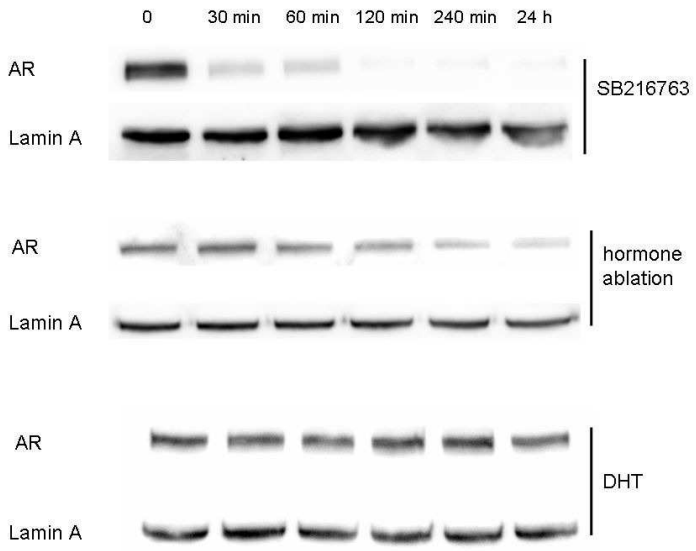


Figure 18: Western blot of nuclear 22Rv1 fractions reveals rapid AR export following GSK-3 inhibition in contrast to androgen ablation. 22Rv1 cells were incubated for 1 hour with 10 nM DHT. GSK-3 inhibitor SB216763 was then added or medium was changed to FBSdcc, respectively. Nuclei were prepared for time periods ranging from 30 minutes to 24 hours, as indicated, and subjected to western blot analysis. AR protein levels were detected by monoclonal mouse anti AR441 (Dako). Detection of Lamin A (polyclonal rabbit, Santa Cruz Biotechnology) served as loading control for nuclear extracts. Incubation of the cells with 10 nM DHT served as control for persisting nuclear localization.

In summary, the experiments indicate that GSK-3 inhibitors like SB216763 do not inhibit nuclear import but dramatically enhance the nuclear export of the AR (Rinnab et al. 2008, Schütz et al., 2010). GSK-3 inhibitor-triggered nuclear export of the AR is a very fast acting mechanism and differs from the relatively slow export mechanism in PCa cells induced by hormone withdrawal (Saporita et al., 2003).

4.4.5.3 Inhibition of GSK-3β induces a CRM1-dependent nuclear export of the AR

In order to guarantee intracellular signalling, transcription factors need to be transported bi-directionally through the nuclear pore complex (NPC) between cytoplasm and nucleus. Whereas small molecules are able to passively diffuse through the NPC, molecules with a greater molecular weight (> 40-60 kDa) are actively transported in an energy-dependent process (Holaska et al., 2001). In general, nucleocytoplasmic shuttling of larger proteins is initiated by the binding of a soluble receptor protein via a nuclear localization signal (NLS) or nuclear export signal (NES) (Holaska et al., 2001). NLS are rich in basic amino acids, the main arrangement being five to eight closely spaced basic amino acids (Savory et al., 1999). The NES, on the other hand, typically contains short hydrophobic activation domains characterized by four critically spaced leucine residues. (Kudo et al., 1999; Liu and de Franco, 2000). Although the mechanisms of nuclear import of steroid receptors are well documented, the mechanisms of export remain still unclear (Saporita et al., 2003; 2007). The best characterized nuclear export pathway uses chromosome region maintenance 1 (CRM1),

a receptor for proteins with leucine rich nuclear export signals. Initially identified as a 115 kDa protein that is essential for the maintenance of the chromosome structure of *Schizosaccharomyces pombe*, CRM1 is now known to be a potent receptor for the nuclear export of NES-containing proteins through the NPC. In the nucleus, CRM1 binds its NES cargo protein in the presence of RanGTP. The resulting trimeric export complex is subsequently transported to the cytoplasm where RanGTP is hydrolysed. Consequently, the trimeric export complex disassociates and CRM1 as well as RanGDP are re-imported into the nucleus (Görlich et al., 1997; Holaska et al., 2001). One very remarkable feature of CRM1 is the great variety of cargo proteins, some of them being cyclin B or the tumor suppressor p53 (Görlich et al., 1999). Although steroid receptors generally lack the classical leucine-rich NESs, there is increasing evidence that some steroid receptors can be exported from the nucleus through a CRM-1 dependent mechanism (Savory et al., 1999; Rimler et al., 2001, 2002; Betanska et al., 2007; Lombardi et al., 2008).

In order to analyze CRM-1 mediated export mechanisms, one usually performs translocation experiments (see 4.4.5.2) in presence/absence of leptomycin B (LMB). Initially identified as a potent antifungal antibiotic, produced by *Streptomyces* spp. (Hamamoto et al., 1983), LMB has been found to directly bind to CRM1. Functionally, LMB prevents the assembly of the trimeric export complex (CRM1, RanGTP, NES-containing protein) by covalently binding to a cysteine residue in CRM1 (Liu and de Franco, 2000). Thus, the Ran-free conformation of CRM1 is stabilized and the association of Ran and the cargo protein is abolished (Görlich et al., 1999). As a result, the nuclear export of the cargo proteins is inhibited (Kudo et al., 1999).

When analyzing whether the fast-acting nuclear export of the AR following GSK-3 inhibition is CRM-1 dependent, Eos-AR translocation experiments (Figure 16 + 17) were repeated in the presence/absence of LMB (see Figure 19). The following data show for the first time that inhibition of GSK-3β promotes fast nuclear export of the AR through a CRM1-dependent mechanism (Schütz et al., 2010).

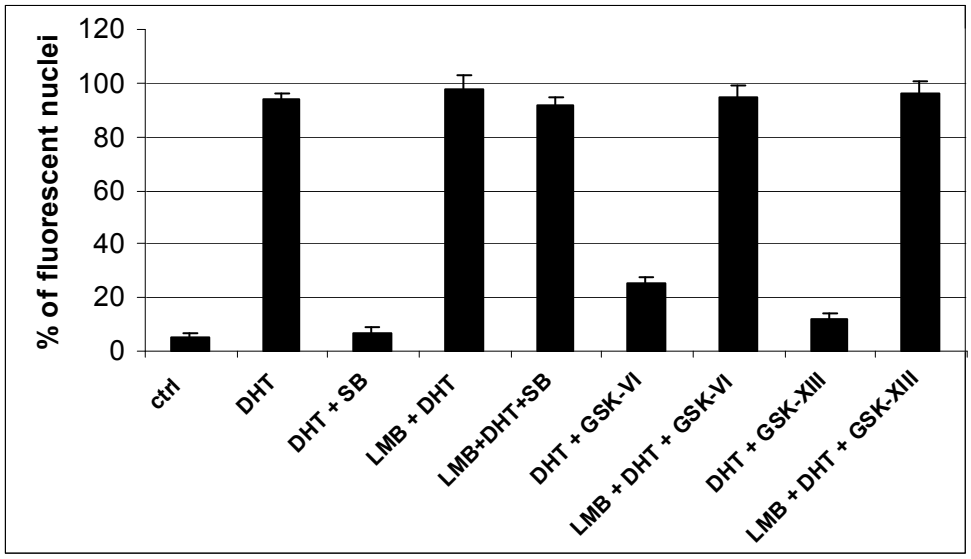


Figure 19: Different GSK-3 inhibitiors induce CRM1-dependent nuclear export of the AR. AR-negative PC3 cells were transfected with pAR-t1EosFP. 24 hours later, cells were treated with/without 1 ng/ml LMB 30 min prior to the addition of 10 nM DHT. 30 min later, GSK-3 inhibitors, SB216763, VI, or XIII, were added at 1 or 10 µM (GSK-VI and XIII). Fluorescent cells were counted 4 hours later by fluorescent microscopy (30 cells/well). Results are expressed as a percent of green fluorescent nuclei +/- SD (Schütz et al., 2010).

Moreover, the CRM1-dependent nuclear AR export is independent of the mode of action of the GSK-3 inhibitor. As shown in Figure 19, three mechanistically different GSK-3 inhibitors, namely maleimide SB216763 (inhibits GSK-3β activation function at Y216), aminopyrazole GSK-inhibitor XIII (ATP-competitive), and chloromethyl-thienyl-ketone GSK-inhibitor VI (non-ATP-competitive inhibitor) are able to rapidly export the AR from the nucleus to the cytoplasm. The nuclear export induced by these three inhibitors could be prevented by LMB in all cases, suggesting that the CRM1-dependent export of the AR is indeed the result of GSK-3 inhibition and not due to eventual unknown side effects of the inhibitors.

4.4.5.4 Localization of a CRM1 binding site on the C-terminus of the AR

Steroid receptors are thought to lack a classical CRM1 binding site. Immunoprecipiation analysis revealed a CRM1-binding site at the C-terminal end of the AR. Taking advantage of the fact that 22Rv1 PCa cells express two AR-isoforms, the CRM1-binding site could be restricted to the C-terminal deleted part of the 79 kDa isoform, corresponding to amino acids 672 to 919 of the AR (Schütz et al., 2010) (see Figure 20).

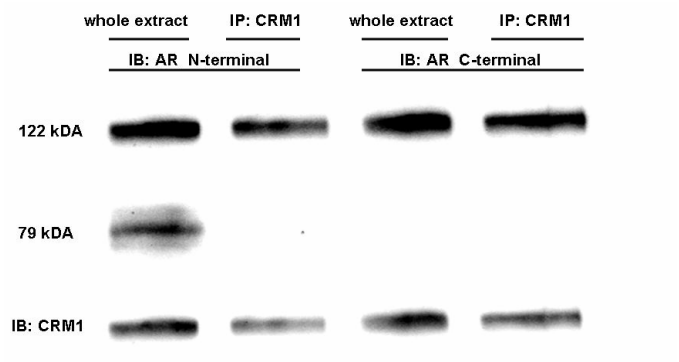


Figure 20: AR binds to CRM1. 22Rv1 cells were cultured under normal conditions (10% FBS) in T25 flasks for 24 h and proteins were collected. 100 µg of total protein extract was immunoprecipitated with 1µg CRM1 antibody. Immunoprecipitates were analyzed by monoclonal antibodies AR441 and EP670Y recognizing the N- terminal or C-terminal end of the AR protein. (Schütz et al., 2010)

To confirm that the CRM1 binding site is truly located at the C-terminal end of the AR, PC3 cells were transfected with an expression construct coding for AR^{Q640X}, an AR deletion mutant recently isolated from a HRPCa-patient (plasmids kindly provided by Prof. Jocelyn Céraline, Strasbourg). This mutant presents a premature stop codon at position 640 and lacks 279 amino acids at the C-terminal end of the receptor. Moreover, AR^{Q640X} displays ligand-independent nuclear localization and exhibits constitutive activities from MMTV-luciferase reporter constructs (Céraline et al., 2004). Transfection of AR-negative PC3 cells with AR^{Q640X} in an AR-dependent probasin reporter gene assay confirmed that AR^{Q640X} is fully active, even in the absence of androgens (see Figure 21). Such permanent AR transactivation could not be prevented by the GSK-3 inhibitors, SB216763, GSK-3 inhibitor XIII, or VI.

A **B**

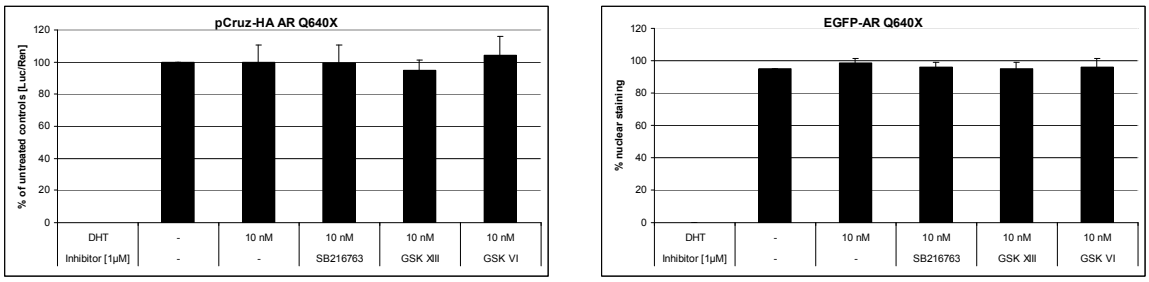


Figure 21: A C-terminally deleted AR mutant (Q640X) is constitutively active and localized in the nucleus. AR-negative PC3 cells were transfected with (A) pCruz-HA AR Q640X and AR-dependent reporter constructs or (B) green fluorescent EGFP AR Q640X. 24 hours after transfection , cells were incubated with/without 10 nM DHT 30 minutes prior to the addition of 1 µM of the GSK-3 inhibitors SB216763, XIII and VI. (A) reportergene assay was measured 24 hours later, (B) nuclear localization was observed four hours later (Schütz, unpublished).

As seen in fluorescence microscopy of AR^{Q640X}-GFP transfected PC3 cells, the mutant AR was predominantly nuclear (>95%) irrespective of the hormonal treatment and/or GSK-3 inhibitor treatment (see Figure 21). Fluorescence microscopy results were confirmed by western blot analysis (see Figure 22) of nuclear extracts from PC3 cells transfected with AR wild type (WT) or AR^{Q640X} (Q640X). The experiments clearly show that an AR mutant lacking the last 279 amino acids at its C-terminus cannot be exported from the nucleus via a CRM1-dependent mechanism, supporting the assumption that a putative CRM1 binding site is located at the AR C-terminus.

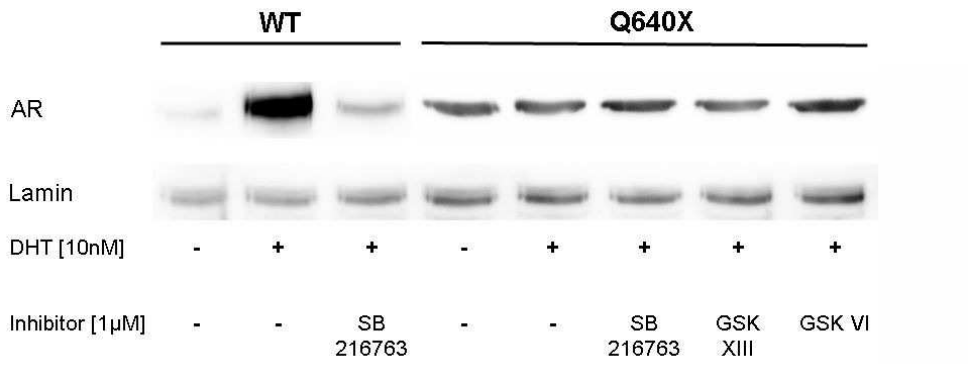


Figure 22: Nuclei prepared from PC3 cells transfected with C-terminally deleted AR-Q640X reveal predominantly nuclear localization in comparison to pSG5-AR WT. AR-negative PC3 cells were transfected with pSG5-AR WT or pCruz-HA AR Q640X. 24 hours later, cells were incubated with/without 10 nM DHT for 30 minutes before the addition of the GSK-3 inhibitors, SB216763 and XIII, or the IKK inhibitors, Wedenolactone and BMS. Cell nuclei were prepared four hours later and subjected to westernblot analysis. AR protein levels were detected by a mouse monoclonal anti-AR441 antibody (Dako). Detection of Lamin A (polyclonal rabbit antibody, Santa Cruz Biotechnology) served as a loading control for nuclear extracts (Schütz SV, unpublished).

4.4.5.5 Identification and characterization of the CRM1-dependent NES on the AR

Recently, Lombardi et al. (2008) identified sequence homologies between amino acids 444 to 456 of ERα and the conserved leucine-rich NES of p53. This sequence, located in the LBD of ERα, was described as a functional, hormone-dependent CRM1-directed nuclear export signal. Remarkably, the region at amino acids 444-456 of ERα is conserved among several steroid receptors (see Figure 23), suggesting that this sequence might be responsible for CRM1-dependent nuclear export of most steroid receptors.

Figure 23: The homology (sequence alignment) between the conserved leucine-rich NES of p53 and the putative NES in steroid receptors. Conserved amino acids are shown in red. The core of the ERα-NES sequence and the leucine enriched sequences which largely resemble a classic NES in the other indicated steroid receptors are underlined (adapted from Lombardi et al., 2008).

Considering a sequence alignment of different steroid receptors with the NES of p53 (see Figure 23), the putative CRM1-dependent NES of the AR is located between amino acids 803-815 with the leucine-enriched region being the sequence ALLL (amino acids 809-812).

Mutations in the core of the ERα-CRM1^{NES} (IILL) significantly impaired estradiol-induced nuclear export of ERα (Lombardi et al., 2008). Consequently, the putative NES of the AR (ALLL) was mutated by site-directed mutagenesis in pSG5-AR (a vector containing an AR wild type: WT), creating three different AR mutants (see Table 3): 1) A single mutant (SM) (SM: L810A) with the first leucine being mutated to alanine; 2) a double mutant (DM) (DM: LL811AA) with the last two leucines being mutated to alanines; and 3) a triple mutant (TM) (TM: LLL810AAA) with all three leucines being converted to alanines.

Table 3: Summary of the mutations created in the putative AR-CRM1-NES

pSG5-AR	amino acid sequence
wild type (WT):	EFLCMK**ALLL**FSI
single mutant (SM): L810A	EFLCMK**A**A**LL**FSI
double mutant (DM): LL811AA	EFLCMK**AL**A**A**FSI
triple mutant (TM): LLL810AAA	EFLCMK**A**A**AA**FSI

As shown by transactivation studies (see Figure 24), the AR wild type construct (WT) was strongly activated by DHT. Transactivation of WT AR after DHT treatment was reduced by 50% after addition of SB216763. In contrast, the reduction of receptor transactivation following SB216763 treatment in the DHT-treated SM was only marginal. Although the overall AR transcriptional activity is strongly decreased in DM, it somehow behaves like the WT, i.e., relatively strong induction of transcriptional activity in presence of androgens and a

50% reduction in DHT induced transcriptional activity after SB216763 treatment. The TM was unable to induce receptor transactivation.

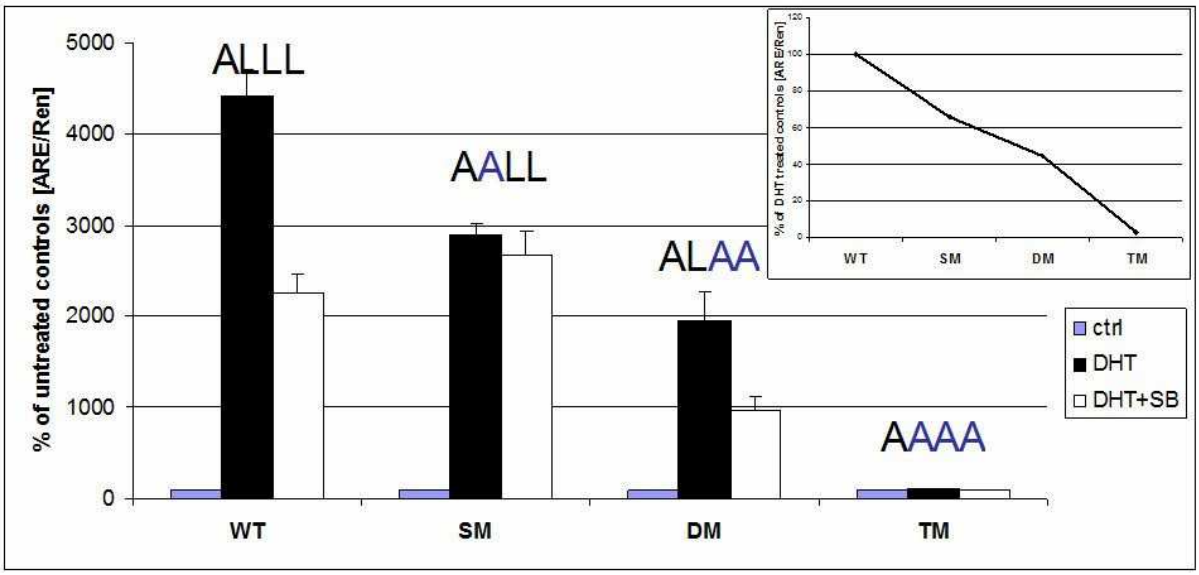


Figure 24: The SM circumvents receptor transactivation after GSK-3 inhibition. AR-negative PC3 cells were transfected with pSG5-AR WT, SM, DM or TM, respectively, and androgen-dependent reporter constructs. 24 hours after transfection, cells were incubated in the presence/absence of 10 nM DHT 30 minutes prior to the addition of 1 µM GSK-3 inhibitor SB216763. A reporter gene assay was carried out 24 hours later to measure receptor transactivation. The insert in the upper right part of the figure shows the transactivation of AR WT or AR mutants in the presence of 10 nM DHT.

Because the mutated NES lies within the hormone binding domain of the AR, the reduction in receptor transactivation (WT > SM > DM > TM) is most likely due to continuously impaired DHT binding (see insert Figure 24). This hypothesis is consistent with data by Jääskeläinen et al. (2006) and Pinsky et al. (1992), who investigated that mutations in this region of the AR-LBD (L812P, Jääskeläinen; S814N, Pinsky) lead to severely impaired AR transactivation and/or androgen insensitivity.

Additional western blot analysis of nuclear extracts from PC3 cells transfected with the AR wildtype, SM, DM, or TM (see Figure 25) revealed that both WT and SM translocated into the nucleus upon DHT treatment. However, SM, in contrast to WT, was unable to perform nucleocytoplasmic shuttling in the presence of SB216763 (see Figure 25). The DM behaves like AR-WT although its effects are less pronounced, whereas the TM seems to completely reside in the cytoplasm, for nearly no nuclear staining could be observed in the western blot.

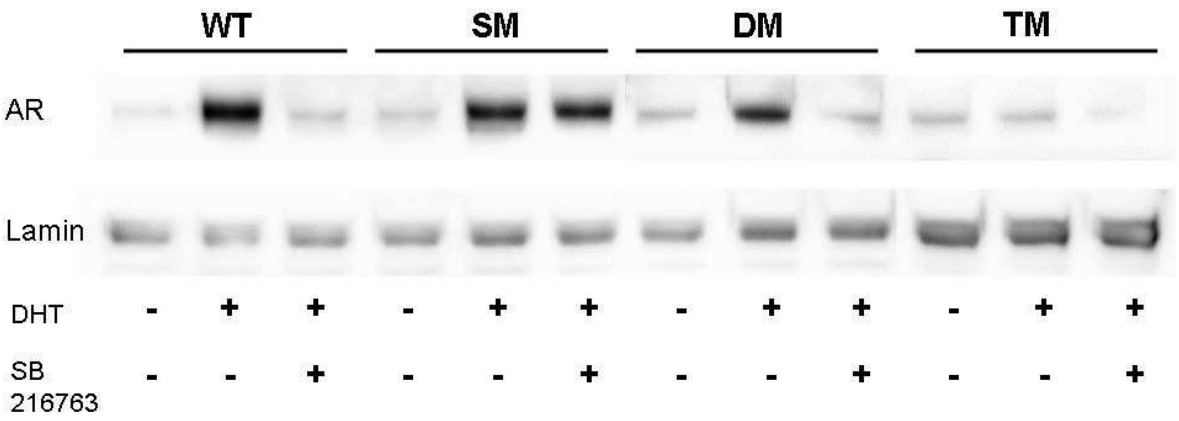


Figure 25: Western blot of nuclear AR-WT or AR-mutant fractions. PC3 cells were transfected with pSG5-AR WT or AR-mutants (SM, DM, and TM). 24 hours after transfection, cells were incubated with/without 10 nM DHT prior to GSK-3 inhibition by SB216763. Nuclei were prepared four hours later and subjected to western blot analysis. AR protein levels were detected by a mouse monoclonal anti-AR441 antibody (Dako). Detection of Lamin A (polyclonal rabbit antibody, Santa Cruz Biotechnology) served as a loading control for nuclear extracts.

Based on the results shown above, a NES^{CRM1} is located in the region of amino acids 803-815 of the AR, with the core amino acid being responsible for nuclear export located at position 810.

The importance of Leucine 810 was emphasized by immunofluorescent staining of PC3 cells transfected with either pSG5-AR (AR-WT) or AR^{L810A} (AR-SM), as shown in Figure 26. Upon DHT-treatment, both AR-WT and AR-SM move into the nucleus. In presence of SB216763, AR-WT is rapidly exported from the nucleus, whereas SB216763 is unable to induce the nucleocytoplasmic shuttling of AR-SM.

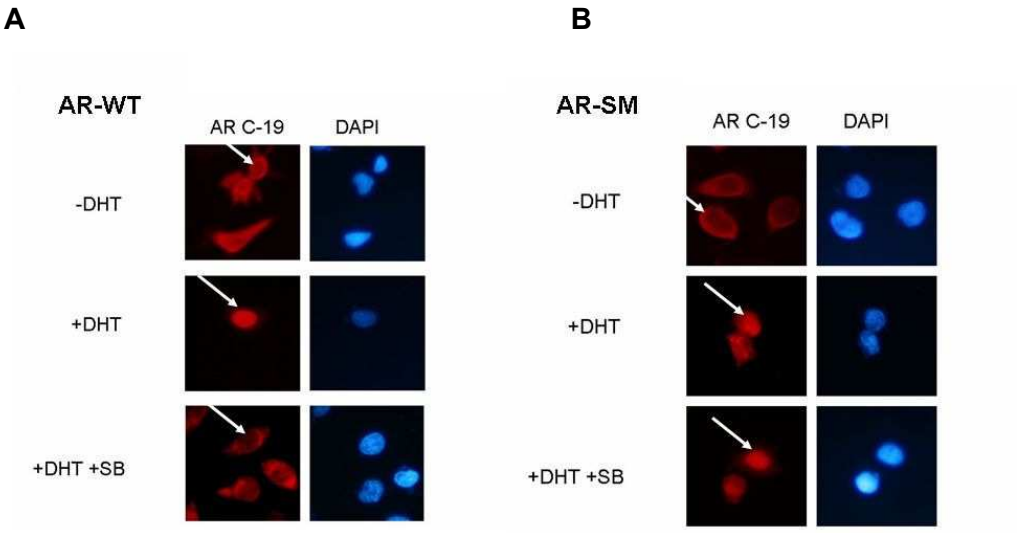


Figure 26: AR-CRM1-SM is not exported from the nucleus upon application of the GSK-3β inhibitor SB216763. PC3 cells were transfected with pSG5-AR WT (A) or SM (B). Following transfection, (after 24h) cells were treated with/without 10 nM DHT followed by the addition of 1 µM SB216763 30 minutes later. The cellular localization of AR (WT/SM) was analyzed four hours later via red immunofluorecence (primary antibody: AR C19, secondary antibody: Alexa Fluor 594 goat anti-rabbit IgG). DAPI-staining served as a control for nuclear localization.

In contrast to long-term inhibition of GSK-3, which induces a proteasomal degradation of the AR protein, short-term inhibition of GSK-3 in PCa cells leads to a rapid CRM1-dependent nuclear export of the AR. Although steroid receptors generally lack leucine-rich sequences which are necessary for CRM1-dependent export, a CRM1-NES could be identified in the LBD of the AR (aa 803-815). CRM1-NES is responsible for the fast nuclear export of the AR following GSK-3 inhibition, and this mechanism largely differs from the slow AR export mechanism following androgen withdrawal. Therefore, the mode of AR export appears to be dependent upon physiological conditions. A similar phenomenon was described by Itoh and colleagues (2002) who discovered two distinct export mechanisms for the GR, a fast CRM1-dependent and a slow CRM1-independent mechanism following dexamethasone withdrawal.

Although a reason as to why the AR is exported by two very different mechanisms remains largely unknown, the CRM1-mediated export of p53 has been described to be necessary in the regulation of transcription factor activity (Görlich et al., 1999). In this context, CRM1-mediated export of p53 favours subsequent cytoplasmic degradation of p53, thereby modulating p53 activity. In PCa cells, GSK-3 could serve as a key element in the regulation of AR signalling by simultaneously controlling AR localization and AR stability.

4.4.6 Inhibition of GSK-3β by SB216763 diminishes PCa cell proliferation

The maleimide SB216763 has been shown to downregulate AR transactivation and AR protein levels (Rinnab et al., 2008). In addition, the GSK-3 inhibitor SB216763 demonstrated antiproliferative effects in the AR-positive PCa cells LNCaP (29% inhibition) and 22Rv1 (32 % inhibition) as presented in Figure 13. The proliferation rate of AR-negative PC3 cells remained unaffected by pharmacological GSK-3 inhibition, suggesting a role for GSK-3 in AR-mediated proliferation.

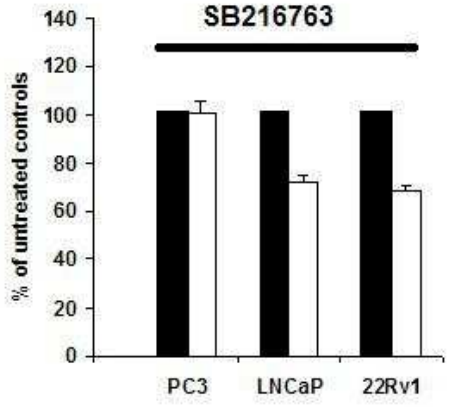


Figure 13: Effects of GSK-3 inhibitor SB216763 on the proliferation of PCa cells. PC3, LNCaP and 22Rv1 prostate cancer cells were seeded in 96-well plates and allowed to adhere overnight. Subsequently, medium was changed, and cells were grown for 72 hours in RPMI 1640, supplemented with 2.5% FBS and antibiotics in the presence/absence of 10-μM SB216763. Proliferation was measured by means of a colorimetric MTT assay. Results are expressed as % of untreated controls, which was set at 100%. (Rinnab et al., 2008)

5. Modulation of AR activity by IKK

5.1 The NF-κB signalling pathway

Abnormalties in signalling pathways that are involved in the control of cell proliferation and apoptosis have frequently been described to be responsible for the development of various cancers (Gasparian et al., 2002a). Apart from the WNT signalling pathway (see Chapter 4.3), the deregulation of the nuclear factor kappa B (NF-κB) signalling pathway has been implicated in the pathogenesis of pancreatic cancer (Wilson et al., 2008), breast cancer (Weitsman et al., 2006), and prostate cancer (Gasparian et al., 2002b).

Originally, NF-κB was identified as an important mediator of the immune system. In particular, members of the NF-κB family control the transcription of antimicrobial effector molecules and cytokines in response to various pathogens. Moreover, NF-κB is involved in the regulation of cellular differentiation, cell survival, and cell proliferation (Hayden et al., 2006; 2008), especially within cells which carry out immune responses in mammals. Therefore, NF-κB transcription factors are crucial regulators of the inflammatory, innate, and adaptive immune responses.

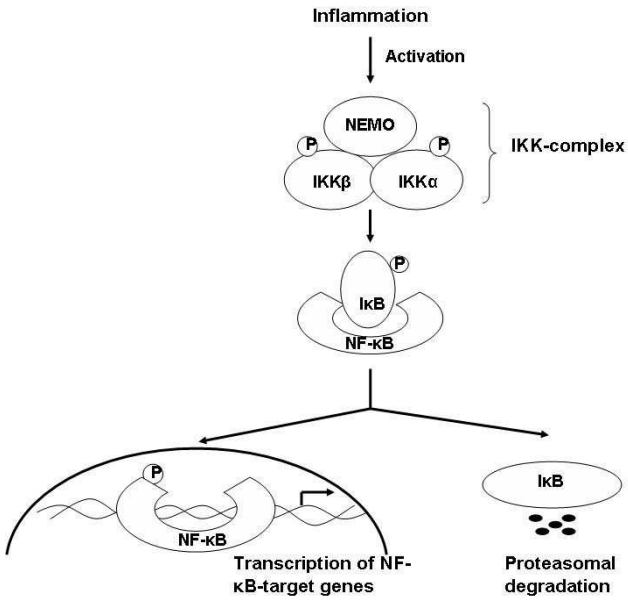


Figure 27: NF-κB signalling pathway. Primarily NF-κB is located in the cytoplasm inactivated by IκB proteins. Inflammatory stimuli activate the IκB complex (IKKα, IKKβ, NEMO) which phosphorylates IκB proteins, subsequently marking them for proteasomal degradation. “Freed” NF-κB further translocates to the nucleus, thereby activating the transcription of NF-κB target genes.

In resting cells, NF-κB is located in the cytoplasm in an inactive state associated with inhibitory IκB proteins (see Figure 27). This interaction blocks the ability of NF-κB to bind to DNA. Various extracellular inducers, some of them being inflammatory cytokines, UV light, reactive oxygen species, or bacterial and viral toxins activate the IκB kinase (IKK) complex, which consists of two catalytic subunits IKKα (IKK1) and IKKβ (IKK2) and a regulatory NEMO (IKKγ) subunit with additional adaptor functions. The activated IKK complex phosphorylates

IκB proteins on two conserved serine residues within their N-terminal area, subsequently leading to IκB ubiquitinylation and degradation by the 26S proteasome (Gasparian et al., 2002a). This proteasomal degradation of the inhibitory IκB proteins releases unmasked NF-κB and promotes its translocation into the nucleus where it binds to its recognition site on the DNA and supports the expression of various pro-inflammatory or anti-apoptotic gene products (Palkowitsch et al., 2007).

5.2 IKK in human cancers

Numerous human cancers are characterized by constitutive NF-κB activation, some of them being human leukaemia and lymphomas, as well as cell lines from solid tumors including breast, ovarian, thyroid, pancreatic, and prostate (Gasparian et al., 2002b). In pancreatic cancers, constitutive IKK activity plays a key role in the regulation of cell survival and cell cycle progression (Wilson et al., 2008). With respect to prostate cancer, constitutive NF-κB activation and thus continuous DNA binding is a result of aberrant activation of IκB kinases (IKKs) leading to increased phosphorylation and a shorter half-life of IκBα (Gasparian et al., 2002b). Such constitutive NF-κB activation not only protects cancer cells from apoptotic cell death, but may even enhance their growth activity. Recently, IKKα was shown to be the main catalytic IKK-subunit that is required for PCa metastasis (Luo et al., 2007). Consequently, the major pharmacological approaches targeting NF-κB in PCa cell lines include either repression of the IKKs or blocking of the proteasomal degradation of IKKs (Gasparian et al., 2009). Most interestingly, IKK has been shown to phosphorylate ERα in mammary cancer cell lines, thereby modulating ERα activity (Weitsman et al., 2006). Potential effects of IKK-inhibition on AR signalling have not been studied yet. Consequently, the effects of a pharmacological inhibition of either IKKα, IKKβ or both catalytical IKK subunits on the androgen signalling cascade in prostate cancer cell lines were investigated. Three very potent pharmacological IKK inhibitors were chosen for this purpose: (1) Wedelolactone (7-Methoxy-5,11,12-trihydroxy-coumestan), the naturally isolated active ingredient of the herbal medicine *Eclipta alba* and a selective and irreversible inhibitor of IKKα and IKKβ activity that inhibits NF-κB mediated gene transcription by blocking phosphorylation and degradation of IκBα; (2) BMS-345541 (4-(2´-Aminoethyl)amino-1,8-dimethylimidazo[1,2-a]quinoxyline), an allosteric site-binding inhibitor of IKKβ with anti-inflammatory properties; and (3) SC-514, a reversible, ATP-competitive inhibitor of IKKβ with anti-inflammatory properties, but lacking an inhibitory effect on the phosphorylation and activation of the IKK complex.

5.3 Role of IKK in the regulation of AR signalling in human PCa cells

5.3.1 IKK inhibitors modulate AR transactivation

In order to determine the effects of functional inhibition of IKK on AR transactivation, androgen-dependent reportergene assays were monitored in 22Rv1 cells incubated with different pharmacological IKK inhibitors. In the presence of Wedelolactone, AR signalling was dose-dependently inhibited (see Figure 28). Inhibitory effects reaching 28% AR signalling inhibition could already be measured at a concentration of only 0.1 µM Wedelolactone. In the presence of 5 µM Wedelolactone, AR transactivation was reduced by 91%. Similar results were achieved with BMS-345541, reaching 81% inhibition of AR signalling at a concentration of 5 µM. Although SC-514 is said to be a reversible ATP-competitive inhibitor of IKK-β, SC-514 had no significant influence on AR signalling inhibition, even at concentrations ranging from 1 µM to 15 µM.

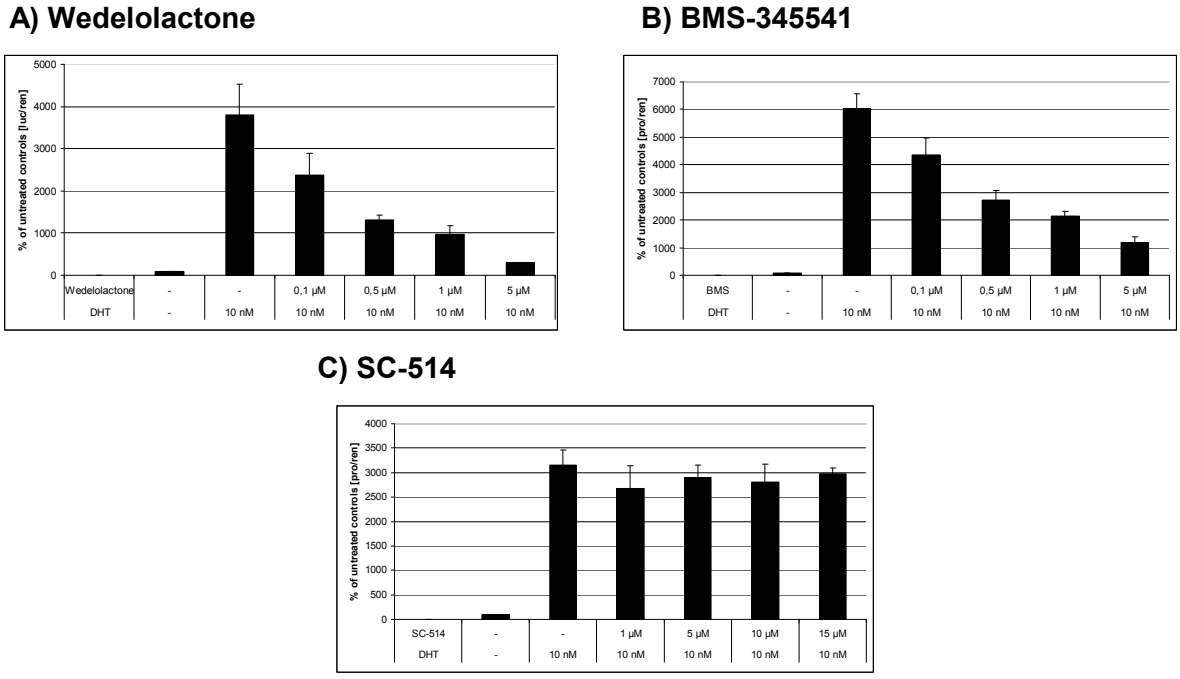


Figure 28: Modulation of AR-dependent reporter gene activity by pharmacological IKK inhibitors. AR-positive 22Rv1 cells were incubated with different pharmacolocigal IKK inhibitors, Wedelolactone, BMS-345541, and SC-514 in the presence/absence of 10nM DHT for 24h. AR transcriptional activity was measured using an AR reportergene assay. Results are expressed as means of at least three separate experiments in % of untreated controls (without DHT, without inhibitor)

Studies of Luo et al. (2007) and Gasparian et al. (2002a) indicate that IKKα is the more important catalytical IKK subunit in the regulation of PCa cell growth. With respect to AR transactivation in PCa cells, BMS-345541, a selective inhibitor targeting solely IKKβ emerged as potent as Wedelolactone which inhibits both catalytic IKK subunits. As SC-514 doesn't inhibit the phosphorylation and activation of the IKK target complex, it can be assumed that the inhibitory effects of Wedelolactone and BMS-345541 on AR signalling are based on

reduced AR phosphorylation. Therefore, effects of IKK inhibition on AR phosphorylation were examined using western blot analysis.

5.3.2 Inhibition of IKK downregulates the phosphorylation of the AR on Ser 308

From *in vitro* studies on the ERα, it is known, that IKKα is responsible for the phosphorylation of ERα on serine residue 118 (S118) (Weitsman et al., 2006). Sequence alignments reveal that the corresponding site on the AR is serine residue 308 (S308) (Schütz, unpublished). As shown in Figure 29, AR-S308 phosphorylation is indeed decreased upon IKK inhibition with Wedelolactone and BMS-345541. Interestingly, the whole AR protein remains stable and is not degraded. Whereas Wedelolactone causes dephosphorylation of the AR on S308 at a concentration of 1 µM, BMS-345541 achieved a similar extent of dephosphorylation at concentrations ranging from 3 µM to 5 µM (see Figure 29). Wedelolactone inhibits both catalytic IKK subunits (IKK α and β), whereas BSM-345541 only functionally inactivates IKK β. The results shown in Figure 29 support the assumption, that IKKα is the more important catalytic subunit being responsible for AR-S308 phosphorylation. These data are in agreement with studies on the ERα, where IKKα was found to be responsible for ERα-S118 phosphorylation (Weitsman et al., 2006). In contrast to the effects seen with Wedelolactone and BMS-345541, AR-S308 phosphorylation is not influenced by SC-514 (see Figure 29). Thus, we hypothesized that it is indeed IKK phosphorylation status which influences the AR signalling cascade.

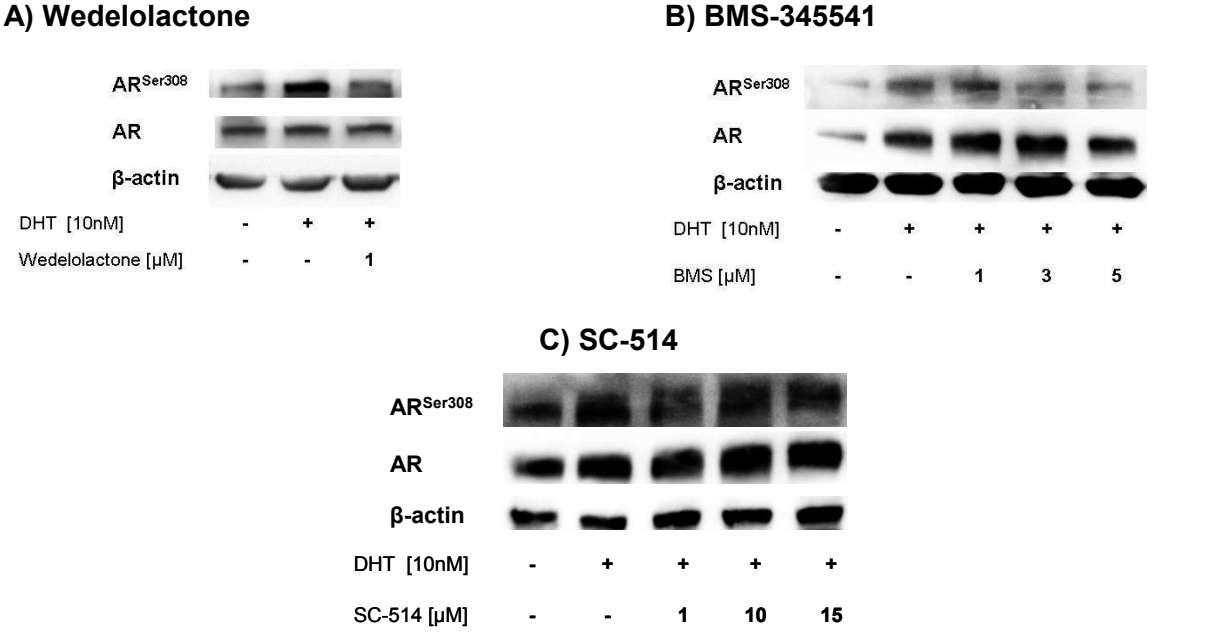


Figure 29: Effects of pharmacological IKK inhibitors on AR protein phosphorylation on S308. 22Rv1 cell extracts incubated with the IKK inhibitors Wedelolactone (A), BMS-345541 (B) and SC-514 (C) were analyzed by western blot with antibodies directed against AR phosphorylated on S308 (S308: polyclonal rabbit antibody; Santa Cruz), overall AR (monoclonal mouse antibody AR441, Dako) and β-actin antibody (Abcam) serving as loading control. Shown is one representative western blot out of at least three separate experiments.

There is experimental evidence, that phosphorylation of ERα on serine 118 (pS118) is an indicator for a functional ligand-dependent ERα signalling pathway in breast cancer cells (Weitsman et al., 2006). According to these observations, pS118 seems to be involved in ERα target gene transcription. In this respect, IKKα has been proven to be at least, in part, involved in this estrogen-mediated S118 ERα-phosphorylation (Weitsman et al., 2006). AR phosphorylation on S308 decreases in parallel to AR transactivation when using IKK-inhibitors Wedelolactone and BMS-345541. Although the effects of S308 are still unknown, it is most likely that AR phosphorylation on S308, like ERα phosphorylation on S118, is indeed necessary for functional ligand-dependent receptor signalling.

5.3.3 IKK inhibitors do not affect AR dimerization

As the pharmacological IKK inhibitors Wedelolactone and BMS-345541 decrease AR phosphorylation and AR transactivation, the possible effects of IKK inhibition on AR dimerization were analyzed by M2H (mammalian two hybrid). As presented in Figure 30, pharmacological IKK inhibitors have no influence on AR dimerization. Upon stimulation with DHT, AR dimerization increases to 694 ± 76% in comparison to the untreated control (100%). Neither Wedelolactone, nor BMS-345541, nor SC-514 were able to impede AR dimerization. Contrarily AR dimerization is slightly increased upon inhibition of the IKK complex by Wedelolactone, BMS-345541, or SC-514 (interaction: 726 ±86%, 900 ± 189% and 770 ± 163%, respectively). However, these differences were not statistically significant ($p > 0.05$).

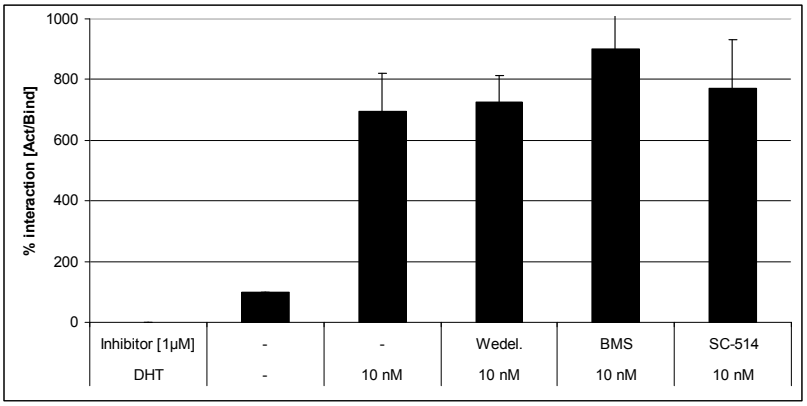
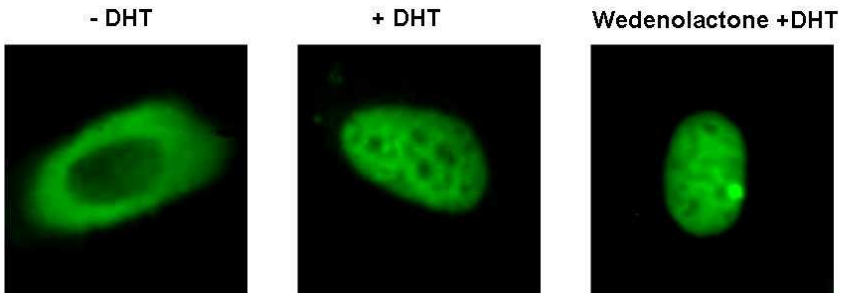


Figure 30: Mammalian two hybrid assay. AR-negative PC3 cells were transfected with AR-ACT and AR-BIND and Gal4 reporter to carry out mammalian two hybrid assays. Subsequently, cells were incubated for 24 hours with 1 µM of the IKK inhibitors Wedelolactone, BMS-345541 and SC-514 in the presence of 10nM DHT. Luciferase activity was measured using the dual-luciferase system (Promega). Results are means of 3 separate experiments and are expressed in % interaction AR-ACT/BIND.

5.4.3 Inhibition of IKK does not interfere with nuclear translocation of the AR

Furthermore, the consequences of the IKK inhibition on AR translocation were tested. AR-negative PC3 cells were transfected with green fluorescent pAR-t1EosFP (see Figure 31). In the absence of DHT, Eos-AR was predominantly located in the cytoplasm (4.7% nuclear staining), whereas upon androgenic stimulation, a translocation of the AR fusion-protein from the cytoplasm to the nucleus could be observed (96.7% nuclear staining). Preincubation of the cells with three pharmacological IKK inhibitors did not prevent nuclear import of Eos-AR (Wedelolactone 96.3%; BMS-345541 94%; SC-514 93.7% nuclear staining). Moreover neither of the applicated IKK inhibitors was able to promote nuclear export of the AR in cells pretreated with DHT. As pharmacological IKK inhibitors do not alter AR localisation, the AR-S308 phosphorylation is most likely linked to AR target gene transcription, rather than to AR localisation.

A)



B)

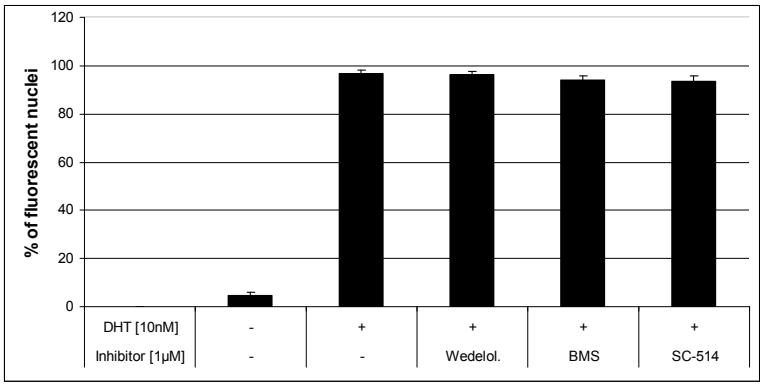


Figure 31: Modulation of nuclear translocation of the AR by pharmacological IKK inhibitors. (A) Fluorescence microscopy of AR-Eos transfected PC3 cells treated without DHT, with 10 nM DHT, and pre-treated with 1µM Wedenolatone prior to DHT. Figures on pre-treatment with 1 µM BMS-345541 or SC-514 are not shown due to reasons of clarity. (B) Fluorescent cells were counted by fluorescent microscopy (30 cells/well). Results are expressed in percent of green fluorescent nuclei +/- standard deviation (SD).

5.3.5 Effects of IKK inhibitors on the proliferation of PCa cells

The NF-κB pathway is known to be constitutively active in AR-positive as well as in AR-negative HRPCa cells (Gasparian et al., 2002b). Expression of dominant negative IKKα-mutants could dramatically reduce PCa cell growth and metastasis in these cells (Gasparian

et al., 2002b). In this respect, it is important to mention that these growth inhibitory effects are less pronounced in PCa cells still depending on androgens for growth and survival, like in androgen sensitive LNCaP cells. On the basis of these observations, the effects of Wedelolactone, BMS-345541, and SC-514 on PCa cell growth were examined.

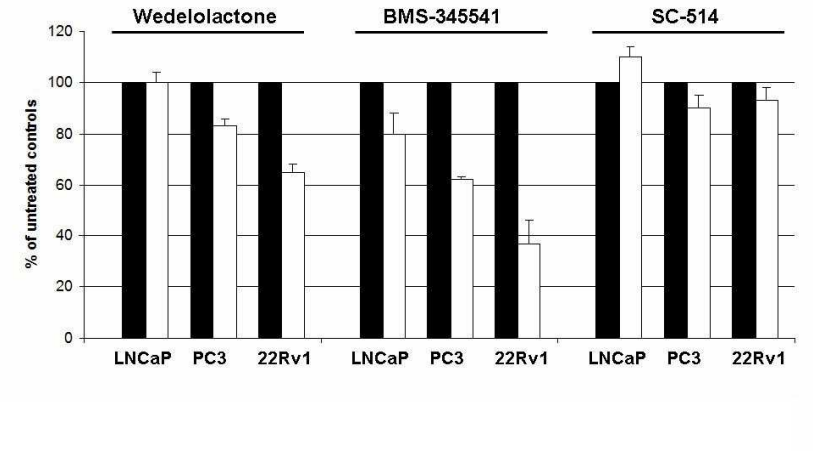


Figure 32: Effects of IKK-inhibitors on prostate cancer cell growth. AR-positive cells (22Rv1, LNCaP) and AR-negative cells (PC3) were seeded in 96-well plates and allowed to adhere overnight. Subsequently, medium was changed and cells were grown for 72 hours in RPMI 1640 supplemented with 2.5% FBS and antibiotics in the presence/absence of 5 µM Wedelolactone/BMS-345541 or 15 µM SC-514. Proliferation was measured by means of a colorimetric MTT assay. Results are means of three separate experiments and are expressed in % of untreated controls, which were set at 100%.

As shown in Figure 32, both IKK inhibitors, Wedelolactone and BMS-345541, were able to reduce HRPCa cell proliferation in 22Rv1 (inhibition: 35% and 63%, respectively) and PC3 (inhibition: 17% and 38%, respectively). In contrast, the growth-inhibitory effects of IKK inhibitor SC-514 on all PCa cells were neglectable. With the exception of BMS-345541 (20% inhibition), that has been shown to have a higher toxicity than Wedelolactone and SC-514, the effects of IKK-inhibitiors on the proliferation of the androgen-sensitive LNCaP cells were only marginal. This is in agreement with Gasparian et al., (2002b), showing that HRPCa cells are more prone to IKK inhibition. The present results support this assumption for the not well characterized 22Rv1 HRPCa cell line.

In summary, pharmacological inhibitors targeting the phosphorylation and activation of the IKK complex (Wedelolactone and BMS-345541) are able to dose-dependently reduce AR transcriptional activity which is paralleled by a decrease in AR phosphorylation on S308. This is consistent with previous data (Weitsman et al., 2006) which showed that IKKα is also necessary for hormone-dependent ERα signalling by phosphorylating ERα-S118. On the contrary, neither AR dimerization nor AR nuclear translocation were influenced by inhibition of the catalytical subunits of the IKK complex. In addition, PCa cell growth could be reduced by application of Wedelolactone and BMS-345541, reaching more pronounced effects in the HRPCa cells, PC3 and 22Rv1.

6. Concluding remarks and future prospects

As PCa is one of the most serious diseases in elderly man, current research focuses on the development of effective new therapies for PCa. Although most patients suffering from PCa initially respond to androgen withdrawal therapy, PCa almost invariably progresses to a hormone refractory state of the disease within 18-24 months. In recent years, it has become obvious that cell growth and survival of HRPCa largely depend on the cellular content of the AR protein as well as on AR function. Alterations in the AR protein, AR expression, and/or AR activity by protein kinases are some of the most common factors leading to hormone-independent PCa (Salas et al., 2004).

The present work highlights important roles of the protein kinases glycogen GSK-3 and IKK in AR signalling of PCa cells. Both kinases have been shown to modulate AR transactivation and AR phosphorylation. In addition, GSK-3 and IKK are both involved directly or indirectly in PCa cell proliferation and survival. In contrast to IKK, a special feature of GSK-3 is the control of AR localisation, thereby influencing the intracellular fate of the AR protein. On many occasions, pharmacological inhibition of serine/threonine kinases has been shown to have a negative impact on AR signalling. Although, inhibition of GSK-3 as well as IKK downregulates AR transactivation, both kinases affect different levels of AR signalling. Therefore, GSK-3 and IKK inhibitors have potential future roles as therapeutic agents in the treatment of PCa.

Some GSK-3 inhibitors have successfully entered clinical trials, showing low cytotoxic side-effects in the treatment of type II diabetes or neurological malignancies, such as Alzheimer's disease and bipolar disorders. Based on our results, GSK-3 inhibitors appear to be the most promising compounds for the treatment of PCa in the near future.

7. References

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8. Articles reprinted in this thesis

Original articles

Abstracts

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Die Rolle des Androgenrezeptors im hormonrefraktären Prostatakarzinom

Molekulare Grundlagen und experimentelle Therapieansätze

In fast allen westlichen Industrienationen ist eine deutliche Zunahme des Prostatakarzinoms (PCA) zu verzeichnen. Nach Angaben der „American Cancer Society" (http://www.cancer.org) erkrankten im Jahr 2007 in den USA rund 218.890 Männer an einem PCA. Dies entspricht in etwa 29% aller neu aufgetretener Karzinome bei Männern in den USA. An ihrem Krebsleiden verstarben 26.059 Patienten. In Deutschland sieht die epidemiologische Datenlage ähnlich aus: Das PCA war 2007 mit rund 48.650 Neuerkrankungen jährlich die häufigste Tumorerkrankung des älteren Mannes und stellt die zweithäufigste Krebstodesursache bei Männern in Deutschland dar (http://www.rki.de). Eine kurative chirurgische Behandlung des PCA ist nur dann möglich, wenn der Tumor in einem frühen Stadium entdeckt wird. Bei fortgeschrittenen Tumoren steht eine palliative Behandlung im Mittelpunkt.

Das PCA ist der Prototyp eines androgenabhängigen Tumors. Die Hormonabhängigkeit der meisten PCA-Zellen wird hierbei durch Androgenentzug therapeutisch genutzt. Obwohl die Mehrzahl der Patienten anfänglich gut auf eine antiandrogene Therapie anspricht, kommt es nach einem Zeitraum von durchschnittlich 2 Jahren zur Tumorprogression mit der Bildung von hormonrefraktären PCA-Zellen. Dies stellt eine der Hauptursachen für die hohe Mortalitätsrate des fortgeschrittenen PCA dar. Eine wesentliche Frage ist daher, welche Mechanismen die Proliferation und das Überleben von PCA-Zellen bei extrem geringen Androgenspiegeln ermöglichen. Während in vitro der Verlust des Androgenrezeptors (AR) der vorherrschende Mechanismus für die Entwicklung einer Hormoninsensitivität ist, ergaben In-vivo-Untersuchungen, dass sowohl bei hormonsensitiven Primärtumoren der Prostata, als auch bei Metastasen hormonrefraktärer PCA die Expression des AR nicht nur weitgehend erhalten bleibt, sondern sogar oftmals verstärkt ist [1, 2, 3, 4].

Der Einsatz neuer molekular- sowie zellbiologischer Methoden bei der Untersuchung hormonrefraktärer PCA-Zellen führte in den letzten Jahren zu einer Vielzahl neuer Erkenntnisse, welche sowohl eine verbesserte Aussage über den Verlauf erlauben, sowie zu neuen Therapiekonzepten führen werden. In den folgenden Kapiteln werden Veränderungen des AR bzw. AR-Signalweges aufgezeigt, die als Ursache für die Entwicklung hormonrefraktärer PCA (HRPCA) diskutiert werden. Basierend auf diesen Erkenntnissen werden in einem 2. Teil neue, sich fast ausschließlich in einem experimentellen Stadium befindliche Therapiekonzepte vorgestellt und diskutiert.

Aufbau und Funktion des Androgenrezeptors

Der AR ist ein nukleärer Rezeptor und gehört zur Superfamilie der Steroidhormonrezeptoren. Diese Superfamilie ist entwicklungsgeschichtlich sehr alt und beinhaltet eine Vielzahl verschiedener Rezeptoren wie den Östrogenrezeptoren (ER), den Progesteronrezeptoren (PgR) sowie die Rezeptoren für Vitamin A und D (RAR, RXR, VDR), aber auch Rezeptoren wie den Ecdysonrezeptor (EcR) bei Insekten [5, 6]. Das AR-Gen befindet sich auf dem X-Chromosom und kodiert für ein aus 919 Aminosäuren bestehendes Rezeptorprotein den AR. Strukturell gliedert sich der AR in drei Domänen:

- der aminoterminalen (-NH_3) Transaktivierungsdomäne,
- einer zentralen DNA-Bindungsdömäne, welche größtenteils aus zwei sog. Zinkfingern besteht sowie
- einer carboxyterminalen (-COOH) Ligandenbindungsdomäne.

DNA- sowie Ligandenbindungsdomäne sind durch die sog. „Hinge-Region" gekoppelt, welche ein für den AR notwendiges nukleäres Lokalisationssignal beinhaltet (**Abb. 1**). Der AR ist ein hormoninduzierbarer Transkriptionsfaktor. In Abwesenheit von Androgenen befindet sich der AR in einem inaktiven Zustand

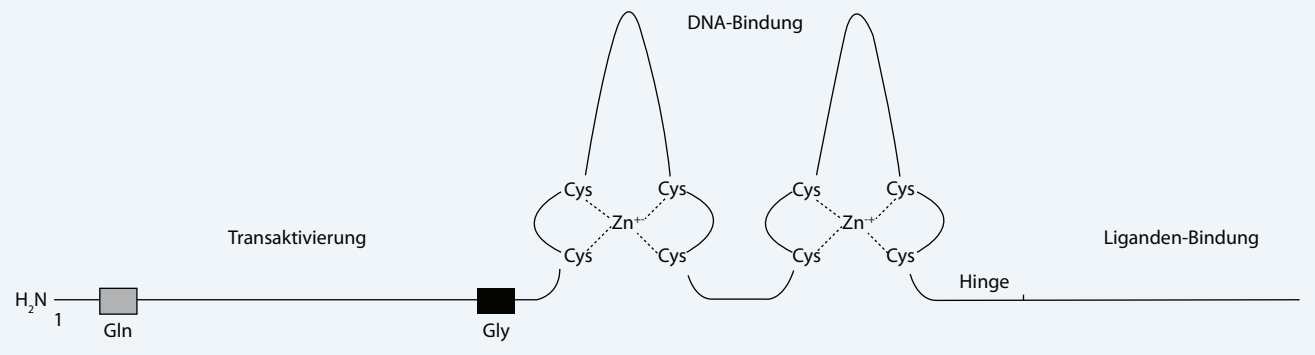

Abb. 1 ▲ Aufbau des Androgenrezeptors

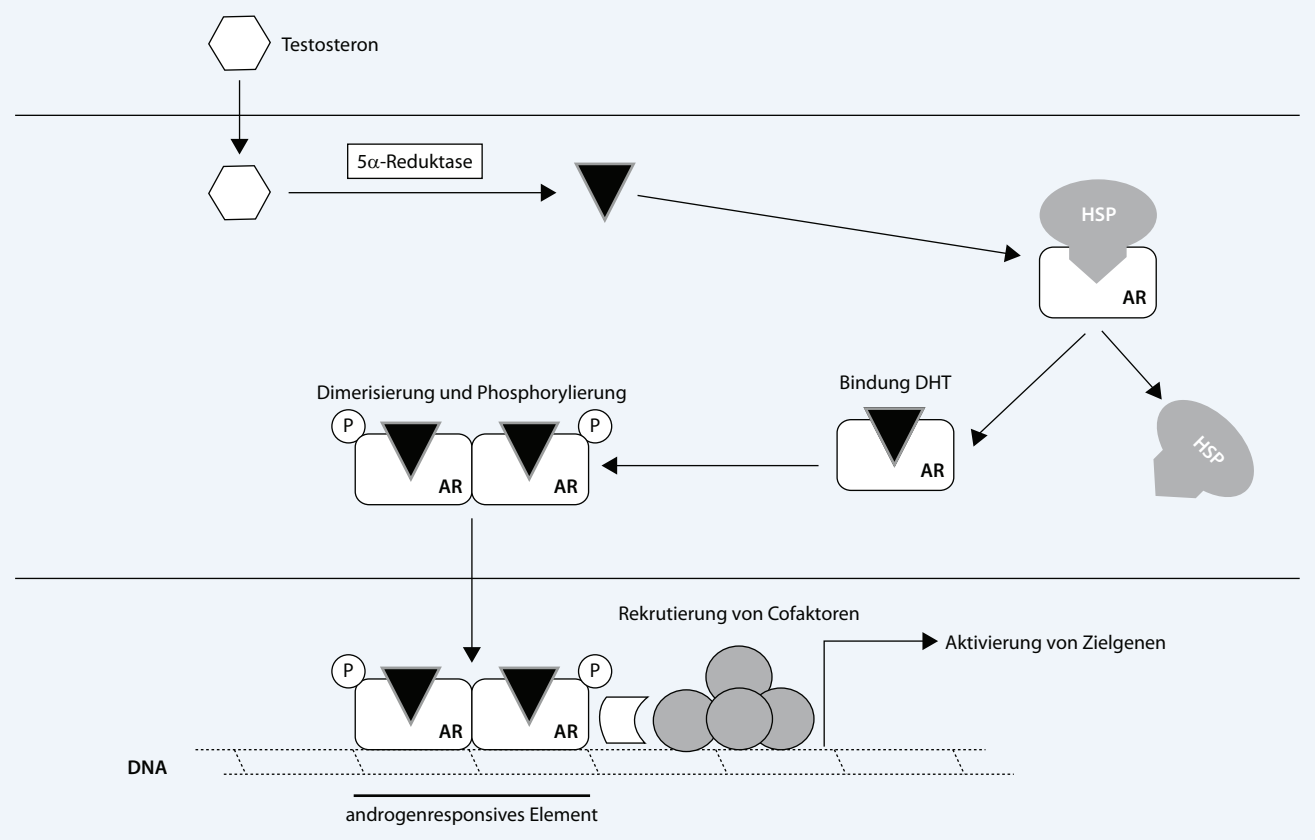

Abb. 2 ▲ Androgenrezeptorsignalweg: Testosteron gelangt in die Prostatakarzinomzelle und wird durch die 5α-Reduktase zu Dihydrotestosteron (DHT) umgewandelt. Die Bindung von DHT an den AR führt zur Phosphorylierung und Aktivierung des AR. In weiterer Folge dimerisiert der AR mit einem weiteren AR-Protein und wandert in den Zellkern. Ein AR-Kofaktorkomplex bindet an sog. „androgen response elements" (ARE) der DNS und aktiviert so androgenabhängige Zielgene

im Zytoplasma und ist an einen sog. Hitzeschockprotein- (HSP-)Komplex gebunden, der den Rezeptor stabilisiert und die Bindung der Androgene ermöglicht.

Die Bindung von Androgenen führt zu einer Dimerbildung des AR und begünstigt dessen Transport in den Zellkern (Translokation) [7]. Im Zellkern bindet das AR-Dimer an spezifische DNA-Sequenzen, den sog. „androgen response elements" (ARE) und aktiviert bzw. inhibiert über eine Reihe von Kofaktoren die Ablesung (Transkription) bestimmter Gene (genomische Androgenantwort [8], **Abb. 2**). Des Weiteren bestehen Hinweise, dass der AR die Transkription von Genen auch indirekt durch Bindung und Kooperation über andere Transkriptionsfaktoren vermitteln kann [9].

Rolle des AR in der gesunden Prostata und im PCA

Die gesunde Prostata entwickelt sich unter dem Einfluss von Androgenen und benötigt im adulten Zustand für ihre Funktion und ihr Wachstum einen kontinuierlichen Androgenstimulus. Die Androgenwirkung begünstigt jedoch auch die Entstehung bzw. das Wachstum von Prostatatumoren. Beim disseminierten fortgeschrit-

tenen PCA wird die Hormonabhängigkeit der Karzinomzellen durch Hormonentzug, wie z. B. nach Orchiektomie oder durch medikamentöse Applikation synthetischer Antiandrogene, therapeutisch genutzt. Bei einer solchen Therapie wird die androgene Signalkette unterbrochen, was zur Induktion der Apoptose hormonabhängiger Zellen führt. Leider kommt es während des Androgenentzugs bei der Mehrzahl der Patienten zur Bildung von hormonrefraktären PCA-Zellen, die eine der Hauptursachen für die hohe Mortalitätsrate beim fortgeschrittenen PCA darstellen. Eine wesentliche Frage ist daher, welche Mechanismen die Proliferation und das Überleben von PCA-Zellen bei extrem geringen Androgenspiegeln ermöglichen. Obwohl das humane PCA sowohl in seiner Entstehung als auch in seinem Verlauf sehr heterogen ist, scheint der AR in den meisten Fällen eine Schlüsselrolle zu spielen.

Der AR im hormonrefraktären PCA

Verlust des AR

Die Progression eines PCA vom hormonabhängigen zum hormonunabhängigen Wachstum wurde ursprünglich als eine klonale Selektion von AR-negativen Zellen infolge der Hormonablationstherapie erklärt. Diese Annahme basierte im Wesentlichen auf dem „Dunning-Ratten-Tumormodell" bei dem eine komplette Androgeninsensitivität mit einem Verlust des AR einherging. Die Theorie wurde durch die Beobachtung unterstützt, dass die ersten humanen Tumorzelllinien von fortgeschrittenen PCA selten einen AR exprimierten. In vivo wird beim Menschen allerdings nur bei ca. 30% der HRPCA ein Verlust des AR beobachtet, was die Relevanz des „Dunning-Ratten-Tumormodells" für das humane PCA in Frage stellt [2].

Strukturelle Veränderungen des AR

Polymorphismen

Im Bereich der Transaktivierungsdomäne des AR-Proteins befinden sich zwei unterschiedlich lange polymorphe Regionen mit einer variablen Anzahl der Aminosäuren Glutamin (Gln) bzw. Glycin (Gly)

Zusammenfassung · Abstract

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Die Rolle des Androgenrezeptors im hormonrefraktären Prostatakarzinom. Molekulare Grundlagen und experimentelle Therapieansätze

Zusammenfassung

Die Entstehung hormonrefraktärer Prostatakarzinomzellen während einer Hormonablationstherapie stellt die Hauptursache für den Tumorprogress und die hohe Mortalitätsrate des fortgeschrittenen Prostatakarzinoms (PCA) dar. Während in vitro der Verlust des Androgenrezeptors (AR) der vorherrschende Mechanismus für die Entwicklung einer Hormoninsensitivität ist, zeigen In-vivo-Untersuchungen, dass die Expression des AR in Zellen hormonrefraktärer PCA weitgehend erhalten bleibt oder sogar gesteigert ist. Die im Hinblick auf die in westlichen Industrienationen kontinuierlich steigende Anzahl an PCA durchgeführten molekularbiologischen bzw. zellbiologischen Untersuchungen führten zur Entdeckung einer Vielzahl neuer Faktoren/Mechanismen, die bei der Entstehung hormonrefraktärer PCA eine Rolle spielen. Diese Erkenntnisse sollten in weiterer Folge zu neuen Therapiekonzepten führen bzw. solche unterstützen.

Schlüsselwörter

Prostatakarzinom · Androgenrezeptor · Wachstumsfaktoren · Experimentelle Therapiekonzepte

Role of androgen receptors in hormone-refractory prostate cancer. Molecular basics and experimental therapy approaches

Abstract

The development of hormone-refractory prostate cancer cells is one of the major causes for the progression and high mortality rates in advanced prostate cancer (PCA). While the loss of the androgen receptor (AR) is the predominant mechanism for development of a hormone-insensitive disease in vitro, the first in vivo studies showed that the AR is still expressed or is even overexpressed in hormone-refractory PCA. In view of the increasing cases of PCA in the industrialized Western countries, a series of cell and molecular biological studies has led to the identification of various new factors and mechanisms that play a role during the development of hormone-refractory tumors. These findings should lead to the development of new therapeutic strategies.

Keywords

Prostate cancer · Androgen receptor · Growth factors · Experimental therapeutic approaches

Tab. 1 Regulation der AR-Transaktivierung und Expression durch Zytokine und Peptidwachstumsfaktoren

Wachstumsfaktor/Zytokin	AR-Transaktivierung	Inhibition der Transaktivierung durch Antiandrogene	AR-Protein	Literatur
bFGF	?	?	Vermindert	Cronauer et al. 1999 [88]
EGF	++	Ja	Vermindert	Culig et al. 1994 [31] Mizokami et al. 1992 [89] Henttu et al. 1993 [90]
HB-EGF	++	?	Vermindert	Adam et al. 2002 [91]
IGF-1	++	Ja	?	Culig et al. 1994 [31]
IL-1	?	?	Vermindert	Culig et al. 1998 [92]
IL-6	++	Ja	Erhöht	Hobisch et al. 1998 [93], Lin et al. 2001 [20]
KGF	+	Ja	?	Culig et al. 1994 [31]
Oncostatin M	++	Nein	?	Godoy-Tundidor et al. 2002 [94]
Wnt-3a	±	Ja	?	Salas et al. 2004 [51], Cronauer et al. 2005 [48]

Tab. 2 Signalwege und Mechanismen hormonrefraktären Wachstums in PCA-Zellen

Signalweg	Mechanismen	Effekt(e)
AR-unabhängig	Verlust des AR, AR-Mutation, Selektion von Tumorstammzellen	Tumor wächst androgenunabhängig
Prosmisker AR	AR-Mutationen	Tumor überlebt durch erweiterte Ligandenspezifität des AR
Hypersensitiver AR	AR-Amplifikation, AR-Mutationen, erhöhte 5α-Reduktaseaktivität	Tumor kann trotz niedrigster Androgenkonzentrationen überleben
Outlaw-AR	Aktivierung des AR durch Wachstumsfaktoren, Zytokine sowie Wachstumsfaktorrezeptoren	Tumor kann durch nicht-steroidale Aktivierung des AR überleben

sog. Polyglutamin bzw. Polyglycinrepeats (**Abb. 1**). So führen extrem lange Abschnitte von Glutaminrepeats im AR (Gln>40) zu einer bulbospinalen Muskelatrophie (SBMA, Kennedy-Syndrom), einer neurodegenerativen Erkrankung, welche neben einer Muskelatrophie mit einer Gynäkomastie, Hodenatropie sowie verminderter Libido und in Fertilität einhergeht [10]. Die Bedeutung der Glutaminrepeats für das PCA ist unklar, zumal deren Länge eine ethnische Variabilität aufweist (Gln-Mittelwerte: Afroamerikaner 18, Kaukasier 21, Asiaten 22). So deuteten frühere Studien darauf hin, dass Männer mit kurzen Glutaminrepeats (Gln<18) ein höheres Risiko haben, bereits in jüngeren Lebensjahren an einem PCA zu erkranken [11, 12, 13, 14, 15]. Ob die Länge der Glutaminrepeats im AR jedoch tatsächlich mit erhöhtem PCA-Risiko assoziiert ist, wird in jüngster Zeit kontrovers diskutiert [16].

Wie die Polyglutaminrepeats wird auch die Rolle kurzer Polyglycinrepeats in der Transaktivierungsdomäne des AR bei der Entstehung von PCA widersprüchlich diskutiert. So zeigten einige Untersuchungen ein erhöhtes PCA-Risiko bei Patienten mit Gly<14/16, andere Untersuchungen erzielten widersprüchliche Daten [15, 17, 18].

Mutationen

Bisher wurde eine Reihe von AR-Mutationen aus Tumorzellen von PCA-Patienten isoliert, jedoch ergaben mehrere Studien, dass die Häufigkeit von AR-Mutationen in PCA relativ gering ist [19, 20]. AR-Mutationen wurden in fast allen Domänen des AR beschrieben. Die am häufigsten betroffene Region ist dabei die Ligandenbindungsdomäne (**Abb. 1**). Im Gegensatz zu den Wildtypformen sind AR-Mutationen in der Ligandenbindungsdomäne oft in der Lage neben Androgenen wie schon erwähnt auch andere Steroidhormone zu binden [21]. Die drei am häufigsten beschriebenen Mutationen in diesem Bereich sind die AR-Mutanten T877A (Aminosäureaustausch Threonin > Alanin), T877S (Threonin > Serin) sowie H874Y (Histidin > Tyrosin) [22]. Wie erste Untersuchungen zeigten sind die AR-Mutanten T877A, T877S sowie H874Y neben Androgenen (Miboleron, DHT, Andiostendinon) auch stark durch Östradiol und Progesteron aktivierbar. Darüber hinaus sind T877A, T877S und H874Y ebenfalls durch die Antiandrogene Cyproteronacetat sowie Hydroxyflutamid aktivierbar. Des Weiteren wies H874Y eine deutlich erhöhte Transaktivierbarkeit durch Medroxyprogesteron als der Wildtyp-AR auf [22]. Androgenrezeptoren mit einem derartig erweiterten Ligandenspektrum werden auch als „promiske AR“ bezeichnet [23, 24]. Interessanterweise werden sowohl der Wildtyp-AR als auch die mutierten AR-Proteine T877A, T877S und T874Y durch Bicalutamid gehemmt. Die unterschiedliche Aktivierbarkeit der oben beschriebenen AR-Mutanten durch verschiedene Liganden wird durch eine, je nach Mutation unterschiedliche, Rekrutierung verschiedener Kofaktorkomplexe (s. unten) erklärt [22].

Zu den interessantesten (wenngleich auch eher seltenen) AR-Mutationen gehören Mutationen im Bereich der Hinge-Domäne. Veränderungen in diesem Bereich können zu einer verstärkten Transaktivierung des AR-Signals führen, obschon die DNA-Bindung des mutierten AR bzw. dessen Fähigkeit in den Kern zu wandern dramatisch vermindert ist [25]. Die Analyse von Mutationen in der Hinge-Domäne zeigt eindeutig, dass dieser relativ kleinen Domäne eine entschei-

dende Rolle bei der Regulation der AR-Funktion zukommt [25]. In den letzten Jahren wurden weitere AR-Mutationen in PCA-Zellen gefunden. Eine Liste der bisher beschriebenen AR-Mutationen findet sich in der „AR Mutation Database" (http://androgendb.mcgill.ca).

Regulation der Expression des AR-Proteins

Durch seine zentrale Stellung bei der Progression des PCA kommt der Regulation der Expression des AR-Proteins eine entscheidende Bedeutung zu. Sowohl eine Erhöhung als auch eine Erniedrigung der intrazellulären AR-Proteinkonzentration konnten in verschiedenen PCA nachgewiesen werden. In ca. 20–30% der fortgeschrittenen PCA liegt eine Amplifikation des AR-Gens vor [3]. Interessanterweise führt eine Amplifikation des AR-Gens nicht notwendigerweise zu einer Erhöhung des AR-Proteins [7]. So konnten Gregory et al. [26] eine erhöhte AR-Stabilität in hormonunabhängigen PCA-Zelllinien im Vergleich zu hormonabhängigen Zelllinien nachweisen, was somit auch ohne Genamplifikation zu einem Anstieg des intrazellulären AR-Proteins führt. Darüber hinaus konnte gezeigt werden, dass das Zytokin Interleukin-6 (IL-6), welches in fortgeschrittenen Prostatatumoren erhöht ist, die Expression des AR zu steigern vermag (**Tab. 1**).

Erste Untersuchungen an AR-positiven PCA-Zelllinien zeigten, dass sowohl eine Überexpression des Wildtyp-AR sowie mutierter Formen des AR es den PCA-Zellen erlaubt, selbst bei extrem geringen Androgenspiegeln, zu überleben und zu proliferieren [27]. Überaschenderweise wirkten typische AR-Antagonisten wie *Bicalutamid*, *Cyproteronacetat* sowie *Flutamid* in diesen androgenhypersensitiven Zellen als Agonisten [27]. Eine weitere, im Zusammenhang mit androgenhypersensitiven PCA-Zellen interessante Beobachtung ist die Tatsache, dass rekurrente fortgeschrittene PCA während eines Hormonentzugs die Fähigkeit entwickeln können, geringe Mengen an testikulären Androgene wie Testosteron und DHT aus zirkulierenden adrenalen Androgenen bzw. Cholesterol zu synthetisieren und somit hypersenitive Tumorzellen möglicherweise stimulieren [28].

Obwohl in der überwiegenden Mehrheit der hormonrefraktären Tumoren eine Heraufregulierung des AR zu beobachten ist, weisen ca. 20% der Tumoren eine Herunterregulierung des AR-Proteins auf. Neben dem Verlust des AR-Gens kann hier eine Methylierung des AR-Promoters zu einer Inhibition der AR-Expression/-Synthese führen [29]. Ein weiterer möglicher Mechanismus ist die Verminderung der AR-Proteinstabilität durch Peptidwachstumsfaktoren wie dem in der Prostata häufig vorkommenden basischen Fibroblastenwachstumsfaktor (bFGF, FGF-2) oder dem heparinbindenden epidermalen Wachstumsfaktor (HB-EGF). Beide Faktoren erhöhen das Wachstum von PCA-Zellen, obschon sie gleichzeitig den AR intrazellulär herunterregeln (**Tab. 1**). In vivo konnte darüber hinaus ein Anstieg von bFGF in Seren von PCA-Patienten während der Progression der Erkrankung beobachtet werden [30].

Regulation der AR-Funktion

Aktivierung des AR durch Wachstumsfaktoren, Zytokine und HER-2

Neben der Regulation der AR-Expression können Wachstumsfaktoren/Zytokine den AR unabhängig von Androgenen aktivieren. Die ligandenunabhängige Aktivierung des AR durch Wachstumsfaktoren/Zytokine sowie deren membrangebundenen Rezeptoren wird auch als „outlaw mechanism" bezeichnet. Outlaw-Aktivierungen des AR durch „Insulin like growth factor-1" (IGF-1), den epidermalen Wachstumsfaktor (EGF) sowie den Keratinozytenwachstumsfaktor (KGF) wurden erstmals von Culig et al. [31] beschrieben. Des Weiteren scheinen auch einige Zytokine [z. B. IL-6 sowie Oncostatin M (OSM)] bei der ligandenunabhängigen Aktivierung des AR eine Rolle zu spielen [32]. Es wird angenommen, dass diese Stimulation v. a. für die Differenzierung der Karzinomzellen von Bedeutung ist. Darüber hinaus kann eine Deregulation/Überexpression von Wachtumsfaktorrezeptoren wie dem HER-2 (human epidermal growth factor receptor 2) den AR ebenfalls in Abwesenheit von Androgenen aktivieren bzw. die Effekte niedriger Androgenkonzentrationen synergistisch verstärken [33, 34], (**Tab. 1**).

Die Mechanismen wie verschiedene Wachstumsfaktoren/Zytokine, die den AR aktivieren, sind nur unzureichend erforscht. Es wird angenommen, dass jene Signalwege, welche durch Wachstumsfaktoren aktiviert werden, oftmals über sog. mitogenaktivierbare Proteinkinasen (MAPK) AR-Proteine phosphorylieren und somit in weiterer Folge den AR aktivieren bzw. dessen Aktivierung begünstigen können. Dabei vermindern einige Wachtumsfaktoren wie EGF die AR-Protein-Konzentration, obwohl sie ein starkes AR-vermitteltes Signal induzieren (**Tab. 1**). Die meisten dieser „Outlaw-Aktivierungen" des AR können durch synthetische Antiandrogene wie Bicalutamid inhibiert werden (**Tab. 1**). Die einzige Ausnahme bildet hier das dem Interleukin verwandte OSM. In Anwesenheit von OSM wirkte das Antiandrogen Bicalutamid agonistisch d. h. als Aktivator des AR [32]. Inwiefern „Outlaw-Aktivierungen" des AR durch Wachstumsfaktoren in vivo eine Rolle spielen ist bisher weitgehend ungeklärt.

Modulation der AR-Funktion durch Koregulatoren

Neben der Rezeptorexpression sowie der jeweiligen Hormonkonzentration wird die Aktivität von Steroidrezeptoren entscheidend durch sog. Kofaktoren beeinflusst. Als Kofaktoren werden Proteine definiert, welche mit nukleären Rezeptoren interagieren und die Transaktivierung von Zielgenen verstärken (Koaktivatoren) bzw. vermindern (Korepressoren) ohne die basale Transkriptionsrate des Rezeptors zu beeinflussen [35]. Bisher wurde eine Vielzahl von Koregulatoren des AR beschrieben (http://www.androgendb.mcgill.ca), [8]. Im Wesentlichen gliedern sich Kofaktoren von Steroidrezeptoren entsprechend ihrer Funktion in zwei Gruppen:

- Komodulatoren, welche die Transkription regulieren, indem sie mit dem Steroidrezeptor im Promotorbereich der Zielgene interagieren (Typ 1) sowie
- jene, die die Ligandenbindung, die Rezeptorstabilität sowie Dimerisie-

rungsvorgänge am AR modulieren (Typ 2).

Es wird angenommen, dass eine Deregulation von Komodulatoren des AR den Verlauf der PCA-Erkrankung entscheidend beeinflussen kann. So wurde eine Überexpression der Kofaktoren TIF-2 (transcriptional intermediary factor 2) sowie SRC-1 (steroid receptor coactivator 1) bei einigen fortgeschrittenen, rekurrenten PCA beschrieben [26]. Des Weiteren konnte eine Steigerung der Expression des Kofaktors p300 während der Hormonablation nachgewiesen werden [36].

Experimentelle Studien zeigen, dass eine Dysregulation von Kofaktoren sowohl die Empfindlichkeit als auch die Spezifität von Wildtyp bzw. mutierten AR entscheidend beeinflussen kann. So verstärkt der Komodulator ARA-54 (AR-associated) die Transaktivierung normaler sowie mutierter Formen des AR in Anwesenheit von Androgenen [37]. ARA-54 vermag die Transaktivierung eines mutierten AR (AR T877R) auch in Gegenwart des Antiandrogens *Hydroxyflutamid* zu verstärken [37].

Zu den am besten untersuchten Koregulatoren des AR gehört β-Catenin. Unter normalen Bedingungen ist der größte Teil des zellulären β-Catenins im Bereich der Zellmembran an E-Cadherin, einem Protein das homotypische Zell-Zell-Verbindungen vermittelt, gebunden. Nur ein kleiner Teil des β-Catenins befindet sich frei im Zytoplasma. Ein Multiproteinkomplex, der die sog. Glykogensynthasekinase 3β (GSK-3β) enthält, führt zu einem kontinuierlichen Abbau des freien β-Catenins im Zytoplasma. Einer der zentralen Schritte des sog. kanonischen WNT-Signalweges ist die Regulation des β-Catenin-Abbaus. So führt eine Aktivierung des kanonischen WNT-Signalwegs zu einer Inhibition der GSK-3β und somit zu einer Verminderung des Abbaus von β-Catenin. Dieses „freie" β-Catenin akkumuliert intrazellulär und interagiert in weiterer Folge mit Transkriptionsfaktoren (z. B. TCF-4, T-cell-factor) im Zellkern und aktiviert dort eine Vielzahl von Zielgenen [38, 39].

Über die Funktion des kanonischen WNT-Signalweges im PCA ist bisher wenig bekannt. Am besten untersucht ist der WNT-Signalweg bei der Entstehung von Kolonkarzinomen. In fast allen Kolonkarzinomen ist eine Dysregulation bzw. Hyperaktivität des kanonischen WNT-Signalweges an der Entstehung des Tumors beteiligt [40]. So blockiert die Aktivierung des WNT-Signalwegs in Kolonzellen die Differenzierung und Apoptosefähigkeit reifender Kolonozyten und verleiht ihnen einen stammzellähnlichen Charakter mit der Fähigkeit zu uneingeschränkter Proliferation [41, 42]. In einem Mausmodell konnte nachgewiesen werden, dass eine stabile Überexpression von β-Catenin sowohl eine epitheliale Hyperplasie als auch eine intraepitheliale Neoplasie in der Prostata induzieren kann [43, 44].

Neben der Schlüsselfunktion bei der Vermittlung WNT-induzierter Signale vermag β-Catenin in Prostatazellen auch mit dem AR zu interagieren. In Anwesenheit von Androgenen bindet β-Catenin im Bereich der Hormonbindungsdomäne des AR und steigert hier die transkriptionellen Effekte von TIF-2 und führt somit zu einer Verstärkung androgeninduzierter Signale [45, 46, 47]. Neuere Untersuchungen in PCA-Zellinien deuten daraufhin, dass unter androgenablatierten Bedingungen ein WNT/β-Catenin induziertes Proliferationssignal in PCA-Zellen eher über den AR als über TCF vermittelt wird [48].

Modulation der AR-Funktion durch weitere Faktoren

Stickstoffmonoxid. Chronische Entzündungsprozesse erhöhen das Krebsrisiko sowie den Verlauf von Tumorerkrankungen. Ein in vielen Entzündungsherden dominierendes Enzym ist die sog. induzierbare Stickstoffmonoxidsynthase (iNOS, inducible nitric oxide synthase), die hohe Konzentrationen an Stickstoffmonoxid (NO) über längere Zeiträume synthetisieren kann. Neben akuten und chronischen Entzündungsreaktionen wurde eine erhöhte Synthese von NO durch iNOS bei einer Vielzahl von pathophysiologischen Prozessen wie Autoimmunerkrankungen sowie bei verschiedenen Karzinomen, so auch dem PCA nachgewiesen [49]. Erste In-vitro-Untersuchungen in AR-positiven PCA-Zellen zeigten, dass NO in der Lage ist AR-vermittelte Signale zu unterdrücken indem es zu einer Nitrosierung der Zinkfingerstrukturen des AR führt. Diese Modifikation vermindert in weiterer Folge die Fähigkeit des AR an die DNA zu binden und somit ein Signal zu induzieren [49].

Glykogensynthasekinase 3β (GSK-3β). Ein weiteres interessantes Enzym in hormonrefraktären PCA-Zellen ist die GSK-3β. Neben seiner Rolle beim kontrollierten Abbau von β-Catenin weisen neuere Untersuchungen daraufhin, dass die GSK-3β über die Fähigkeit verfügt, verschiedene Steroidrezeptoren direkt zu phosphorylieren und somit deren Funktion bzw. Stabilität entscheidend zu beeinflussen [50, 51, 52, 53].

Proteinkinase CK2. Die Proteinkinase CK2 (früher Caseinkinase 2) ist ein weiterer Faktor, der die androgene Signalvermittlung beeinflussen kann [54]. Es handelt sich dabei um eine ubiquitär vorkommende Serin-/Threoninkinase, die vielfältige Funktionen in der Regulation von Zellproliferation und Apoptose ausübt [55]. Eine Hemmung der CK2 durch synthetische sowie pflanzliche Inhibitoren (Tetrabromobenzotriazol, Antisense-DNS, Apigenin) hat sowohl in vitro als auch in tierexperimentellen Modellen eine Apoptoseinduktion gezeigt [56, 57]. Darüber hinaus wurde die CK2 kürzlich als prognostischer Marker für das PCA beschrieben, wobei die Lokalisation der katalytischen CK2α-Untereinheit signifikant mit dem Tumorgrad korreliert [58].

P53-Tumorsuppressorprotein. P53 ist ein Transkriptionsfaktor, der die Expression von Proteinen reguliert, die als Antwort auf DNA-Schädigungen in den Zellzyklus eingreifen bzw. an der DNA-Reparatur beteiligt sind. Bisher war bekannt, dass eine Überexpression von P53 die Signalübertragung bei einer Reihe von Steroidrezeptoren beeinflusst. So konnte auch in PCA Zellen gezeigt werden, dass P53 ein AR-vermitteltes Signal inhibiert [59]. Umso überraschender sind neuere Erkenntnisse, denen zufolge auch eine Verminderung der P53-Expression bzw. Funktion die androgene Signalkette in PCA-Zellen ebenfalls hemmt [59]. So deutet einiges darauf hin, dass ein aus-

balanciertes Verhältnis zwischen AR und P53 für ein androgenabhängiges Wachstum von PCA-Zellen notwendig ist. Störungen in diesem Gleichgewicht könnten eine entscheidende Rolle bei der Entwicklung androgeninsensitiver PCA-Zellen spielen [59].

Nicht-genomische Androgenantwort

Der AR wird normalerweise als induzierbarer Transkriptionsfaktor beschrieben, der im Zellkern an regulatorische DNA-Sequenzen bestimmter Gene bindet und deren Transkription reguliert (genomische Androgenantwort). Einige In-vitro-Arbeiten konnten jedoch zeigen, dass ein hormonaktivierter AR bereits im Zytoplasma verschiedene Signalwege (z. B. Wachstumsfaktorsignalwege) aktivieren kann (nicht genomische Androgenantwort, [60]).

Im Vordergrund dieser Untersuchungen steht die für viele Wachstumsfaktorsignalwege notwendige Aktivierung sog. mitogenaktivierbarer Proteinkinasen (MAPK) wie ERK1 und ERK2 [60]. Im Gegensatz zur genomischen Androgenantwort des AR, welche erst Stunden nach der Hormonbehandlung beobachtet werden kann, sind nicht-genomische Effekte wie die Phosphorylierung von MAPK bereits nach wenigen Minuten messbar [60]. Inwiefern eine nicht-genomische Androgenantwort in vivo eine Rolle spielt, ist jedoch bisher weitgehend ungeklärt. Interessanterweise konnte eine verstärkte nicht-genomische Aktivierung von ERK-Proteinen in künstlich in vitro gealterten PCA-Zellen beim Übergang von androgensensitiven zu androgenunabhängigen Prostatakarzinomellen beobachtet werden [61]. Da ERK-Proteine die Zellteilung in Gang setzen können, ist es möglich, dass ihre Aktivierung durch den AR zumindest z. T. für die Proliferation von Tumorzellen verantwortlich gemacht werden kann.

Lokale Erhöhung der Androgene

Eine weitere Möglichkeit für PCA-Zellen den medikamentösen Androgenentzug zu überleben ist die erhöhte lokale Produktion von Androgenen. Da die pharmakologische bzw. chirurgische Hormonablation das im Serum zirkulierende Testosteron und zunächst auch das Serum-PSA reduziert, wurde angenommen, dass die Gewebespiegel der Androgene ebenfalls im Rahmen einer Hormonablationstherapie sinken. Interessanterweise fallen bei einer Androgenablationstherapie die Serumtestosteronwerte um etwa 95% ab, während die Konzentration des Dihydrotestosteron (DHT) im prostatischen Gewebe hingegen nur um 60% abnimmt [24]. Die Tatsache, dass lokal erhöhte Konzentrationen an DHT die Tumorentstehung und den Verlauf beeinflussen, wird durch Studien in verschiedenen ethnischen Gruppen unterstützt. So variiert die Häufigkeit latenter PCA zwischen Japanern und US-Amerikanern nur geringfügig, während die Frequenz klinisch apparenter PCA in Japan 8-mal geringer ist. Im Gegensatz zu US-Amerikanern führt bei Asiaten eine häufige Änderung der Aminosäuresequenz des 5α-Reduktasegens Typ 2 (Aminosäureposition 89: Valin gegen Leucin) zu einer deutlichen Aktivitätsabnahme des Enzyms. Die verminderte Aktivität der 5α-Reduktase und der damit verbundenen reduzierten Umwandlung von Testosteron zu DHT wird als eine mögliche Erklärung für das niedrigere PCA-Risiko dieser Population herangezogen [62].

Verminderung der Apoptoserate

Ein wichtiger, bisher nicht diskutierter Weg zur Entwicklung von hormonrefraktärem bzw. androgenunabhängigem Tumorwachstums ist die Aktivierung antiapoptotischer Signalkaskaden wie die Heraufregulierung des antiapoptotisch wirksamen BCL2-Proteins. Die antiapoptotischen Mechanismen verhindern einerseits den Zelltod nach Androgendeprivation bzw. ermöglichen das Überleben von PCA-Zellen, welche den AR verloren oder herunterreguliert haben. Eine Aktivierung von antiapoptotischen Signalen, welche die Wirkung der Androgendeprivation umgeht, wird deshalb auch als „Bypassweg“ bezeichnet (**Tab. 2**), [24].

Tumorstammzellen

Bisher beruht das klinische Vorgehen bei der Krebserkrankung noch auf der Annahme, dass alle Zellen eines Tumors überwiegend die gleichen Eigenschaften besitzen und zu seinem Wachstum beitragen. Neue Erkenntnisse legen jedoch nahe, dass Karzinomzellen innerhalb eines Tumors (ähnlich wie normale Gewebe) hierarchisch gegliedert sind, d. h. mit einer pluripotenten Tumorstammzelle an der Spitze und davon abgeleiteten, differenzierteren Tumorzellen darunter. Es wird angenommen, dass eine geringe Anzahl dieser Tumorstammzellen das Tumorwachstum aufrecht erhält [63]. Unterstützt wird diese Theorie durch tierexperimentelle Beobachtungen an Mäusen, bei denen eine Androgendeprivation zu einer rapiden Involution der Prostata führt. Werden die ursprünglichen Hormonspiegel wieder hergestellt, so regeneriert sich das Organ fast vollständig. Es wird daher angenommen, dass das Organ sich unter der Kontrolle von Stammzellen wieder regeneriert. Diese Stammzellen gehören zu einer Unterpopulation der basalen Prostataepithelzellen (ca. 1%), die in Abwesenheit von Androgenen überleben können, deren Tochterzellen jedoch zu androgenabhängigen Zellen differenzieren [64].

Charakteristisch für Tumorstammzellen der Prostata ist die erhöhte Expression der Membranproteine CD44, Integrin $\alpha_2\beta_1$ sowie CD133 [65]. Während CD44 und Integrin $\alpha_2\beta_1$ eine wichtige Rolle bei Zell-Zell- bzw. Zell-Matrix-Kontakten spielen, ist die Funktion von CD133 in PCA-Zellen weitgehend ungeklärt. Legt man der Entstehung des PCA eine Tumorstammzelle zugrunde, so würden als Folge einer Androgenablation überwiegend androgeninsensitive Stammzellen bzw. Tumorstammzellen überleben. Infolge des durch die Ablation entstandenen Selektionsdruckes würden sich aus den Tumorstammzellen wieder „normale“ bzw. auf niedrige Androgenspiegel angepasste Tumorzellen differenzieren. Die Hypothese, der zufolge das Tumorwachstum durch eine geringe Anzahl von Tumorstammzellen, die sich sozusagen im Hintergrund verbergen, kontrolliert wird, wird als „lurker cell hypothesis“ (to lurk = auf der Lauer liegen) bezeichnet (**Tab. 2**).

Experimentelle Ansätze und therapeutische Zukunftsperspektiven

Entwicklung neuer Antiandrogene

Antiandrogene wie Flutamid besitzen im Vergleich zu DHT eine sehr niedrige Affinität zum AR und müssen deshalb in relativ hohen Dosierungen verabreicht werden [66]. Da reine Antiandrogene wie *Flutamid*, *Bicalutamid* sowie *Nilutamid* mit toxischen bzw. v. a. hepatotoxischen Nebenwirkungen in Verbindung gebracht werden, besteht ein zunehmendes Interesse an hochaffinen und somit effektiveren Antiandrogenen. Erste steroidale, auf DHT basierende synthetische Antiandrogene, die eine hohe Affinität zum AR besitzen, die Aktivierung des AR-Dimers jedoch verhindern (**Abb. 2**), befinden sich in Erprobung [66].

Inhibition der AR-Proteinexpression und Destabilisierung des AR-Proteins

Neben der Entwicklung neuer Antiandrogene bestehen erste experimentelle Ansätze zur Verminderung des AR-Proteins in PCA-Zellen durch Gentherapie. So wurde in vitro der AR in PCA-Zelllinien mittels sog. Antisense-DNS bzw. mittels siRNA (short interfering RNA) erfolgreich herunterreguliert [67, 68]. Ein System zum Einbringen der siRNA in Tumoren (microbubble-enhanced ultrasound delivery system) wurde von den oben genannten Autoren bereits in einem Tiermodell experimentell getestet [69]. Eine weitere Möglichkeit, den AR intrazellulär zu vermindern, ist die Destabilisierung des synthetisierten Proteins durch Inhibition der ihn stabilisierenden Hitzeschockproteine (HSP [70]). Ein Multichaperonkomplex, bestehend aus HSP70, HSP90 und Ko-Chaperonen garantiert die dreidimensionale Struktur ihrer sog. Klientenproteine (wie z. B. dem AR) und stabilisiert ihn im Zytoplasma (**Abb. 2**). Die Aktivität des Multichaperonkomplexes ist in vielen Tumorzellen besonders hoch [71]. So führt die Behandlung von PCA-Zellen mit dem HSP90-Inhibitor 17-Allylamino-17-demethoxy-Geldanamycin (17-AAG) zu einer dramatischen Verminderung der AR- sowie der HER-2 Proteinexpression in vitro [72]. Der HSP90-Inhibitor 17-AAG befindet sich bereits bei einigen Karzinomarten, so auch beim PCA in klinischer Erprobung [73].

Inhibition von Wachstumsfaktorsignalwegen

Wechselwirkungen zwischen Wachtumsfaktorsignalwegen und dem AR scheinen eine entscheidende Rolle bei der Entwicklung hormonrefraktärer Tumoren zu spielen und stehen im Fokus der Entwicklung neuer Therapieansätze. Durch eine gezielte Inhibition verschiedener membranständiger Wachstumsfaktorrezeptoren bzw. nachgeschalteter Signalproteine sind neben den eigentlichen Wachstumsfaktorsignalkaskaden auch die sog. Outlaw-Aktivierung des AR bzw. die nicht-genomische Androgenantwort betroffen. So führt z. B. eine Inhibition der membranständigen Rezeptortyrosinkinase HER-2 mittels monoklonaler Antikörper zu einer Verminderung androgeninduzierter Signale in vitro [74].

Inhibition von Rezeptortyrosinkinasen (RTK)

Die Familie der RTK umfasst eine Vielzahl von transmembranären Rezeptoren für sog. Peptidhormone. Binden Peptidhormone wie z. B. Insulin oder EGF an ihren jeweiligen Rezeptor, so wird dieser zunächst durch seine intrazelluläre Kinaseuntereinheit autophosphoryliert und der Rezeptor damit aktiviert. In weiterer Folge stimuliert der Rezeptor nachgeschaltete intrazelluläre Signalmoleküle. Die Inhibition von RTK-induzierten Signalen kann einerseits durch monoklonale Antikörper wie *Herceptin/Trastuzumab* erfolgen, welche extrazellulär an den Rezeptor binden und andererseits mittels Inhibitoren der Kinaseuntereinheit wie *Gefinitib/Iressa* bzw. PKI-166, die die Aktivität der RTK vermindern. Darüber hinaus wird die Möglichkeit der Inhibition RTK-nachgeschalteter Effektormoleküle wie dem RAS-Protein in PCA-Zellen untersucht [75, 76].

Obwohl erste In-vitro- sowie tierexperimentelle Studien die Wirksamkeit der RTK-Inhibitoren Gefinitib sowie Trastuzumab bei der Behandlung von PCA zeigen konnten [74, 77], verliefen erste klinische Phase-II-Studien enttäuschend. Sowohl mit Gefinitib als auch mit Trastuzumab konnte bisher keine nennenswerte Verbesserung bei der Therapie von HRPCA-Patienten erzielt werden [78, 79, 80]. Interessanter sind die vorläufigen Daten einer Studie, welche die Wirkung des TNFα-Inhibitors Infliximab bei 7 Patienten mit HRPCA und symptomatischen Knochenmetastasen untersuchte. Bei gutartigen entzündlichen Prozessen stimuliert TNFα die Expression von IL-6, welches den AR in PCA-Zellen aktivieren kann. Gegen Ende der Therapie (12 Tage, 5 mg/kg des monoklonalen Antikörpers Infliximab) war ein Patient schmerzfrei, bei den anderen 6 kam es während der Behandlung zu einer signifikanten Schmerzabnahme. Die Schmerzabnahme korrelierte mit einer Abnahme des IL-6-Spiegels. Relevante Nebenwirkungen wurden nicht beobachtet [81].

Kanonischer WNT-Signalweg

Ein weiterer neuer Ansatz bei AR-positiven PCA ist die Inhibition der GSK-3β, einem Vermittlermolekül des kanonischen WNT-Signalweges. Bisher wurde die Funktion der GSK-3β im PCA v. a. unter dem Aspekt der Modulation des freien β-Catenins untersucht. Es stellt sich jedoch zunehmend heraus, dass die GSK-3β ein multifunktionelles Enzym ist, welches über die Fähigkeit verfügt, verschiedenste Steroidrezeptoren wie den Glukokortikoidrezeptor (GR), den AR sowie den ER direkt zu phosphorylieren und deren Funktion bzw. Stabilität zu beeinflussen [50, 51, 52, 53, 82, 83].

Erste Untersuchungen in PCA-Zelllinien, die die Auswirkungen einer Modulation der GSK-3β-Expression bzw. -Funktion auf androgeninduzierte Signale analysierten, verliefen widersprüchlich. So konnten 2 Arbeitsgruppen unabhängig voneinander zeigen, dass eine Inhibition der GSK-3β die Fähigkeit des AR-Reportergens zu aktivieren, erhöhte [51, 84]. Im Gegensatz zu diesen Arbeiten zeigten 2 weitere Studien einen negativen Einfluss der GSK-3β auf den AR [83, 85]. Bisher unpublizierte Arbeiten der Autoren mit gegen GSK-3β gerichteter shRNA bzw. pharmakologischen Inhibitoren der GSK-3β zeigen ein sehr komplexes Bild. So sind,

unabhängig vom Wirkungsmechanismus und der Spezifität für GSK-3β, nicht alle pharmakologischen GSK-3β-Inhibitoren in der Lage AR-induzierte Signale zu inhibieren. Generell kann jedoch gesagt werden, dass jene GSK-3β-Inhibitoren, welche die AR-Transaktivierung vermindern auch in der Lage sind, die Expression des AR-Proteins zu reduzieren (Rinnab et al., unpublizierte Daten). Ob und inwiefern die Verminderung des AR-Proteins direkt durch die GSK-3β bzw. indirekt über die Induktion weiterer Enzyme gesteuert wird, wird derzeitig von unseren Arbeitsgruppen untersucht.

Inhibition der AR-Funktion durch NO-freisetzende Verbindungen

In-vitro-Untersuchungen mit NO-freisetzenden NSAID (non steroidal anti-inflammatory drugs) wie NO-Aspirin und NO-Iboprofen zeigten eine hohe Empfindlichkeit verschiedener Tumorzelllinien gegenüber diesen Substanzen. So zeigten PCA-Zelllinien eine deutliche Inhibition der Proliferation sowie eine Induktion von Apoptose nach der Behandlung mit NO-NSAID. Interessanterweise waren AR-positive PCA-Zellen empfindlicher gegenüber den NO-NSAID als AR-negative Zelllinien.

Untersuchungen an verschiedenen Steroidrezeptoren zeigten, dass NO in der Lage ist hormoninduzierte Signale zu unterdrücken. Die Verminderung hormoninduzierter Signale erfolgt durch eine reversible Nitrosierung der für die DNA-Bindung des Steroidrezeptors notwendigen Zinkfingerstrukturen [49, 86, 87]. Diese Modifikation vermindert in weiterer Folge die Fähigkeit des AR an die DNA zu binden und somit ein Signal zu induzieren [49]. Erste experimentelle Studien, inwiefern NO-freisetzende Verbindungen wie NO-NSAID bzw. NO-Steroide bei der Behandlung von HRPCA nutzbar gemacht werden können, werden derzeitig in unserer Arbeitsgruppe getestet (Cronauer et al., unpublizierte Daten).

Fazit für die Praxis

Aktuelle Studien decken eine Reihe von Faktoren auf, die zu einer Potenzierung der AR-Aktivität auch bei niedrigen Androgenspiegeln beitragen oder Antagonisten als Agonisten am AR wirken lassen. Sowohl die vermehrte Expression von AR als auch die Modulierung des AR durch andere Zellsignalproteine sind an der Entwicklung des HRPCA beteiligt. Hier ist in den nächsten Jahren ein besseres Verständnis des komplexen Wechselspiels zwischen Zellsignalwegen und dem AR sowie seiner Koaktivatoren und -repressoren gefragt, um therapeutische pharmakologische Substanzen entwickeln zu können, die das Problem der HRPCA in absehbarer Zeit vielleicht einmal lösen können.

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Inhibition of Glycogen Synthase Kinase-3 in Androgen-Responsive Prostate Cancer Cell Lines: Are GSK Inhibitors Therapeutically Useful?[1,2]

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Abstract

The glycogen synthase kinase 3 (GSK-3) is a serine/threonine kinase widely expressed in mammalian tissues. Initially identified by its ability to modulate glycogen synthesis, GSK-3 turned out to be a multifunctional enzyme, able to phosphorylate many proteins, including members of the steroid receptor superfamily. Although GSK-3 was shown to phosphorylate the androgen receptor (AR), its effects on AR transcriptional activity remain controversial. Analysis of short hairpin RNA (shRNA)–mediated downmodulation of GSK-3 proteins in prostate cancer cells showed a reduction in AR transcriptional activity and AR protein levels. Pharmacological GSK-3 inhibitors such as the maleimide SB216763 or the aminopyrazole GSK inhibitor XIII inhibited AR-dependent reporter gene activity and AR expression *in vitro*. Analysis of androgen-induced nuclear translocation of the AR was performed in PC3 cells transfected with pAR-t1EosFP coding for EosAR, a green fluorescent AR fusion protein. When grown in presence of androgens, EosAR was predominantly nuclear. Incubation with SB216763 before and after androgen treatment almost completely reduced nuclear EosAR. In contrast, the thiazole-containing urea compound AR-A014418 increased rather than decreased AR–expression/function. Although not all GSK inhibitors affected AR–stability/function, our observations suggest a potential new therapeutic application for some of these compounds in prostate cancer.

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Introduction

Prostate cancer (PCa) is very heterogeneous in its etiology and progression, but androgen signaling seems to be a key element in its development and progression. Two important androgens, testosterone and its metabolite dihydrotestosterone, mediate their effects through the androgen receptor (AR), a member of the steroid receptor superfamily. In the early stage of the disease, most patients respond favorably to androgen ablation therapy. However, a major problem of these treatments is the progression of androgen-dependent tumors to a hormone refractory state. Although the molecular basis for this phenomenon is largely unknown, studies of tumor specimens indicate that the AR signaling pathway is still functional in hormone refractory PCa [1]. Transcriptional activity of AR correlated with its phosphorylation status. Alteration of AR phosphorylation by protein kinases in PCa cells may provide a mechanism to circumvent the growth-inhibitory effects caused by androgen ablation.

The glycogen synthase kinase 3 (GSK-3) is a serine/threonine kinase widely expressed in mammalian tissues. In humans, two highly conserved isoforms, GSK-3α and GSK-3β, that share a 97% sequence homology within their kinase domains have been cloned [2]. Initially identified by the ability to modulate glycogen synthesis, GSK-3 turned out to be a multifunctional enzyme, able to phosphorylate many proteins, including members of the steroid receptor superfamily [3–5]. Although GSK-3 has been recently shown to

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[2]This article refers to supplementary materials, which are designated by Tables W1 and W2 and are available online at www.neoplasia.com.

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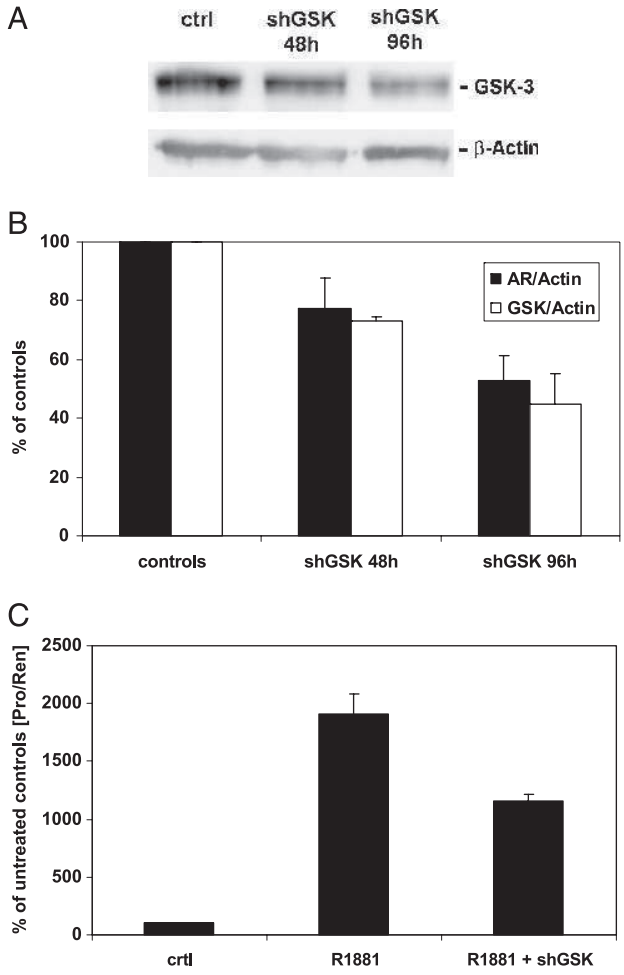

Figure 1. Silencing of GSK-3 in 22Rv1 cells. (A) GSK-3–directed shRNA diminishes GSK-3 protein expression: 22Rv1 cells were transfected with shRNA directed either against GSK-3 (pKD-GSK-3β-v1) or control shRNA (pKD-NegCon-v1). A total of 30 μg of protein extracts from the transfected cells was isolated after 48 and 96 hours and subsequently analyzed by Western blot. (B) Densitometric analysis of GSK-3 and AR-expression after GSK-3 silencing: 22Rv1 cells were transfected with shRNA directed against GSK-3 as described in the Materials and Methods section. Cell extracts were isolated after 48 and 96 hours, respectively. Expression of GSK-3 and AR-protein after GSK-3 silencing was quantified by densitometry using ChemiCapt/Bio1D software from Vilber Lormat. GSK and AR-protein expression were normalized to actin. Results represent the mean of three independently analyzed Western blots and are expressed in % of untreated controls which were set at 100% (% AR/Actin and % GSK/Actin). (C) Silencing of GSK-3 by shRNA diminishes AR-signaling: 22Rv1 were cotransfected with pKD-GSK-3-v1 or pKD-NegCon-v1 and with pGL3Eprob and pRL-tk-LUC. After transfection, cells were grown for 48 hours. Subsequently, cells were incubated with/without 5-nM R1881 for another 24 hours. After androgen treatment, AR-reporter gene activity was determined as described in the Materials and Methods section. Results are expressed as in % of untreated controls (which were set at 100%) ± SD.

phosphorylate AR, its effects on AR-mediated transcriptional activity are discussed controversially [3,6–8].

In this report, we screened different novel pharmacological GSK-3 inhibitors for their effect on androgen action in human AR-positive PCa cells. To elucidate the functional relationship of GSK-3 and AR and to specify the specific role of GSK-3 in the signaling processes related to androgen-dependent AR activation, we used different methodological approaches including RNA interference targeting GSK-3 and various specific chemical inhibitors of GSK-3 function. Here, we show that short hairpin RNA (shRNA)–mediated downmodulation of GSK-3β-protein in 22Rv1 PCa cells exhibit a reduction in AR transcriptional activity and in AR protein levels. Inhibition of GSK activity by chemical inhibitors like the maleimide, SB216763 or the aminopyrazole, GSK inhibitor XIII diminished AR-dependent reporter gene activity and AR expression in 22Rv1 and LNCaP cells. Analysis of androgen induced nuclear translocation of the AR was performed in PC3 cells transfected with an expression plasmid coding for a green fluorescent EosAR fusion protein. Interestingly, treatment of PC3 cells with SB216763 or GSK inhibitor XIII almost completely inhibited androgen-induced nuclear import of the EosAR. When cells were preincubated with synthetic androgen R1881, treatment with SB216763 and GSK inhibitor XIII decreased nuclear localization of EosAR dramatically within 30 minutes after GSK inhibition. To our knowledge, this is the first report to link the nuclear export of the AR to GSK-3 activity. In contrast to SB216763 and GSK inhibitor XIII, the thiazole-containing urea compound AR-A014418, another highly potent GSK-3 inhibitor, increased rather than decreased AR activity and AR expression.

The present results suggest that GSK-3 modulates AR-stability and different AR-functions. Although not all GSK inhibitors affected AR–stability/function, some of these compounds could play a role in new therapeutic approaches for the treatment of PCa.

Materials and Methods

Plasmids

T-cell factor (TCF) reporter plasmids pTopFlash (TOP) and pFopFlash (FOP), containing three copies of wild type or mutant TCF-binding sites upstream of a thymidine kinase minimal promoter driving a luciferase gene and the mammalian GSK-3 siRNA expression plasmid pKD-GSK-3β-v1 and its control plasmid pKD-NegCon-v1 were products of Upstate Biotechnology, Lake Placid, NY. The probasin promoter luciferase reporter plasmid (pGL3Eprob) containing a 267-bp fragment of the rat probasin gene promoter (base positions –256 to + 11) was a gift from Dr. Z. Culig, Innsbruck, Austria. pAR-t1EosFP, an expression vector coding for EosAR, a green fluorescent AR fusion protein, was a generous gift from Dr. J Wiedenmann, Ulm, Germany. pRL-tk-LUC *Renilla reniformis* luciferase reporter plasmid, used as an internal control for transfection efficiencies, was a product of Promega, Mannheim, Germany.

Table 1. GSK-3 *In Vitro* Kinase Assay.

GSK-3 Inhibitor	Concentration (μM)	% Inhibition
SB216763	2.5	33 ± 8
AR-A014418	2.5	95 ± 3
GSK inhibitor XIII	2.5	34 ± 14

Pharmacological GSK-3 inhibitors were mixed with GSK-3 kinase, GSK-3 substrate and ATP-solution as described in the Materials and Methods section. The mixture was subsequently incubated for 30 minutes at 30°C. Kinase reaction was stopped by an equal volume of Kinase-Glo reagent and luminescence was measured after 10 minutes.
% Inhibition was determined as: 100 × (luminescence of GSK-3 inhibitor – positive control)/(negative control – positive control).

Table 2. Effects of GSK-3 Inhibitors on AR and TCF/LEF Signaling and GSK-3 Activity *In Vitro*.

GSK-3β Inhibitor	Class	AR Signaling			TCF-4/LEF Signaling		Kinase Assay
		22Rv1	LNCaP	LNCaP-SSR	22Rv1 (AR+)	PC3 (AR–)	
AR-A014418	Thiazole urea	↑↑	↑↑	↑↑	↑	↑	↓↓
SB216763	Maleimide	↓↓	↓↓	↓	↑↑	↑	↓
GSK inhibitor XIII	Aminopyrazole	↓	↓	↓	↑	ND	↓
pKD-GSK-3β-v1	shRNA	↓	ND	ND	↑↑	ND	—

ND indicates not determined.

GSK-3β Inhibitors

GSK-3β inhibitor AR-A014418 (*N*-4-methoxybenzyl-*N*-5-nitro-1,3-thiazol-2-yl) and GSK-3β inhibitor XIII (5-methyl-1*H*-pyrazol-3-yl-2-phenylquinazolin-4-yl amine) were purchased from Calbiochem, Merck Biosciences, Darmstadt, Germany. SB216763 (3-(2,4-dichlorophenyl)-4-(1-methyl-1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione) was provided by Biomol GmbH, Hamburg, Germany.

Tissue Culture Materials

Androgen receptor–negative PCa cell lines PC3 and the AR receptor-positive LNCaP and 22Rv1 cell lines were purchased from the American Type Culture Collection, Manassas, VA. The AR-positive, hormone refractory PCa cell line LNCaP-SSR [9] was a gift from Dr. M. Burchardt, Hannover, Germany. RPMI 1640 was purchased from Gibco Invitrogen GmbH, Karlsruhe, Germany. Fetal bovine serum (FBS) was a product of Sigma, Taufkirchen, Germany. Steroid-free dextran-charcoal–treated FBS (FBSdcc) was from Biochrom AG, Berlin, Germany, and BioWest, Nuaille, France. Penicillin/streptomycin solution was a product of PAA Laboratories, Linz, Austria. The synthetic androgen methyltrienolone R1881 was provided by New England Nuclear, Dreieichenhain, Germany. All other chemicals were products of Sigma (Taufkirchen, Germany).

Tissue Culture

Cells were routinely maintained in RPMI 1640, supplemented with penicillin/streptomycin and 10% FBS (PC3, LNCaP, and 22Rv1) or 10% FBSdcc (LNCaP-SSR). During experiments, cells were cultured in RPMI 1640 with 2.5% FBSdcc and antibiotics in the presence/absence of the synthetic androgen methyltrienolone R1881.

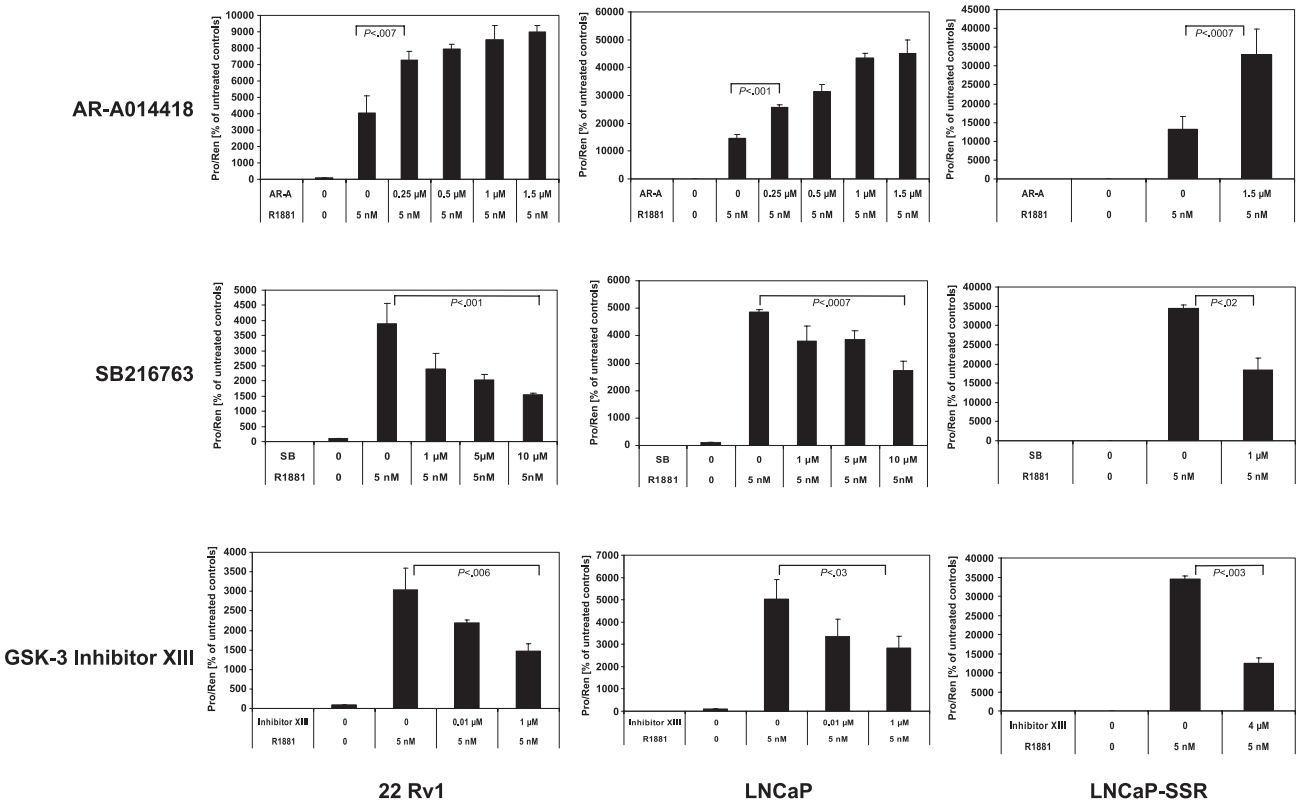

Figure 2. Modulation of AR transactivation by pharmacological GSK-3 inhibitors. AR-receptor–positive cell lines 22Rv1, LNCaP, and LNCaP-SSR were incubated with different pharmacological GSK-3 inhibitors in the presence/absence of 5-nM R1881 for 96 hours. AR transcriptional activity was measured using an AR-reporter gene assay. Results are expressed in % of untreated controls (without R1881, without inhibitors).

Reporter Gene Assays

Androgen receptor signaling and WNT/β-catenin signaling were analyzed by reporter gene assays as recently described [10]. In brief, cells were seeded in 24-well plates and allowed to grow overnight. Subsequently, cells were transiently cotransfected with pGL3Eprob and pRL-tk-LUC (AR signaling) or pTOPFlash/pFOPFlash and pRL-tk-LUC (WNT/β-catenin signaling) using FuGene6 (Roche Diagnostics Corporation, Basel, Switzerland). At 24 hours after transfection, cells were treated with different GSK-3β inhibitors in the presence/absence of the synthetic androgen R1881. Reporter activity was assessed after a 48-hour incubation period using the Dual-Luciferase Reporter Assay System (Promega). Luminescence was measured using Lumat LB 9507, Berthold Technologies, Bad Wildbad, Germany. Firefly luciferase activities of the reporter constructs were normalized by *Renilla* luciferase activities. All experiments were performed at least in triplicates.

GSK-3 In Vitro *Kinase Assay*

GSK-3 activity was determined using the Kinase-Glo Luminescent Kinase Assay Platform from Promega. Human recombinant GSK-3β-kinase was provided by Cell Signaling Technology (New England Biolabs GmbH, Frankfurt am Main, Germany), the GSK-3 substrate representing a part of the hydrophilic loop domain of presenilin 1 was a product of Calbiochem (Merck Biosciences). The Kinase-Glo Luminescent Kinase Assay was performed according to the manufacturer's instructions. In short, 10 μl of GSK-3β inhibitor (concentration, 10 μM) was mixed with 10 μl of GSK-3 kinase (20 ng), 10 μl of a 25-μM GSK-3β substrate solution, and 10 μl of a 1-μM ATP solution. This mixture was subsequently incubated for 30 minutes at 30°C. The kinase reaction was stopped by adding 40 μl of Kinase-Glo reagent. Luminescence was measured after 10 minutes using Lumat LB 9507 (Berthold Technologies).

Nuclear Translocation Assays

Nuclear translocation of AR was analyzed in PCa cells transfected with a green fluorescent AR fusion protein [11]. Therefore, PC3 prostate cancer cells were seeded into 24-well tissue culture plates and allowed to adhere overnight. Subsequently, cells were transfected with the green fluorescent reporter construct pAR-t1EosFP (0.25 μg/well) for 4 hours in RPMI and allowed to grow for another 24 hours in RPMI 1640 supplemented with 5% FBSdcc and antibiotics. Thereafter, cells were grown in RPMI supplemented with 2.5% FBSdcc in the presence/absence of 5-nM R1881 for 6 hours. For each well, 30 fluorescent cells were counted, and AR distribution was categorized as nuclear staining (ncl), cytoplasmatic staining (cyto), or nuclear and cytoplasmatic staining (ncl + cyto). Experiments were done in triplicates. Results are expressed in % ± SD.

Western Blot Analysis

Whole-cell extracts from transfection assays were used for Western blot analysis of the AR protein and GSK-3β protein. Protein concentration of the cell extracts was determined using the BCA–Protein Assay (Pierce, Rockford, IL). Protein extracts (20–30 μg) were electrophoresed in a 10% SDS-PAGE and electroblotted onto nitrocellulose membranes. Nonspecific binding was blocked by incubation with 5% nonfat dry milk in phosphate-buffered saline. Subsequently, membranes were incubated with a rabbit polyclonal AR antibody (1:800; Cell Signaling Technology) or with a mouse monoclonal GSK-3β antibody (1:5000; BD Biosciences Pharmigen, Becton Dickinson GmbH, Heidelberg, Germany). Incubation with mouse monoclonal antibody AC-15 to β-actin (1:9000; Biozol Diagnostica, Eching, Germany) served as a loading control. Immunoreactive bands were detected using peroxidase-labeled antirabbit or antimouse antibodies [1:1500, Goat Anti–Rabbit IgG peroxidase conjugate (Pierce) and 1:5000, Goat Anti–Mouse IgG peroxidase conjugate (Sigma)]. Androgen receptor bands were visualized either by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Buckinghamshire, UK) with a subsequent exposure to an ECL film or by direct detection using a ChemiSmart 500, Vilber Lormat, Marne-la-Vallee, France. Immunoreactive bands were quantified by desitometry using ChemiCapt/Bio1D software from Vilber Lormat.

Cell Proliferation Assay

Cellular proliferation was assessed by means of a colorimetric MTT assay measuring the reduction of tetrazolium salts to formazan

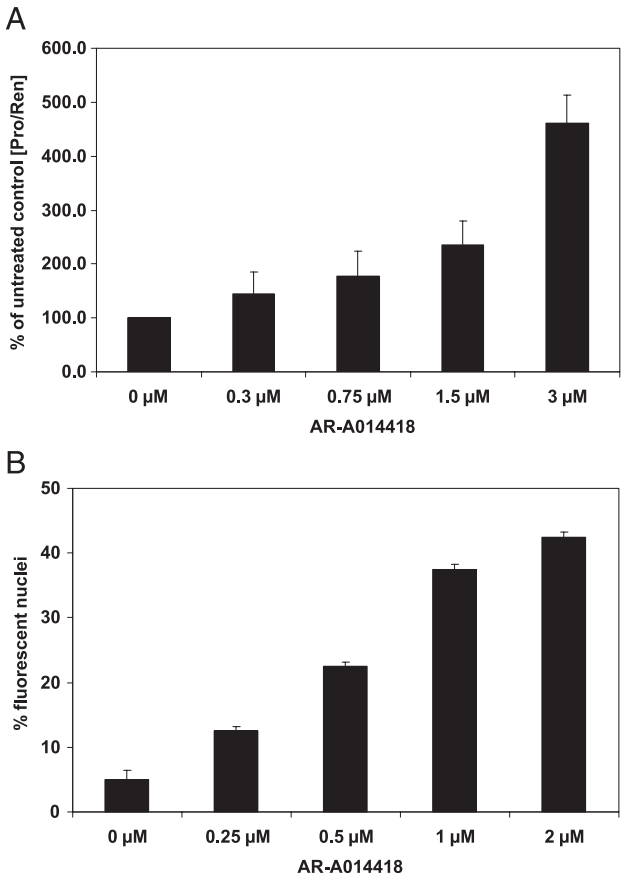

Figure 3. Androgenic properties of the GSK-3 inhibitor AR-A014418. (A) AR-A014418 activates the AR in absence of androgens: 22Rv1 cells were treated for 30 hours with AR-A014418 in the absence of androgens. AR-activity was determined using an AR-reporter gene assay as described in the Materials and Methods section. Results are expressed in % of untreated controls ± SD. (B) AR-A014418 stimulates nuclear translocation of the AR in the absence of androgens: PC3 cells grown in a 24-well plate, were transfected with pAR-t1EosFP, a plasmid coding for a green fluorescent AR-fusion protein. Subsequently, cells were treated with different amounts of AR-A014418 and incubated for 4 hours. After this, green fluorescent cells were counted using a fluorescence microscopy (30 cells/well). Results are expressed in % of green fluorescent nuclei ± SD.

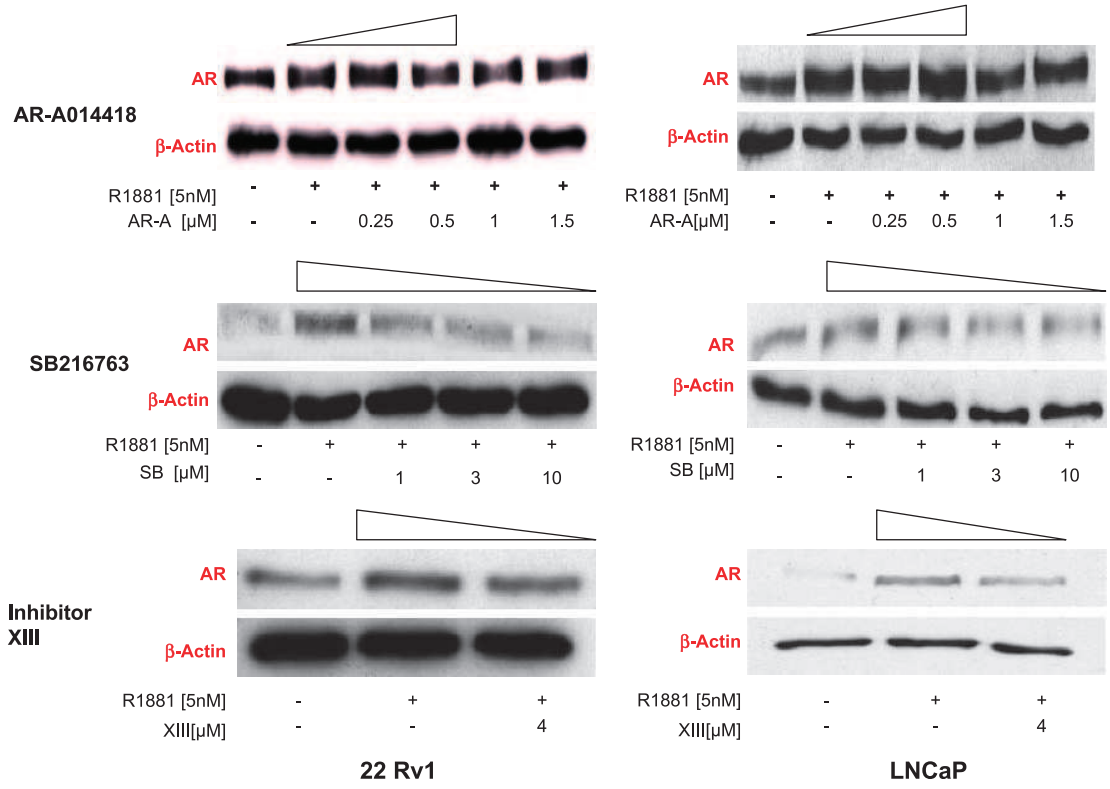

Figure 4. Effects of GSK-3 inhibitors on AR-protein expression. Cell extracts isolated from AR-reporter gene assays were analyzed by Western blot analysis as described in the Materials and Methods section. Inhibition of AR-transactivation by pharmacological inhibitors of GSK-3 was paralleled by a decrease in AR-protein expression.

derivatives by functional mitochondria. The assay was performed as recently described [11].

Statistical Analysis

Data are reported as mean ± SD. Analysis was performed with Student's *t* test with $P < .05$ considered as significant.

Results

GSK-3 Silencing Decreases AR Transcriptional Activity

Previous studies analyzing the effects of GSK-3 on AR signaling using lithium chloride yielded controversial results [6–8]. To determine whether AR transcriptional activity is dependent on GSK-3, we reduced cellular GSK-3 protein levels using a commercial shRNA directed against GSK-3 (pKD-GSK-3β-v1; Upstate). The efficiency to down-regulate GSK-3 was monitored by Western blot analysis (Figure 1, *A* and *B*). Treatment of 22Rv1 cells with GSK-3β–shRNA in the presence of R1881 (5 nM) significantly reduced transcriptional activity of the AR by 50% ($P < .0001$; Figure 1*C*). Inhibition of transcriptional activity was paralleled by a decrease in AR protein as shown by densitometry of three independent Western blots (Figure 1*B*).

Inhibition of GSK-3β Activity Modulates AR Transcriptional Activity

To determine the effects of a functional inhibition of GSK-3 on AR signaling, the AR-positive cells 22Rv1, LNCaP, and the hormone refractory LNCaP subline LNCaP-SSR were treated with different novel pharmacological inhibitors of GSK-3. The capability of the compounds to reduce the enzymatic activity of recombinant human GSK-3β *in vitro* was determined using a commercial luminescent kinase assay (Table 1). The efficiency of the inhibitors to down-regulate GSK-3 activity *in vivo* was determined indirectly using a β-catenin–inducible reporter gene assay. This approach is based on the principle that under normal conditions GSK-3 is a part of a multiprotein complex that targets free intracellular β-catenin for degradation. Inactivation of the enzyme leads to an increase in intracellular β-catenin that acts as a cofactor for the activation of TCF transcription factors. The increase of intracellular β-catenin was measured by a TCF-specific

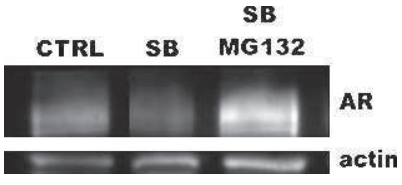

Figure 5. Downmodulation of AR by GSK-3 is due to proteasomal degradation. LNCaP cells were incubated with/without 5 μM of the proteasome inhibitor MG132 for 60 minutes, followed by a SB216763 treatment (final concentration, 1 μM) for another 60 minutes. Subsequently, R1881 (5 nM) was added to the medium, and the cells were grown for another 6 hours. Subsequently, cell extracts were analyzed by Western blot analysis.

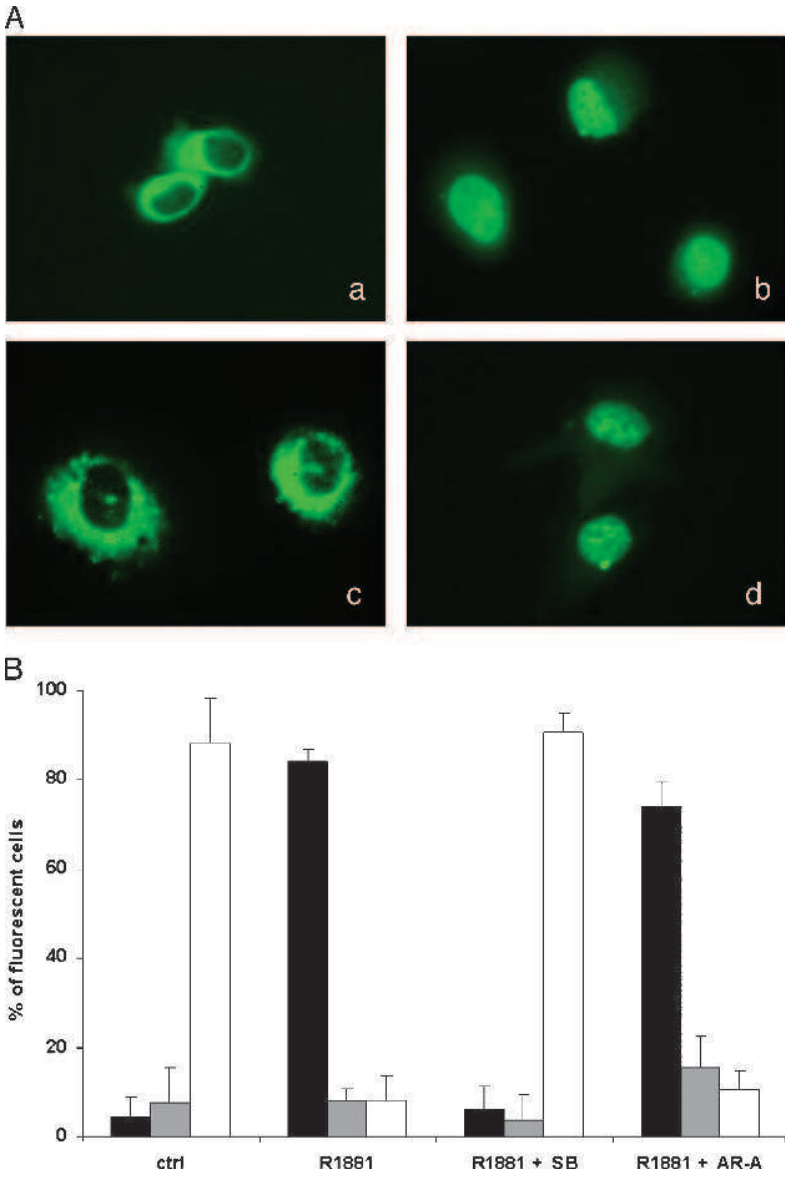

Figure 6. Effects of GSK-3 inhibitors on nuclear translocation of the EosAR. (A) Fluorescence microscopy of pAR-t1EosFP–transfected PC3 cells: (a) absence of androgens, (b) presence of androgens, (c) preincubation with SB216763 before androgen treatment, and (d) pretreatment with AR-A014418 before androgen treatment. (B) Percentage of cells exhibiting predominantly nuclear (black bars) or cytoplasmatic fluorescence (white bars) and even cytoplasmatic and nuclear fluorescence (gray bars).

reporter gene assay (pTOPFlash, pFOPFlash). All GSK-3 inhibitors SB216763, AR-A014418, GSK inhibitor XIII, and shGSK-3β were able to increase intracellular β-catenin, suggesting that they are active *in vivo* (Table 2).

Effects of the inhibitors on AR signaling were monitored by the probasin reporter gene assay (Figure 2). In the presence of the maleimide SB216763, AR signaling was dose-dependently inhibited in all three cell lines. The inhibitory effects were already measurable at a concentration of 1-μM SB216763 ranging from 21% in LNCaP to 38% in 22Rv1 and 46% in LNCaP-SSR (Figure 2). At a concentration of 10-μM SB216763, the transcriptional activity of AR was diminished in LNCaP and 22Rv1 by 40% and more than 60%, respectively (Figure 2). Similar results could be achieved in all three cell lines with the aminopyrazole GSK-3 inhibitor XIII (Figure 2).

Although the GSK-3 inhibitor, AR-A014418, a thiazole-containing urea compound, inhibited GSK-3 activity as did SB216763 or GSK inhibitor XIII (Tables 1 and 2), to our surprise, this inhibitor increased rather than decreased AR signaling in 22Rv1, LNCaP, and LNCaP-SSR in a dose-dependent manner. The most significant increase in AR signaling (>70% in 22Rv1 and LNCaP cells) was already measurable at a concentration of 0.25-μM AR-A014418.

Enhancement of AR Signaling By AR-A014418 Is Due to Androgenic Properties of the Compound

SB216763, GSK inhibitor XIII, and AR-A014418 inhibit GSK-3 function in an ATP-competitive manner (Tables 1 and 2). To explain the increase in AR signaling after AR-A014418 treatment, we tested

whether the compound itself exhibits androgenic properties. Therefore, 22Rv1 cells were treated with increasing concentrations of AR-A014418 in the absence of androgens. Indeed, AR-A014418 increased probasin reporter gene activity in a dose-dependent manner (Figure 3*A*). At an AR-A014418 concentration of 3 μM, androgen-dependent reporter gene activity was increased by >360% compared with the untreated controls (Figure 3*A*). Moreover, AR-A014418 was able to induce nuclear translocation of the green fluorescent fusion protein EosAR in PC3 (Figure 3*B*). In the absence of androgens, approximately 5% of the EosAR was nuclear, increasing to >20% in the presence of 0.5-μM AR-A014418 to more than 40% in presence of 2 μM of the compound. In contrast, the GSK-3 inhibitor SB216763 was unable to induce probasin reporter gene activity and nuclear import of the AR. We conclude that the positive effects of the thiazole-containing urea compound AR-A014418 on AR signaling is due, at least in part, to the androgenic properties of the compound.

Downmodulation of AR Signaling By GSK-3 Inhibitors Is Paralleled By a Degradation of AR protein

Inhibition of GSK-3 has recently been shown to influence estrogen receptor (ER) stability in MCF-7 cells [12]. Similar to the experiments with GSK-3–shRNA, all functional GSK-3 inhibitors, able to inhibit AR signaling, decreased AR proteins in 22Rv1, LNCaP, and LNCaP-SSR PCa cells (Figures 1 and 4). In contrast to SB216763 and GSK inhibitor XIII, AR-A014418 was unable to decrease but increased intracellular AR protein levels in all three cell lines (Table W1). Furthermore, this observation supports the assumption that AR-A014418 acts like an androgen, stabilizing the AR on binding. The down-regulation of AR protein by SB216763 could be rescued by MG-132, an inhibitor of proteasomal degradation, suggesting an important role of GSK-3 in the stabilization of the AR molecule (Figure 5; Table W2).

Inhibition of GSK-3 Activity By SB216763 and GSK inhibitor XIII Modulates Nuclear Translocation of the AR

GSK-3 has been shown to modulate nuclear translocation of various proteins and transcription factors; however, its effect has not been shown in members of the steroid receptor superfamily. In consequence, we tested the effects of a GSK-3 inhibition on the ability of the AR to translocate in the nucleus on androgenic stimulation. Therefore, we transfected the AR-negative PC3 cells with pAR-t1EosFP, a plasmid coding for an AR protein fused to the GFP-like Eos protein [11]. In the absence of androgens, the fusion protein EosAR was predominantly present in the cytoplasm (>90%; Figure 6). On androgenic stimulation, more than 90% of EosAR translocated into the nucleus within 30 minutes (Figure 6*B*). When PC3 cells were incubated with SB216763 before androgen treatment, nuclear import of EosAR was completely inhibited as can be seen by the >90% cytoplasmatic staining of EosAR (Figure 6*B*). Similar results could be obtained with GSK inhibitor XIII and GSK-3–shRNA, although the effects on nuclear translocation were less pronounced (data not shown). Most interestingly, SB216763 was able to induce a nuclear export of EosAR in PC3 cells pretreated with 5-nM R1881. The compound was able to reduce the predominant nuclear localization of EosAR from more than 90% in the presence of R1881 to basal levels (Figure 7). Export of the AR was already measurable after 30 minutes, suggesting an active transport of EosAR from the nucleus to the cytoplasm. In addition, similar experiments in 22Rv1 cells and replacement of pAR-t1EosFP by a GFP-tagged AR construct yielded identical results (data not shown). In contrast, AR-A014418 had no effect on the nuclear export of EosAR (Figure 7).

Effects of SB216763 and AR-A014418 on PCa Cell Growth

On the basis of the previous results, we examined the effects of the GSK-3 inhibitors SB216763 and AR-A014418 on PCa cell growth. When grown in RPMI 1640 supplemented with 2.5% FBS in the presence of SB216763 (10 μM), the proliferation of the AR-positive cell lines 22Rv1 and LNCaP was inhibited by 32% and 29%, respectively (Figure 8*A*). In contrast, the proliferation rate of the AR-negative PC3 cells remained almost unaffected under these conditions. These findings are in perfect agreement with a previous study of Mazor et al. [6], analyzing the effects of SB216763 on the proliferation of PCa cells. Most interestingly, the GSK-3 inhibitor AR-A014418 slightly enhanced the proliferation of the androgen-sensitive LNCaP cells (Figure 8*A*).

In the absence of androgens (2.5% FBSdcc), AR-A014418 stimulated the growth of androgen-responsive LNCaP cells in a dose-dependent manner, reaching its maximum at 1.5 μM of AR-A014418 (126 ± 8% compared with untreated controls which were set at 100%), whereas proliferation of the AR-negative PC3 cells remained unaffected under the same conditions (Figure 8*B*). We conclude that the positive effects of the thiazole-containing urea compound AR-A014418 on the proliferation of LNCaP are due to the androgenic properties of the compound.

Discussion

Prostate cancer is the most commonly diagnosed cancer and the second leading cause of male cancer deaths in the western world. Although PCa is very heterogeneous in its etiology, AR signaling seems to be a key element in its development and progression. As PCa cells depend on androgens for growth and survival antiandrogen therapy involving androgen depletion by orchiectomy, treatment with LHRH-analogues and blockade of AR with antiandrogens are standard treatments for patients experiencing advanced PCa. However, a major clinical problem of these treatments is the progression of androgen-dependent tumors to a hormone refractory state. Although the molecular

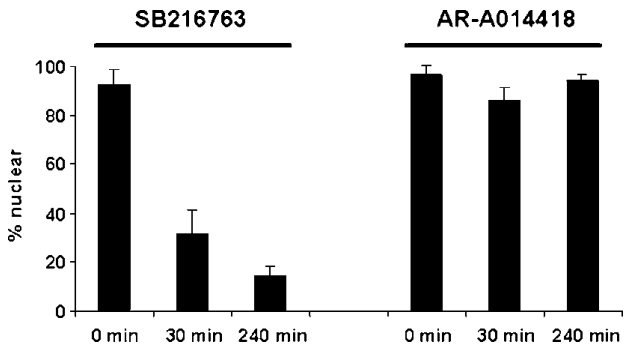

Figure 7. Inhibition of GSK-3 by SB216763 induces a rapid export of EosAR from the nucleus. PC3 cells grown in a 24-well plate were transfected with pAR-t1EosFP. Subsequently, cells were treated with 5-nM R1881 for 30 minutes resulting in a >90% of nuclear EosAR. Subsequently, cells were treated with/without 1-μM SB216763 or 1.5-μM AR-A014418 for 30 and 240 minutes. Fluorescent cells were counted using fluorescence microscopy (30 cells/well). Results are expressed in % of green fluorescent nuclei ± SD.

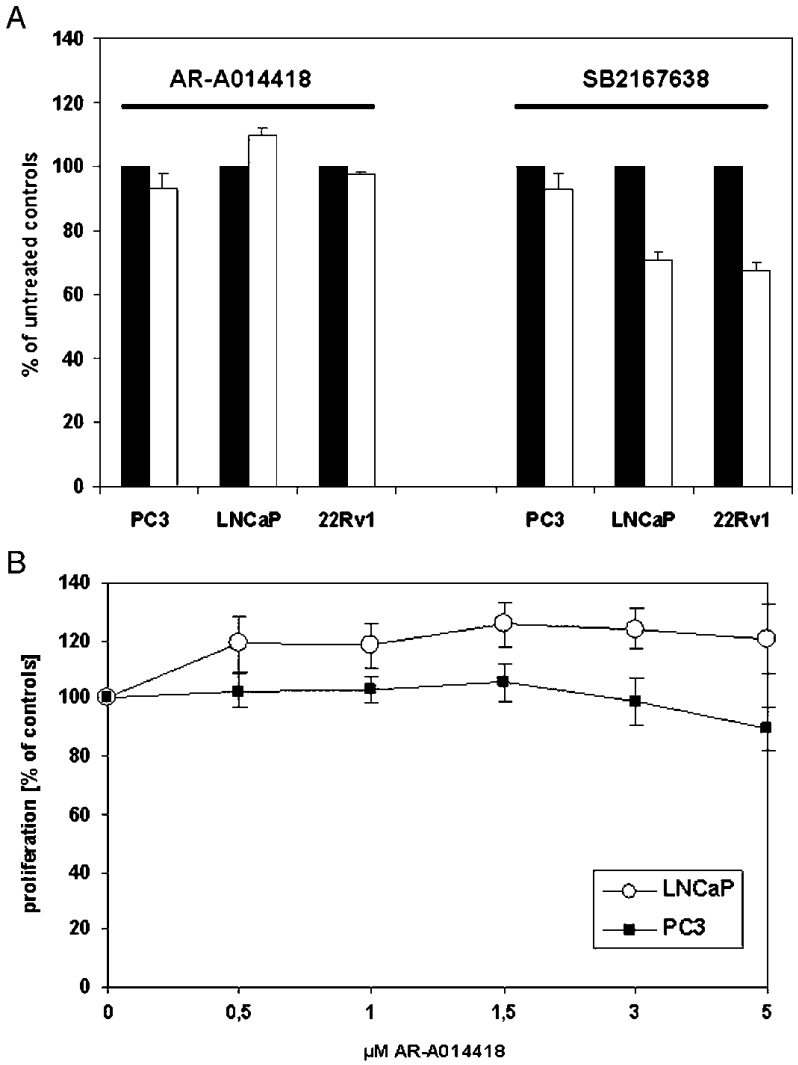

Figure 8. Effects of GSK-3 inhibitors on prostate cancer cell growth. (A) Modulation of cell growth by AR-A014418 and SB216763 in the AR-positive cell lines 22Rv1 and LNCaP: 22Rv1, LNCaP, and PC3 prostate cancer cells were seeded in 96-well plates and allowed to adhere overnight. Subsequently, medium was changed, and cells were grown for 72 hours in RPMI 1640, supplemented with 2.5% FBS and antibiotics in the presence/absence of 10-μM SB216763 or 1-μM AR-A014418. Proliferation was measured by means of a colorimetric MTT assay. Results are expressed in % of untreated controls, which were set at 100%. (B) AR-A014418 stimulates androgen-sensitive LNCaP cells in the absence of androgens: LNCaP (AR+) and PC3 (AR−) prostate cancer cells were seeded in 96-well plates and allowed to adhere overnight. Subsequently, medium was changed, and cells were grown for 96 hours in an androgen-free medium (RPMI 1640, supplemented with 2.5% FBSdcc and antibiotics) in the presence of increasing concentrations of AR-A014418. Proliferation of the cells was measured by means of a colorimetric MTT assay. Results are expressed in % of untreated controls.

basis for this phenomenon is largely unknown, studies of tumor specimens indicate that the AR signaling pathway is still functional in hormone refractory PCa.

The AR is a ligand-dependent transcription factor of the steroid receptor superfamily. Like all steroid receptors, the AR is structurally organized in three different domains: an amino-terminal transactivation domain, a well-conserved central DNA-binding domain with two zinc finger motifs required for DNA binding and a ligand-binding domain at the carboxy-terminal region of the protein. The DNA-binding domain and the ligand-binding domain are linked by the hinge region containing a nuclear export signal [13]. On androgen binding, the AR dimerizes and the AR–ligand complex translocates to the nucleus where it binds the DNA at specific androgen response elements to induce the transcription of androgen-dependent genes. Transcriptional activity of the AR is correlated with its phosphorylation status. Alteration of AR phosphorylation by protein kinases in PCa cells are thought to provide a mechanism enabling the cells to circumvent the growth-inhibitory effects caused by androgen ablation.

The GSK-3 is a serine/threonine kinase widely expressed in mammalian tissues. In humans, two highly homologous forms of GSK-3, GSK-3α and GSK-3β, that share a 97% sequence homology within their kinase domains have been cloned [2]. Initially identified by the ability to modulate glycogen synthesis, GSK-3 turned out to be a multifunctional enzyme, able to phosphorylate many proteins, including members of the steroid receptor superfamily [3–5]. Although GSK-3 has been recently shown to phosphorylate the AR, its effects on AR-mediated transcriptional activity are still discussed controversially. In 2004, four groups independently reported that transcriptional

activity of the AR is regulated by GSK-3. Whereas Salas et al. [3] and Wang et al. [7] reported that GSK inhibits AR transactivation, Mazor et al. [6] and Liao et al. [8] found that GSK-3 activity is required for androgen-stimulated gene expression and, in consequence, that inhibition of GSK-3 represses AR activity in PCa cells.

To elucidate the specific role of GSK-3 in the signaling processes related to androgen dependent AR activation we used RNA interference targeting GSK-3 and three specific chemical inhibitors of GSK-3 function. Depletion of GSK-3–protein by shRNA significantly down-regulated AR transcriptional activity in 22Rv1 cells grown in the presence of R1881 (Figure 1*C*). Moreover, depletion of GSK-3 by shRNA led to a downmodulation of AR protein (Figure 1*B*). Inhibition of GSK activity by chemical inhibitors such as the maleimide SB216763 or the aminopyrazole GSK inhibitor XIII diminished AR-dependent reporter gene activity in 22Rv1, LNCaP, and LNCaP-SSR cells (Figure 2). The reduction of AR transcriptional activity was paralleled by a proteasomal degradation of AR protein as shown for the cell lines 22Rv1 and LNCaP (Figures 4 and 5). These observations are in agreement with a previous study, reporting a downmodulation of AR protein using SB216763 [6]. Furthermore, the results are supported by previous studies on the ER showing that GSK-3 inhibition destabilizes ER protein expression, thereby modulating full transcriptional activity of the receptor [4,12].

Androgen receptor protein expression and function are critical determinants for the survival and proliferation of PCa cells. Indeed, PCa cells tolerate only a narrow range of AR expression and activity [14]. The AR expression is delicately regulated to provide an optimal balance between AR and its cofactors or repressors as has been shown for AR and p53 [15]. Besides its ability to function as an inducible transcription factor, there is experimental evidence from PCa cells that the AR is a licensing factor for DNA replication that must be degraded through a proteasome-dependent pathway during each cell cycle to allow reinitiation of DNA replication in the next cell cycle [16]. Modulation of intracellular AR protein/activity through GSK-3 makes the enzyme an interesting target for therapeutical applications. At present, there are numerous pharmacological GSK-3 inhibitors on the market but not all are able to inhibit AR signaling. As shown in our study, the GSK inhibitor SB216763 decreased AR-functions and AR expression. Downmodulation of AR-function was paralleled by an inhibition of PCa cell growth in the AR-positive cell lines 22Rv1 and LNCaP. In contrast, the highly specific GSK-3 inhibitor AR-A014418 increased rather than decreased AR activity and AR expression in PCa cells. Moreover, AR-A014418 was able to stimulate the proliferation of the AR-responsive LNCaP cells in the absence of androgens (Figure 8*B*). We presume that these effects are due to the androgenic properties of AR-A014418, showing that not all potent GSK-3 inhibitors are suitable *per se* to downmodulate AR activity.

Most interestingly, inhibition of GSK function by SB216763 and GSK-3 inhibitor XIII had a dramatic effect on the nuclear translocation of EosAR, a green fluorescent AR fusion protein (Figures 6 and 7) [11,17]. When PCa cells were treated with SB216763 or GSK inhibitor XIII 2 hours before an androgenic stimulus, the nuclear import of EosAR was almost completely inhibited (Figure 6). PCa cells cotransfected with GSK-3–shRNA and pAR-t1EosFP showed similar results although not that pronounced (data not shown). When PCa cells were preincubated with synthetic androgen R1881, treatment with SB216763 and GSK inhibitor XIII decreased nuclear localization of EosAR from 93% to 32% within 30 minutes after GSK inhibition. To our knowledge, this is the first report to link the nuclear export of the AR to GSK-3 activity.

The abundance of the AR inside the nucleus is tightly regulated by numerous factors that control nuclear import, binding to androgen response element, export and degradation of the AR import, export [11,18]. The fact how GSK-3 modulates nuclear translocation of the AR remains unknown. On the basis of *in silico* data Ser424, Ser514, and Ser650 have been identified as putative AR phosphorylation sites that could be phosphorylated by GSK-3 [19]. Among these, the position Ser650, located proximal to the DNA-binding domain in the hinge region, is the most interesting because it contains a nuclear export signal [13]. Whether Ser650, Ser424, and Ser514 are potential targets of GSK-3 to control either nuclear import/export or AR-stability remains to be elucidated.

In summary, there is experimental evidence that GSK-3 modulates AR-stability and AR-functions. Although not all GSK inhibitors down-regulated AR–stability/function, our observations suggest a potential new therapeutic application for some of these compounds in PCa.

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Table W1. Densitometric Analysis of AR-Protein Levels After Treatment with Pharmacological GSK-3 Inhibitors.

	AR-A014418			SB216763			GSK XIII	
	Control	0.25 μM	0.50 μM	Control	3 μM	5 μM	Control	4 μM
% Control [AR/Actin]	100	107 ± 11	118 ± 6	100	81 ± 8	70 ± 10	100	88 ± 3

LNCaP prostate cancer cells were grown for 48 hours in RPMI 1640, 10% FBSdcc, antibiotics, and 5-nM R1881 supplemented with different pharmacological GSK-3 inhibitors. Subsequently, cell extracts were analyzed by Western blot analysis as described in the Materials and Methods section. Expression of AR-protein levels was quantified by densitometry using ChemiCapt/Bio1D software. Results represent the mean of three different Western blots and are expressed in percent of R1881-treated cells, which were set at 100% (% AR/Actin ± % SD).

Table W2. Densitometric Analysis of AR Protein Levels After Treatment with MG-132 and SB216763.

	Control	SB216763	SB216763 + MG-132
% Control [AR/Actin]	100	61 ± 5	95 ± 7

LNCaP cells were incubated with/without 5 μM of the proteasome inhibitor MG-132 for 60 minutes, followed by a SB216763 treatment (final concentration 1 μM) for another 60 minutes. Subsequently, R1881 (5 nM) was added to the medium, and the cells were grown for another 6 hours. Subsequently, cell extracts were analyzed by Western blot analysis: Immunoreactive bands were quantified by densitometry using ChemiCapt/Bio1D software.
Results represent the mean of four different Western blot experiments. Increase/decrease of AR/Actin protein level is expressed in % R1881-treated controls, which were set at 100%.

ARTICLE
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Inhibition[Q1] of Glycogen Synthase Kinase-3β Promotes Nuclear Export of the Androgen Receptor Through a CRM1-Dependent Mechanism in Prostate Cancer Cell Lines

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ABSTRACT
The androgen receptor (AR) is a ligand-dependent transcription factor belonging to the steroid hormone receptor superfamily. Under normal conditions, in the absence of a ligand, the AR is localized to the cytoplasm and is actively transported into the nucleus upon binding of androgens. In advanced prostate cancer (PCa) cell lines, an increased sensitivity to dihydrotestosterone (DHT), enabling the cells to proliferate under sub-physiological levels of androgens, has been associated with increased stability and nuclear localization of the AR. There is experimental evidence that the glycogen synthase kinase-3β (GSK-3β), a multifunctional serine/threonine kinase is involved in estrogen and AR stability. As demonstrated in the following study by immunoprecipitation analysis, GSK-3β binds to the AR forming complexes in the cytoplasm and in the nucleus. Furthermore, inhibition of GSK-3β activity by pharmacological inhibitors like the maleimide SB216761, the chloromethyl-thienyl-ketone GSK-3 inhibitor VI or the aminopyrazol GSK-3 inhibitor XIII in cells grown in the presence of DHT triggered a rapid nuclear export of endogenous AR as well as of green fluorescent AR-EosFP. The nuclear export of AR following GSK-3β inhibition could be blocked by leptomycin B suggesting a CRM1-dependent export mechanism. This assumption is supported by the localization of a putative CRM1 binding site at the C-terminus of the AR protein. The results suggest that GSK-3β is an important element not only in AR stability but also significantly alters nuclear translocation of the AR, thereby modulating the androgenic response of human PCa cells. J. Cell. Biochem. 9999: 1–10, 2010.

KEY WORDS: GSK-3B; ANDROGEN RECEPTOR; NUCLEAR EXPORT; CRM1

Prostate cancer (PCa) is the most commonly diagnosed neoplasm in elderly man and a major cause of cancer-related deaths in the Western world. In their early stages, most PCas respond to androgen deprivation therapy achieved by surgical or medical castration reflecting the dependence of PCa cells on androgenic stimuli for growth and survival. Although this therapy is initially very effective, almost all tumors relapse to a hormone refractory stage where tumor cells can grow under castrate levels of androgens. The underlying mechanisms for hormone refractory prostate cancer (HRPCa) are poorly understood. In the past it was presumed that the expression of the androgen receptor (AR) is lost in the cells of advanced, hormone-refractory tumors. However, clinical studies demonstrated that the AR is rarely lost in human PCa specimens in vivo, even in those of therapy-refractory tumors [Hobisch et al., 1995]. Both, gene of function mutations as well as AR amplification have been reported in HRPCa. Moreover, it had also been proposed that peptide growth factor or cytokine signaling pathways could activate or enhance AR signaling, especially in the presence of low levels of circulating androgens [Cronauer et al., 2003]. In advanced PCa cell lines, an increased sensitivity to dihydrotestosterone (DHT), enabling cells to proliferate under sub-physiological levels of androgens, has been associated with increased stability and/or nuclear localization of the AR [Umekita et al., 1996; de Vere White et al., 1997; Gregory et al., 1998, 2001; Sweat et al., 1999]. In summary, current pre-clinical and clinical studies imply that the AR is not only expressed but is still transcriptionally active in the majority of recurrent PCa after failure of hormone deprivation therapy [Scher et al., 2004; Snoek et al., 2009].

The AR is a member of the nuclear receptor superfamily and acts as a ligand-dependent transcription factor. The AR protein consists of 919 amino acids and has a molecular weight of around 120 kDa.

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Structurally, the receptor is organized in four different domains: the N-terminal transactivation domain (NTD), the central DNA-binding domain (DBD) containing two zinc-finger motifs, the C-terminal ligand-binding domain (LBD) and the hinge-region which connects DBD and LBD [Cronauer et al., 2003]. In the absence of androgens, the AR is located in the cytoplasm associated with heat shock proteins (HSPs). Upon stimulation with androgens, HSPs dissociate, the AR is phosphorylated, dimerizes, and translocates to the nucleus, where it binds to androgen-responsive elements (AREs) on the DNA and thus regulates transcription of androgen-dependent genes [Feldman and Feldman, 2001].

One potential mechanism by which AR stability and function may be increased is by post-translational modification like phosphorylation. Indeed the AR has been shown to express potential phosphorylation sites at serine, threonine, and tyrosine residues [Gioeli et al., 2002; Wong et al., 2004; Guo et al., 2006]. Alteration of AR phosphorylation by protein kinases are thought to provide a mechanism enabling the cells to circumvent the inhibitory effects caused by androgen ablation therapy [McCall et al., 2008]. There is experimental evidence that the glycogen synthase kinase-3 (GSK-3) is involved in AR signaling [Liao et al., 2004; Mazor et al., 2004; Rinnab et al., 2008]. The GSK-3 is a ubiquitously expressed serine/threonine kinase with orthologs identified in nearly every eukaryontic species. In humans, two highly homologous forms of the enzyme, GSK-3α and GSK-3β have been isolated. These two isoforms were reported to exhibit about 97% sequence homology within their kinase domain [Woodgett, 1990]. Initially identified by its ability to phosphorylate and inactivate glycogen synthase, GSK-3 turned out to be a multifunctional enzyme, able to phosphorylate a broad range of proteins, including members of the steroid receptor superfamily [Rogatsky et al., 1998; Salas et al., 2004; Medunjanin et al., 2005]. As recently shown in vitro, GSK-3β is involved in the regulation of estrogen and AR stability and function [Mazor et al., 2004; Medunjanin et al., 2005; Grisouard et al., 2007; Rinnab et al., 2008].

The following study demonstrates that GSK-3β binds to the AR forming a complex in the cytoplasm that seems to be transported into the nucleus upon androgenic stimulation. Inhibition of GSK-3β activity by structurally and functionally different pharmacological inhibitors triggered a rapid nuclear export of the AR in androgen-stimulated PCa cells. In contrast to the relatively slow export of the AR from the nucleus after androgen withdrawal (>8 h) nuclear export triggered by GSK-3 inhibitors was already detectable after 30 min reaching its maximum after 4 h. The induction of the fast AR nuclear export was independent of the mechanism of action of the GSK-3 inhibitor used and could be blocked by leptomycin B (LMB), the latter suggesting a CRM1-dependent export mechanism. This assumption was furthermore supported by the localization of a CRM1 binding site at the carboxyterminal end of the AR. The results suggest that inhibition of GSK-3β helps target the AR for export from the nucleus and thereby modulates the androgenic response of human PCa cells.

MATERIALS AND METHODS

CHEMICALS

GSK-3β inhibitor SB216763 (3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione) was provided by Biomol GmbH (Hamburg, Germany). GSK-3β inhibitors VI (2-chloro-1-(4,5-dibromo-thiophen-2-yl)-ethanone) and XIII (5-methyl-1H-pyrazol-3-yl-2-phenylquinazolin-4-yl-amine) as well as phosphatase inhibitor cocktail set III were purchased from Calbiochem (Merck Biosciences, Darmstadt, Germany). Protease inhibitors Pevabloc, Aprotenin, Leupeptin, and Pepstatin were purchased from AppliChem GmbH (Darmstadt, Germany). LMB was a product of Alexis Biochemicals (Lörrach, Germany). DHT, ethylene-diamine-tetraacetic acid (EDTA), hydroxyethyl-piperazineethanesulfonic acid (HEPES), dithiotreitol (DTT), and Nonidet-P40 (NP-40) were provided by Sigma–Aldrich GmbH (Taufkirchen, Germany). Formalin and glycerol were products of Roth GmbH (Karlsruhe, Germany). Bovine serum albumin (BSA) was provided by PAA Laboratories GmbH (Pasching, Austria). All other chemicals, if not specified, were purchased from Sigma–Aldrich GmbH.

PLASMIDS

pGL3Eprob, a probasin promoter luciferase reporter plasmid, including a 267-bp fragment of the rat probasin gene promoter (base positions −256 to +11), was kindly provided by Dr. Zoran Culig (Innsbruck, Austria). *Renilla reniformis* luciferase reporter Plasmid (pRL-tk-LUC), used as an internal control for transfection efficiencies, was purchased from Promega (Mannheim, Germany). Plasmid pAR-t1EosFP, an expression vector coding for the green fluorescent AR-fusion protein AREos, was a gift from Dr. Jörg Wiedenmann (Ulm University, Germany).

ANTIBODIES

Mouse monoclonal AR antibody (AR 441), directed against the N-terminal domain of the AR, was purchased from Dako GmbH (Hamburg, Germany). Rabbit monoclonal antibody (Clone EP670Y) directed against the C-terminus of the AR was a product of Epitomics (Biomol). C-terminally directed AR antibody (AR C19, polyclonal rabbit antibody), rabbit polyclonal Lamin A antibody (H-102), and rabbit polyclonal antibody (H300) to CRM1 were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Anti-GSK-3β (27C10) was a product of Cell Signaling Technology (Frankfurt a. M., Germany). Monoclonal mouse antibodies to phosphoserine (clone 1C8) and phosphothreonine (clone 1E11) were products of NanoTools Antikoerpertechnik GmbH (Teningen, Germany). Mouse monoclonal antibody AC-15 to β-actin and goat anti-mouse IgG peroxidase conjugate were provided by Sigma–Aldrich GmbH. Horseradish peroxidase-labeled goat anti-rabbit antibody was a product of Pierce (Rockford, IL).

CELL CULTURE

AR-positive LNCaP and 22Rv1 PCa cell lines, and AR-negative PC3 cells were provided by the American Type Culture Collection (Manassas, VA). RPMI-1640, phosphate-buffered saline (PBS), and penicillin/streptomycin solution were products of PAA Laboratories GmbH. Fetal bovine serum (FBS) and steroid-free dextran-charcoal-treated FBS (FBSdcc) were obtained from BioWest (Nuaille, France). Cell culture plastic ware was purchased from Sarstedt (Nürmbrecht, Germany) or Nunc (Langenselbold, Germany).

22Rv1, LNCaP, and PC3 PCa cells were routinely cultured in RPMI-1640, supplemented with 1% penicillin/streptomycin (v/v),

and 10% FBS (v/v). During experiments, cells were maintained in RPMI-1640 with 2.5% FBSdcc (v/v) and antibiotics in the presence/absence of DHT and the respective inhibitors.

REPORTER GENE ASSAYS

Androgen receptor signaling was analyzed by an AR-specific reporter gene assay as recently described [Rinnab et al., 2008]. In brief, AR-positive 22Rv1 cells were transiently co-transfected with pGL3Eprob and pRL-tk-LUC using FuGene HD (Roche Diagnostics Corporation, Basel, Switzerland). Twenty-four hours after transfection, cells were treated with different GSK-3 inhibitors in the presence/absence of 10 nM DHT. Reporter gene activity was assessed after a 24-h incubation period using the Dual-Luciferase Reporter Assay [Rinnab et al., 2008].

NUCLEAR TRANSLOCATION ASSAYS

Nuclear translocation of AR was analyzed in PC3 cells transfected with pAR-t1EosFP coding for a green fluorescent AR-Eos-fusion protein (AR-EosFP) [Cronauer et al., 2007]. Twenty-four hours after transfection, cells were treated with/without leptomycin, DHT and GSK-3 inhibitors, SB216763, GSK-inhibitor VI or GSK-inhibitor XIII for 4 h. Subsequently, fluorescent cells were counted and AR distribution was categorized as nuclear staining, cytoplasmic staining or staining in both compartments. For reasons of clarity only the percentage of nuclear staining ± standard deviation (SD) is shown in the corresponding graphical figure.

PREPARATION OF NUCLEAR AND CYTOSOLIC EXTRACTS

Monolayers from cells grown in T25 flasks were lysed in 500 µl buffer A (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.05% NP-40, pH 7.9) supplemented with phosphatase and protease inhibitors. Subsequently cells were scraped thoroughly out of the flask and left on ice for 10 min. Cell lysates were centrifuged at 3,000*g* for 10 min at 4°C in a tabletop centrifuge. Supernatants containing the cytosolic fraction were collected and transferred into a separate tube. Residual pellets containing the nuclei were resuspended in 93.5 µl buffer B (5 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 26% glycerol (v/v), pH 7.9) and 6.5 µl of 4.6 M NaCl and vortexed for 30 s at highest settings. After incubation on ice for another 30 min, nuclear lysates were centrifuged at 15,000*g* for 30 min at 4°C to remove nuclear debris. Protein concentrations of cytosolic and nuclear extracts were determined using the BCA-Protein Assay (Pierce).

PREPARATION OF IMMUNOPRECIPITATES

GSK-3β/AR complexes of nuclear/cytoplasmatic fractions (containing 100 µg protein) were immunoprecipitated with 1 µg rabbit anti-GSK-3β (27C10) while gently rotating at 4°C for 1 h on a roller mixer (Stuart ST6, VWR International, Darmstadt, Germany). After that, 20 µl of Protein A/G-Sepharose (Santa Cruz Biotechnology) were added and the mixture was incubated overnight at 4°C while rotating. Subsequently, sepharose beads were washed three times with PBS and resuspended in 40 µl sample buffer (100 mM Tris–HCl, 3% SDS, 10% glycerol, 2% β-mercaptoethanol, 1 mg/ml bromphenol blue). Twenty microliters of this solution were subjected to Western blot analysis.

CRM1/AR complexes: Whole cell extracts of LNCaP and 22Rv1 cells (containing 100 µg of protein) were immunoprecipitated with 1 µg rabbit polyclonal CRM1-antibody (H300) according to the procedure described above.

Serine/threonine phosphorylation of the AR was analyzed in whole cell extracts of 22Rv1 cells. Therefore the cells were grown for 30 min in the presence/absence of 1 µM SB216763 followed by a 60 min incubation with/without 10 nM DHT. Whole cell extracts (containing 250 µg protein) were immunoprecipitated with 1 µg AR-C19 antibody, directed against the C-terminal end of the AR, as described above.

WESTERN BLOT ANALYSIS AND IMMUNODETECTION

Cell extracts or immunoprecipitates were electrophoresed in a 10% SDS–PAGE and electroblotted onto nitrocellulose membranes as recently described [Rinnab et al., 2008]. AR protein was detected using AR-specific antibodies recognizing either the N- or C-terminal epitopes of the AR (N-terminal: AR441 monoclonal mouse, 1:1,000 and C-terminal: AR rabbit monoclonal antibody, Clone EP670Y, 1:2,000). AR phosphorylation was analyzed in AR immunoprecipitates using monoclonal mouse antibody to phosphoserine (clone 1C8, 1:100) or monoclonal mouse antibody to phosphothreonine (clone 1E11, 1:100). Incubation with rabbit Lamin A antibody (H-102, 1:1,000) or mouse monoclonal antibody AC-15 to β-actin (1:20,000) served as a loading control for nuclear or cytoplasmatic fractions, respectively. Immunoreactive bands were visualized using horseradish peroxidase-labeled goat anti-rabbit antibody (1:2,000) or goat anti-mouse antibody (1:5,000) as recently described [Rinnab et al., 2008].

STATISTICAL ANALYSIS

All experiments were carried out at least three times. Data are reported as mean ± SD. Analysis was performed with Student's *t*-test with $P < 0.05$ considered as significant.

RESULTS

INTERACTION OF GSK-3β AND AR IN PROSTATE CANCER CELLS

GSK-3β has been found in the cytoplasm as well as in the nucleus of different cell lines [Salas et al., 2004; Medunjanin et al., 2005; Grisouard et al., 2007]. Moreover, in whole cell lysates from AR- and GSK-3-over-expressing cells, GSK-3β was shown to bind to the AR [Mazor et al., 2004]. In order to analyze whether GSK-3 binding to the AR depends on androgenic stimuli, cytoplasmic and nuclear fractions of AR-positive human PCa-cell lines LNCaP and 22Rv1 were subjected to co-immunoprecipitation (IP) analysis in the presence/absence of androgens. As shown in Figure 1, in the absence of androgens, co-immunoprecipitates of GSK-3β/AR were predominantly found in the cytoplasm although complex formation was also observed in the nucleus. In contrast, when adding DHT to the cells, the strongest GSK–AR signals could be detected in the nuclei of both cell lines whereas cytoplasmic co-immunoprecipitates were decreasing under these conditions (Fig. 1). In summary, these findings strongly suggest that GSK-3 and AR form complexes both in the cytoplasm as well as in the nucleus and that a possible co-transport of both proteins could not be excluded.

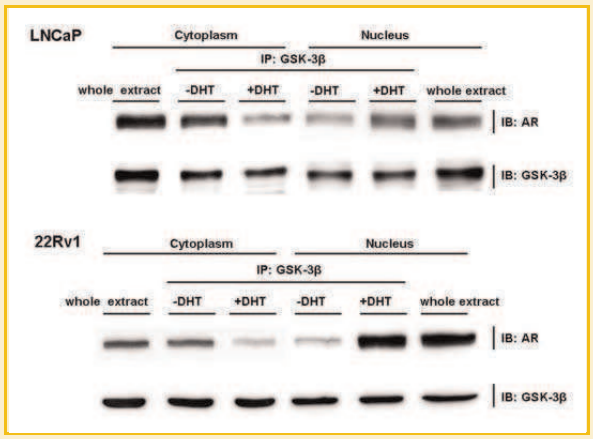

Fig. 1. GSK-3β binds to the AR. Analysis of AR–GSK-3 interaction by IP of nuclear and cytosolic cell fractions: LNCaP and 22Rv1 cells were grown in T25 flasks for 24 h in RPMI-1640 supplemented with 10% FBS (v/v) and antibiotics. After that, the medium was replaced by RPMI-1640, 10% FBSdcc (v/v), and antibiotics for another 24 h. Subsequently, cells were grown in the presence/absence of 10 nM DHT for 4 h. One hundred micrograms of nuclear/cytosolic protein extracts was subjected to immunoprecipitation (IP) using an antibody directed against GSK-3β followed by Western blot analysis with anti-AR and anti-GSK-3β antibodies (IB) as described in the Materials and Methods Section. IP: GSK-3 (anti-GSK-3β, 27C10), IB: AR (anti-AR, AR441) or GSK-3β (anti-GSK-3β, 27C10 serving as loading control for IP).

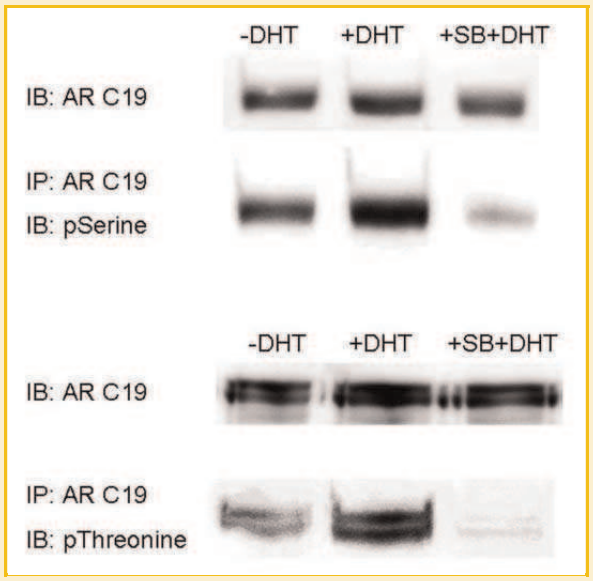

Fig. 2. SB216763 prevents serine and threonine phosphorylation of the AR 22Rv1 cells were cultured in T25 flasks for 24 h under standard conditions (RPMI-1640 supplemented with 10% FBS (v/v) and antibiotics). Thereafter medium was replaced and cells were grown for 24 h under steroid free conditions (RPMI-1640, 10% FBSdcc, antibiotics). Subsequently, cells were pre-incubated with/without 1 μM SB216763 30 min before addition of 10 nM DHT. Proteins were collected 1 h after addition of DHT. Tow hundred fifty micrograms of total protein extract was immunoprecipitated with 1 μg AR-C19 antibody. Immunoprecipitates were analyzed by monoclonal antibodies recognizing phosphoserine or phosphothreonine residues.

INHIBITION OF GSK-3 BY SB216763 PREVENTS SERINE/THREONINE PHOSPHORYLATION OF THE AR IN 22RV1 CELLS

The finding that GSK-3 interacts with the AR in the cytoplasm as well as in the nucleus prompted us to analyze the effects of GSK-3 on AR phosphorylation. As can be seen in Figure 2 treatment of the 22Rv1 cells with DHT induces AR phosphorylation on serine- and threonine sites. Pre-treatment of the cells with the GSK-3 inhibitor SB216763 leads to a dramatical inhibition of both serine and threonine phosphorylation of the AR in vivo, indicating that GSK-3 plays an important role in AR phosphorylation.

INHIBITION OF GSK-3 TRIGGERS A RAPID NUCLEAR EXPORT OF FULL LENGTH AR PROTEIN VIA A CRM1-DEPENDENT MECHANISM

As previously shown by our group, GSK-3 inhibitors SB216763 and XIII were able to induce a rapid nuclear export (>30 min) of transiently over-expressed AR-EosFP in DHT-treated PC3 cells [Rinnab et al., 2008]. The underlying mechanism for this phenomenon remained largely unknown. In order to determine, whether the nuclear export of the AR by GSK-3 inhibitors depends on the most prominent export receptor, the chromosome region maintenance 1 (CRM1), nuclear translocation experiments were repeated in the presence/absence of LMB (Fig. 3A). LMB prevents the interaction between a specific nuclear export signal (NES) of a protein and the CRM1 receptor and, therefore, inhibits nuclear export of target proteins. In PC3 cells transfected with AR-EosFP, the receptor protein was predominantly located in the cytoplasm in the absence of androgens. Upon androgenic stimulation, AR-EosFP was rapidly transported to the nucleus (Fig. 3B) reaching 94% nuclear staining after 30 min of treatment with DHT. The latter could be almost completely reversed by the addition of SB216763 (7% nuclear staining after a 240 min SB216763 treatment) (Fig. 3B). Most interestingly SB216763 triggered export of the AR could be prevented by pre-treatment with LMB (92% nuclear staining) (Fig. 3B), indicating a CRM1 dependent export mechanism. In order to ascertain that the rapid export of the AR is not due to unexpected side effects of SB216763 we inhibited the GSK-3 by the aminopyrazol GSK-3 inhibitor XIII. As seen in Figure 3B nuclear export of AR-EosFP could also be achieved when using GSK-3 inhibitor XIII instead of SB216763 (Fig. 3B).

SB216763 and GSK-3 inhibitor XIII operate in an ATP-competitive manner [Meijer et al., 2004]. In consequence we analyzed the effects of a specific, non-ATP competitive GSK-3β inhibitor, the chloromethyl-thienyl-ketone (GSK-3 inhibitor VI) on the nuclear export of the AR. Like SB216763 and XIII, the GSK-3 inhibitor VI was able to induce a rapid export of AR-EosFP from the nucleus (Fig. 3B). Moreover, the effects of GSK-3 inhibitor VI could be reversed by pre-incubation with LMB (Fig. 3B). The observation that structurally and mechanistically different GSK-3 inhibitors are able to induce an LMB reversible nuclear export of Eos-AR strongly supports the assumption that inhibition of GSK-3 activity is responsible for the nuclear export of AR by a CRM1 dependent mechanism (Fig. 3B). It is interesting to note that, in contrast to GSK-3β-inhibition, androgen withdrawal caused only a slow and LMB insensitive nuclear export of the AR suggesting two distinct export mechanisms for the AR in PCa cells (see Supplemental Data).

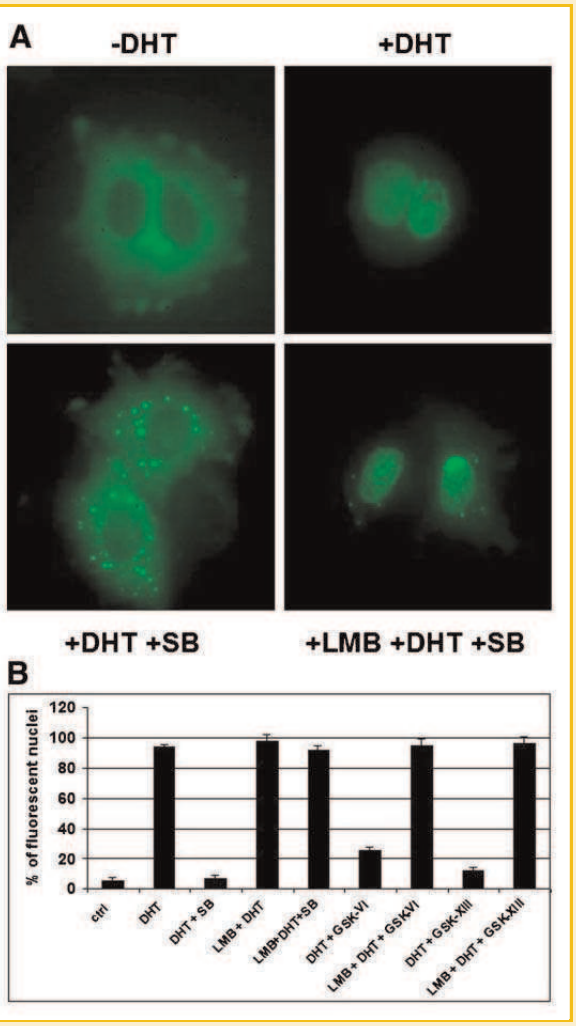

Fig. 3. Nuclear export of AR due to GSK-3 inhibition is mediated by a CRM1-dependent mechanism. AR-negative PC3 cells were seeded in 24-well plates and allowed to adhere overnight. Cells were transfected with pAR-t1EosFP and allowed to grow for another 24 h under steroid free conditions. Thereafter, cells were treated with/without 1 ng/μl LMB 30 min prior to the addition of 10 nM DHT. Thirty minutes later, GSK-3 inhibitors, SB216763, VI, or XIII, were added at 1 or 10 μM (GSK-VI and XIII). AR-EosFP localization was measured 4 h later by fluorescence microscopy. (A) Localization of AR Eos-FP. (B) Fluorescent cells were counted by fluorescent microscopy (30 cells/well). Results are expressed as a percent of green fluorescent nuclei ± SD.

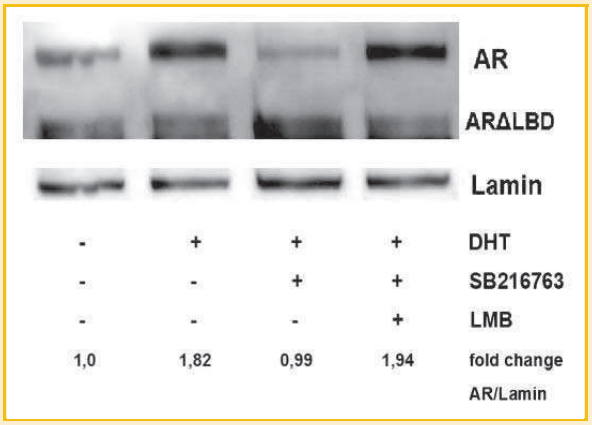

Fig. 4. Nuclear export of endogenous AR following GSK-3 inhibition is mediated by a CRM1 dependent mechanism. 22Rv1 cells were seeded in T25-flasks and allowed to grow overnight. Twenty-four hours after seeding, medium was changed to FBSdcc. Forty-eight hours after seeding, cells were pre-treated with 1 ng/μl LMB if necessary for 30 min, before addition of 10 nM DHT. After 30 min, SB216763 was added at 1 μM. Four hours later, cytoplasmic and nuclear fractions were collected as described in the Materials and Methods Section. Nuclear AR (full length AR and ARΔDBD) was detected by Western blotting with the AR441 antibody and Lamin A as a loading control. AR and Lamin A-signals were quantified and expressed as fold-change AR/Lamin.

The finding that different GSK-3 inhibitors are able to enhance a rapid nuclear export of transiently over-expressed AR-EosFP prompted us to ascertain that this effect is also true for endogenously expressed AR. Therefore, nuclei prepared from AR-positive 22Rv1 were studied by Western blot analysis (Fig. 4). 22Rv1 cells express two AR variants, a mutated full length AR with duplicated exon 3 (122 kDa) and a shortened 79 kDa AR variant termed ARΔLBD lacking the LBD in the C-terminal part of the AR [Tepper et al., 2002]. Whereas ARΔLBD exhibits constitutive nuclear localization and DNA binding, these functions remain androgen dependent in the full length AR [Tepper et al., 2002; Hartel et al., 2004].

Treatment with DHT dramatically increased nuclear full length AR in cells (AR/Lamin = 1.82) as compared to cells grown in the absence of DHT (AR/Lamin = 1.00). When adding SB216763 to DHT-treated cells, endogenous AREx3dup was rapidly exported from the nucleus reaching the basal levels of DHT-untreated cells (AR/Lamin = 0.99). SB216763 induced nuclear export of the AR could be completely inhibited by the CRM1 inhibitor LMB (AR/Lamin = 1.94) (Fig. 4). These results are in perfect agreement and support the observations previously made in PC3 cells transfected with the wild-type AR-EosFP (Fig. 3).

In contrast to the export of endogenous AR as well as transiently transfected AR-EosFP, the nuclear localization of ARΔLBD remained unaffected by GSK-3 inhibition (Fig. 4). Additional experiments with another truncated AR mutant (AR^{Q640X}) yielded similar results (data not shown). Based on these observations we hypothesized that the C-terminal end of the AR is necessary for a CRM1 mediated nuclear export of the receptor.

A CRM1-BINDING SITE IS LOCATED AT THE C-TERMINAL END OF THE AR

In order to be transported out from the nucleus via the CRM1/exportin-1 system, proteins must contain a specific leucine-rich NES that is recognized by the export receptor CRM1. To identify the functional domains of the AR that interact with CRM1, we performed an IP analysis on whole cell lysates from 22Rv1 cells (Fig. 5A). As seen above, 22Rv1 express a full length as well as an LBD-lacking AR isoform. When analyzing the CRM1 co-immunoprecipitates with AR antibodies directed against the C-terminal (EP670Y) or N-terminal (AR441) part of the AR only the full length AR was detectable (Fig. 5A). The absence of the ARΔLBD signal at

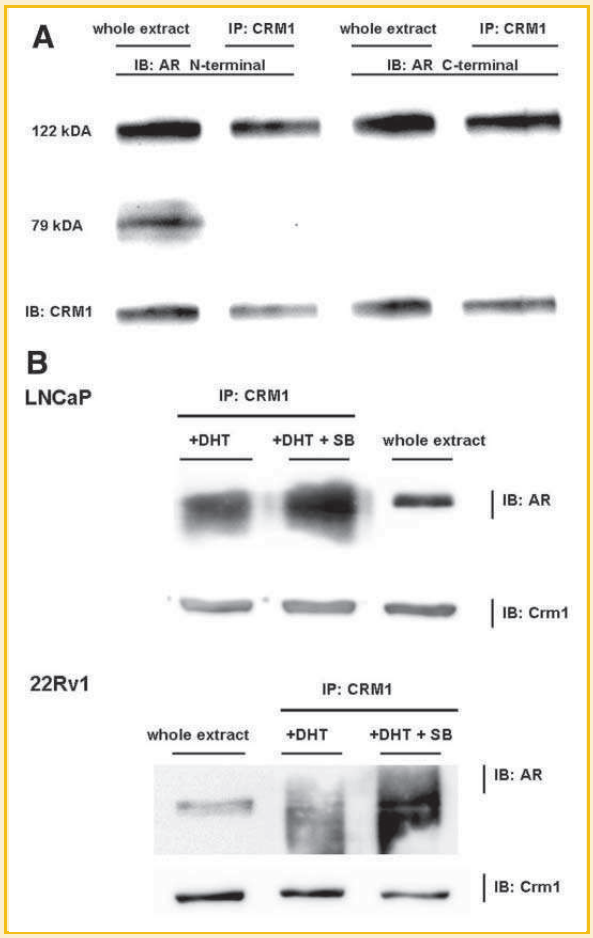

Fig. 5. AR binds to CRM1. A: 22Rv1 cells were cultured under normal conditions (10% FBS) in T25 flasks for 24 h and proteins were collected. One hundred micrograms of total protein extract was immunoprecipitated with 1 μg CRM1 antibody. Immunoprecipitates were analyzed by monoclonal antibodies AR441 and EP670Y recognizing the N-terminal or the C-terminal end of the AR protein. (B) LNCaP and 22Rv1 cells were cultured under normal conditions in T25 flasks for 24 h. Subsequently cells were incubated in the presence/absence of 1 μM SB216763. Proteins were collected 24 h later. One hundred micrograms of total protein extract was immunoprecipitated with 1 μg CRM1 antibody. Immunoprecipitates were analyzed using AR antibody (AR441).

79 kDa in the immunoprecipitates performed with an antibody directed against the N-terminus of the AR suggests that a putative CRM1 binding site is located at the C-terminal end of the AR. Moreover, based on the translocation experiments with AR^{Q640X} we conclude that the putative CRM1 binding site is located in the LBD between amino acid 640–919 of the AR.

INHIBITION OF GSK-3*B* INCREASES CRM1/AR INTERACTION

The finding that inhibition of GSK-3β induced a rapid CRM1 mediated nuclear export of the AR in PCa-cell lines prompted us to study the interaction between AR and CRM1 in the presence/absence of the GSK-3 inhibitor SB216763. As shown by co-IP analysis of whole cell lysates from LNCaP cells, there is an increased interaction of the AR with CRM1 in SB216763 treated cells in comparison to untreated control cells (Fig. 5B). In 22Rv1 cells the interaction between CRM1 and the AR following GSK-3-inhibition is even more pronounced than in LNCaP cells (Fig. 5B).

INHIBITION OF SB216763-INDUCED NUCLEAR EXPORT BY LMB RESTORES AR SIGNALING

As shown previously, inhibition of GSK-3 activity diminishes AR transactivation [Mazor et al., 2004; Rinnab et al., 2008]. To test whether the inhibition of AR transactivation by SB216763 could be rescued by LMB, 22Rv1 cells were subject to DHT treatment in an androgen-dependent reporter gene assay. Treatment of 22Rv1 cells with 10 nM DHT for 5 h almost doubled AR transactivation compared to untreated controls (Fig. 6). Transcriptional activity of AR was significantly lower in cells additionally treated with SB216763 (130% in DHT/SB216763-treated cells vs. 182% in DHT treated cells). The inhibition of AR transactivation by the GSK-3 inhibitor SB216763 could be completely reversed by pre-treatment with LMB (211% in DHT/SB216763/LMB-treated cells vs. 130% in DHT/SB216763-treated cells) (Fig. 6). Taken together this experiment clearly demonstrates that inhibition of the CRM1-mediated export machinery, leads to enhanced AR signaling in human PCa cells.

DISCUSSION

Prostate cancer is the prototype of an androgen-dependent tumor. In the absence of androgens, the AR predominantly resides in the cytoplasm. Upon ligand binding, the receptor rapidly shuttles into the nucleus. Conversely, in advanced HRPCa cell lines, the ability to

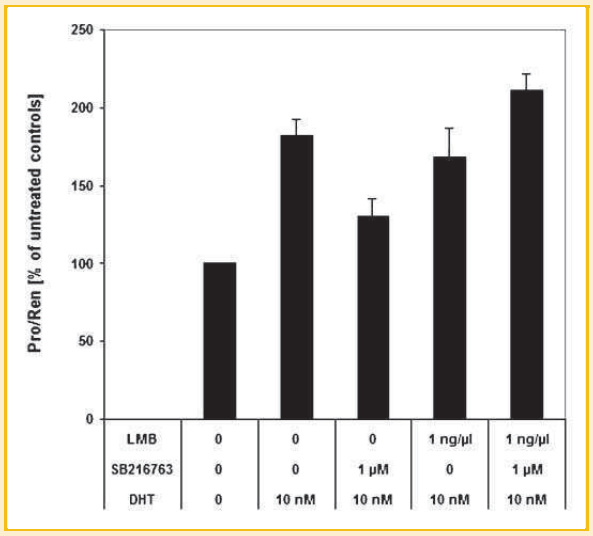

Fig. 6. LMB rescues AR-transcriptional activity in 22Rv1 treated with SB215673 AR-positive 22Rv1 cells were transfected with Probasin and Renilla luciferase reporter plasmids and incubated with 1 ng/μl CRM1-inhibitor LMB for 30 min, followed by DHT-treatment (10 nM) for another 30 min. Cells were treated with 1 μM SB216763 for 5 h. AR transcriptional activity was measured by a reporter gene assay as described in the Materials and Methods Section. Results are expressed as a percent of untreated controls ± SD.

proliferate under sub-physiological levels of androgen has been associated with increased nuclear localization of the AR even in the absence of androgens [Gregory et al., 1998, 2001; Saporita et al., 2007].

Various studies have offered insights into the nuclear import of the AR. A bipartite nuclear localization sequence (NLS) has been identified in the DNA binding domain and hinge region of the AR [Zhou et al., 1994]. This NLS utilizes the classical importin pathway for transport through the nuclear pore complex [Savory et al., 1999; Freedman and Yamamoto, 2004]. In addition, a less defined NLS is present in the LBD [Jenster et al., 1992; Poukka et al., 2000]. Although the mechanism responsible for the nuclear import of steroid receptors is well documented, the mechanisms of AR export remain largely unknown [Saporita et al., 2003; Shank et al., 2008; Nguyen et al., 2009]. In this paper, we demonstrate that GSK-3β, a multifunctional serine/threonin kinase interacts with the AR (Fig. 1) thereby phosphorylating the receptor on serine and threonine residues (Fig. 2). Most interestingly, pharmacological inhibition of GSK-3β activity targets the activated AR for a rapid export from the nucleus thereby down-regulating AR transactivation in human PCa cells (Figs. 3, 4, and 6). The nuclear export following GSK-3 inhibition could be inhibited by LMB, the latter suggesting a CRM1-dependent mechanism (Figs. 3 and 4).

As shown previously, there is a physical interaction of GSK-3β and the AR [Mazor et al., 2004; Salas et al., 2004]. Based on IP studies, we demonstrate that GSK-3β binds the AR both in the absence and presence of androgens and co-immunoprecipitates are detectable in the cytoplasm as well as in the nucleus (Fig. 1). As the detection of co-immunoprecipitates is very strong in the cytoplasm in the absence of androgens as well as in the nucleus upon androgenic stimulation (Fig. 1), a possible co-transport of AR and GSK-3 could be assumed. Although the precise binding site for GSK-3β on the AR has still to be determined, there is evidence that GSK-3β binds to the N-terminal as well as to the C-terminal part of the AR [Salas et al., 2004]. Inhibition of GSK-3 activity by the maleimide SB216763 triggered a rapid nuclear export of endogenous AR as well as transiently over-expressed green fluorescent AR-EosFP in 22Rv1 and PC3 cells (Figs. 3 and 4). Similar results were obtained when incubating AR-EosFP-expressing cells with the aminopyrazole GSK-3 inhibitor XIII and the chloromethyl-thienyl-ketone GSK-3 inhibitor VI (Fig. 3B). In all cases, the nuclear export of the AR protein induced by SB216763 and GSK-3 inhibitor XIII could be rescued by LMB (Fig. 3B). In contrast to the maleimide SB216763 that has been shown to inhibit the activating phosphorylation site tyrosine 216 of GSK-3 [Lochhead et al., 2006], the aminopyrazole moiety of GSK-3 inhibitor XIII blocks the ATP-binding site of GSK-3 [Pierce et al., 2005]. However, both inhibitors operate in an ATP-competitive manner [Meijer et al., 2004]. Therefore we used GSK-3 inhibitor VI, a non-ATP competitive GSK-3β whose inhibitory activity is related to the irreversible binding to a key cysteine residue present in the ATP-binding site of GSK-3, which is relevant for modulation of GSK-3 activity [Perez et al., 2009]. Like SB216763 and GSK-3 inhibitor XIII, GSK-3 inhibitor VI was able to dramatically enhance the nuclear export of AR-EosFP in PC-3 cells (Fig. 3B). This export could be inhibited by pre-incubation of the cells with LMB.

Other non-ATP dependent modes of GSK-3 inhibition like GSK-3 silencing or over-expression of kinase dead GSK-3 were taken into consideration, but were not used for the following reasons: (1) In contrast to the fast acting pharmacological GSK-3 inhibitors, GSK-3 silencing is too slow to analyze nuclear export in real time. An increase in cytoplasmic AR after long-term treatment with siRNA/shRNA does not allow to discriminate between a reduced nuclear import or an increased export of the receptor protein. (2) The use of dominant negative (kinase dead) GSK-3 constructs was not an option as over-expression of kinase dead or dominant negative GSK-3 constructs was shown to impede nuclear entry of GSK-3 [Meares and Jope, 2007]. Changes in the nuclear/cytoplasmic GSK-3 ratio may have unpredictable side effects on the AR localization as AR and GSK-3 interact and eventually co-migrate into the nucleus. In consequence we focused on the inhibition of GSK-3 by structurally and mechanistically different GSK-3 inhibitors. To summarize, all inhibitors triggered a rapid nuclear export of endogenous as well as transiently over-expressed AR-EosFP in 22Rv1 and PC3 cells. Nuclear export of the AR following GSK-3 inhibition was abrogated by LMB, an inhibitor of the CRM1/exportin-1 system (Figs. 3B and 4).

CRM1 is one of the best characterized receptors responsible for the nuclear export of proteins expressing a leucine-rich NES. In order to induce nuclear export, CRM1 has to build a trimeric complex with RanGTP and an NES-containing protein [Liu and DeFranco, 2000]. The interaction of CRM1 with LMB selectively abolishes its ability to bind NES in proteins [Kudo et al., 1999]. Although it is generally accepted that most steroid receptors lack a prototypical leucine-rich NES, there is experimental evidence that steroid receptors can be exported through a CRM1-dependent mechanism [Rimler et al., 2001, 2002; Itoh et al., 2002]. In agreement with these observations, an NES with limited homology to the canonical CRM1 site was recently found in the C-terminal end of the estrogen receptor [Lombardi et al., 2008].

The assumption that a functional putative CRM1 binding site is also located in the LBD of the AR is supported by co-IP analysis of CRM1 with different AR isoforms (Fig. 4A). 22Rv1 are known to express two AR isoforms, a mutated and extended AR (122 kDa) and a C-terminally deleted 79 kDa isoform termed ARΔLBD. When analyzing the CRM1 immunoprecipitates with antibodies recognizing the N/C-terminal end of the AR, only the full-length AR, but not the ARΔLBD isoform, was detectable (Fig. 4A). This observation clearly shows that CRM1 interacts with a sequence located in the C-terminal end of the AR. In addition, treatment of LNCaP as well as 22Rv1 cells with SB216763 enhanced CRM1-binding to the AR in both cell lines, although CRM1/AR binding was more pronounced in 22Rv1 as compared to LNCaP cells (Fig. 5B). This difference could be due to an inactivating Serine 9-phosphorylation of GSK-3 in LNCaP reported by several authors [Mazor et al., 2004; Salas et al., 2004]. However, GSK-3 activity is not fully inhibited in LNCaP. There is experimental evidence that the extent of GSK-Ser9 phosphorylation in LNCaP can still be regulated leading to a modulation of various physiological functions [Liao et al., 2003; Kumar et al., 2004; Liao, 2004[Q4]; Liu et al., 2008; Rinnab et al., 2008, Supplemental Data].

Based on these results we postulate that a functional GSK-3 dependent CRM1-binding site is located in the LBD of the AR.

Although we did not identify the exact localization of this putative NES^{CRM1} on the AR, it functionally differs from the leptomycin-insensitive NES^{AR}, recently identified by Saporita et al. (2003). Under these circumstances, it is interesting to note that androgen withdrawal, in contrast to GSK-3β inhibition, causes a slow and leptomycin-insensitive nuclear export of the AR (Supplemental Data) [Poukka et al., 2000; Tyagi et al., 2000; Saporita et al., 2003]. A similar phenomenom was described for the glucocorticoid receptor (GR). As shown by Itoh et al. [2003][Q5], UV-induced nuclear export of the GR is dependent on the activity of the c-JUN N-terminal kinase (JNK), a stress inducible mitogen activated protein kinase. The UV/JNK-induced rapid export of the GR can be inhibited by LMB, indicating that CRM1 plays a pivotal role in stress-induced nuclear export of the GR. In contrast to these findings, nuclear export of the GR induced by dexamethasone-withdrawal is relatively slow and remains unaffected by LMB treatment [Itoh et al., 2002]. In summary the AR as well as the GR studies suggest that steroid receptors may be targeted to different export systems depending on the physiological conditions. Therefore, the mechanism by which GSK-3β interferes with the CRM1 machinery needs to be elucidated.

AR transcriptional function depends on the nuclear localization of the receptor. In HRPCa cells, a considerable great amount of AR is located in the cell nucleus even upon androgen ablation [Gregory et al., 1998, 2001]. The predominant nuclear localization of AR in HRPCa requires mechanisms directing ligand-independent nuclear import and/or means of retaining AR in the nucleus in the absence of androgens. As shown in this study, inhibition of GSK-3β activity leads to a rapid CRM1-dependent nuclear export of the AR. Once in the cytoplasm, the AR is prone to proteosomal degradation [Rinnab et al., 2008]. Defining enzymes that affect components of the AR export machinery could provide new strategies for the therapy of advanced HRPCa.

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Supplemental Data

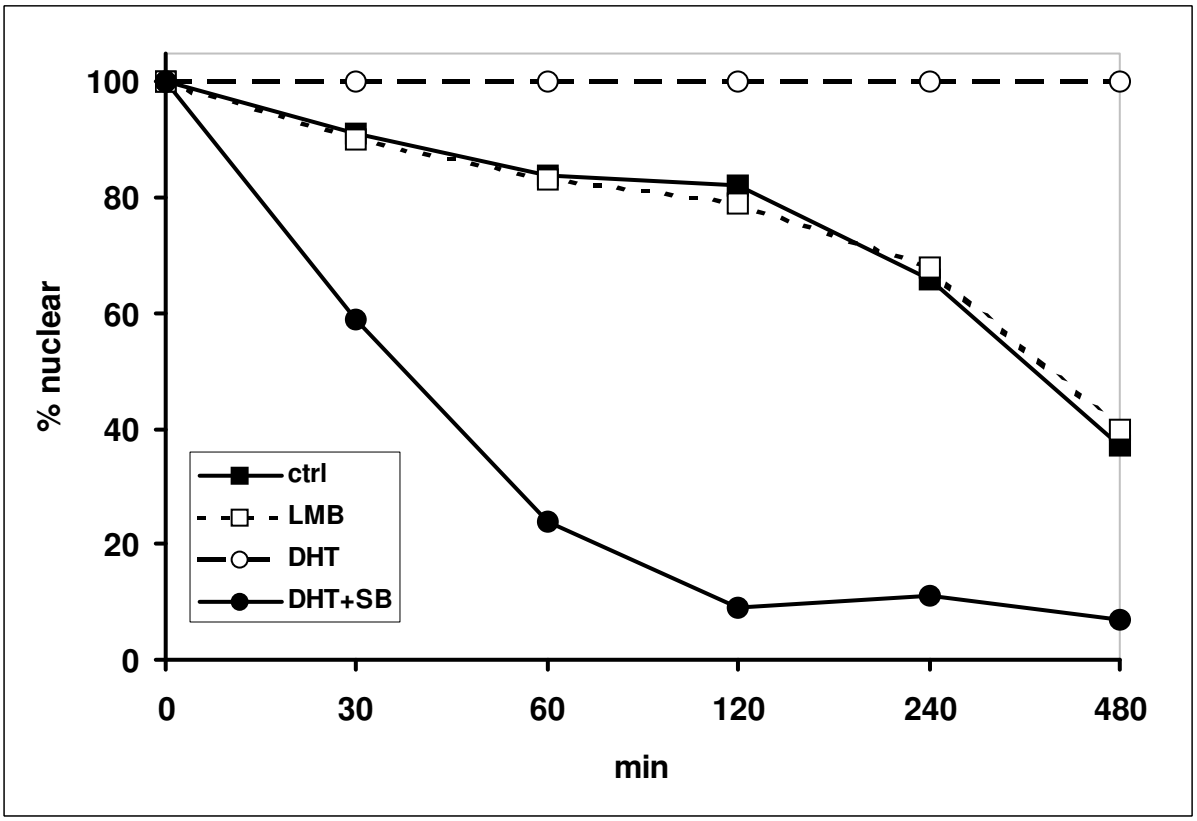


Fig. 1S: Androgen-withdrawal, in contrast to GSK-3 inhibition, causes slow LMB-insensitive nuclear export of the AR.

AR-negative PC3 cells were seeded in 24 well plates and allowed to adhere overnight. Subsequently cells were transfected with pAR-t1EosFP and allowed to grow for another 24 hours under steroid free conditions. Thereafter cells were treated for 1 hour with 10 nM DHT. After that medium was removed and cells treated as indicated (ctrl: absence of DHT; LMB: 1 ng/µl Leptomycin B; DHT: 10 nM DHT; DHT + SB: 10 nM DHT + 1 µM SB216763). Results are expressed in % of untreated controls (ctrl).

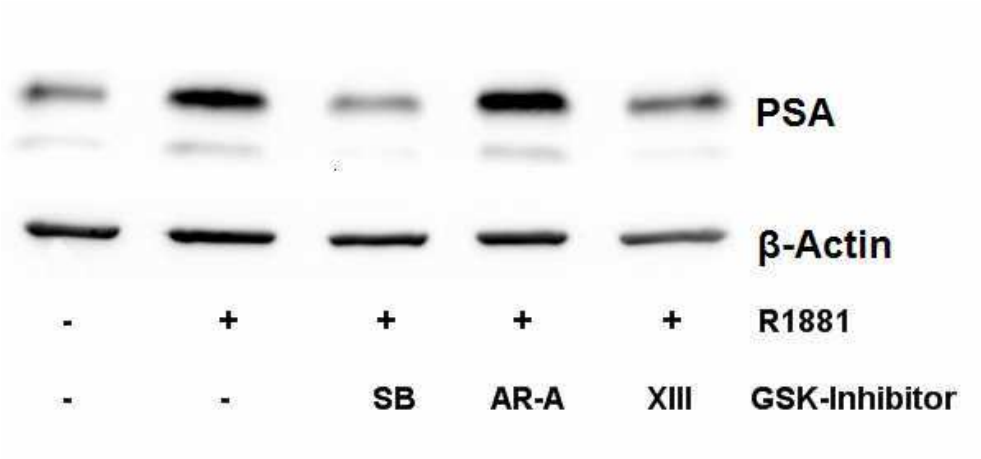


Fig. 2S: Modulation of endogenous PSA-levels by different pharmacological GSK-3 inhibitors

LNCaP cells were seeded in 6 well plates and allowed to adhere overnight. Subsequently medium was replaced and cells were grown under steroid free conditions for 24 hours. Thereafter cells were incubated with 1 µm of the GSK-3 inhibitors SB216763 and GSK XIII in the presence of R1881 (10 nM) for another 24 hours. The GSK-3 inhibitor AR-A014418 recently shown to exhibit strong androgenic effects that dominate over its GSK-inhibitory properties (Neoplasia 10, 624-633, 2008) served as control. Endogenous levels of PSA were detected by western blotting with Anti-PSA antibody (CHYH2, Santa Cruz Biotechnology) and anti β-Actin antibody as loading control.

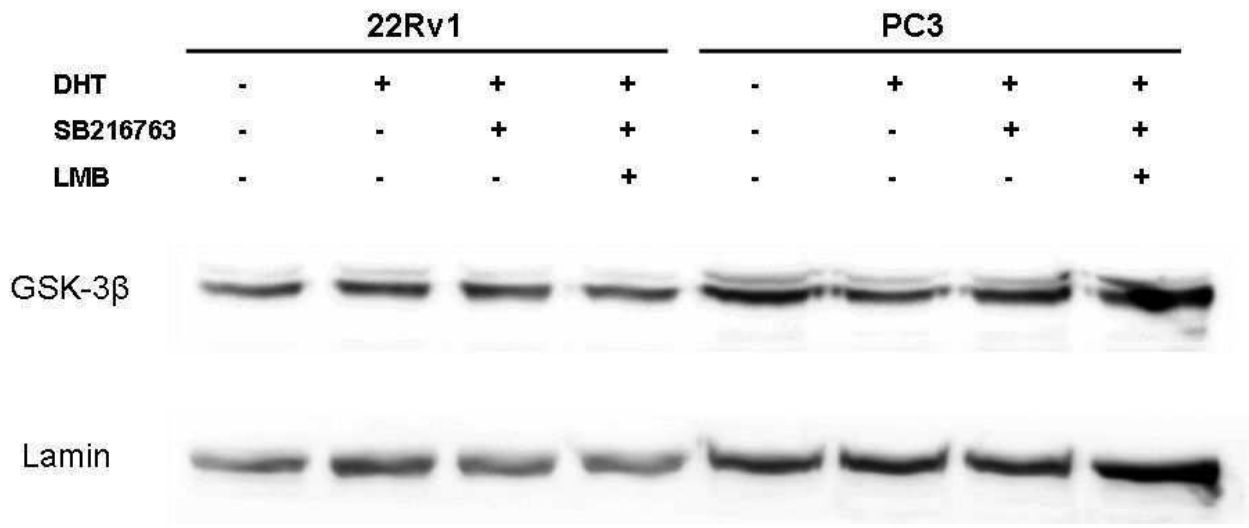
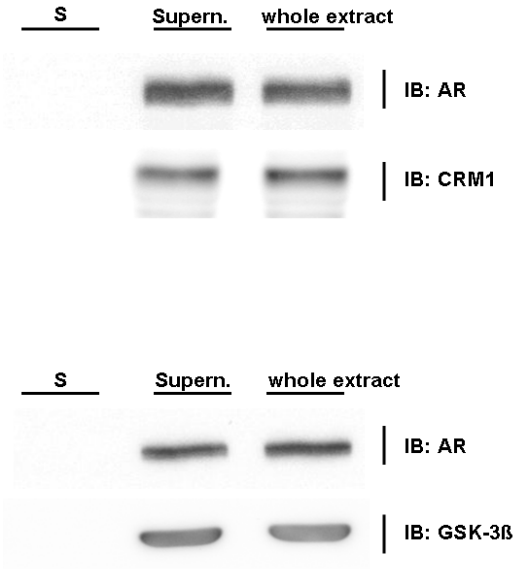


Fig. 3S: GSK-3β is not exported by a CRM1 dependent mechanism after treatment with the GSK-3 inhibitor SB216763.

22Rv1 and PC3 cells were seeded in 25T-flasks and allowed to grow overnight. 24 hours later, medium was changed to FBSdcc. 48 hours after seeding, cells were pre-treated with 1 ng/µl LMB if necessary for 30 minutes, before addition of 10 nM DHT. After 30 minutes, SB216763 was added at 1 µM. 4 hours later, cytoplasmic and nuclear fractions were collected as described in the Materials and Methods. Nuclear AR was detected by Western blotting with the AR441 antibody and Lamin A as a loading control.

A



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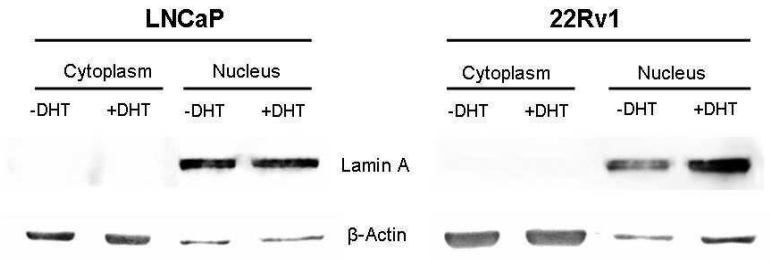

Fig. 4S: Controls for immunoprecipitation (IP) and cell fractionation procedure

A. Immunoprecipitation controls: LNCaP cells were cultured in 25T flasks for 24 hours. Subsequently protein extracts were prepared as described in material and methods. 100 µg of total protein extract was precleared with sepharose for 1 hour (Supern: supernatant after preclearing; S: sepharose after preclearing).

B. Quality control of nuclear/cytoplasmic fractionation procedure: LNCaP and 22Rv1 cells were seeded in T25 flasks for 24 hours in RPMI-1640 with 10% FBS (v/v) and antibiotics. Subsequently, cells were grown in RPMI-1640, 10% FBSdcc (v/v) and antibiotics for another 24 hours. Subsequently, cells were grown in the presence/absence of 10nM DHT for 4 hours. 25 µf of nuclear/cytosolic protein extracts were subjected to western blotting using antibodies directed against Lamin A and β-Actin.

60. Kongress der Deutschen Gesellschaft für Urologie e.V.

24. – 27. September 2008, Internationales Congresscenter Stuttgart

Inhibition of Glycogen Synthase Kinase-3 by Different Novel Inhibitors in Androgen-Responsive Prostate Cancer Cell Lines

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Objectives: The glycogen-synthase-kinase-3 (GSK3) is a serine/threonine kinase initially identified by its ability to modulate glycogen synthesis. However, GSK3 is able to phosphorylate many proteins, including members of the steroid receptor superfamily. Although GSK3 has been shown to phosphorylate the androgen receptor (AR) its effects on AR-mediated transcriptional activity are discussed controversially.

Material and Methods: AR- and TCF-signalling were measured by reporter-gene assays, AR-protein expression by western blotting. Translocation studies were done using fluorescence microscopy.

Results: Silencing of GSK3 in prostate cancer cells (PCa) by shRNA showed a 50% reduction in AR transcriptional activity and AR-protein levels. Therfore we tested the effects of different GSK3-inhibitors on AR-signaling. The capability of the compounds to inhibit GSK3 was monitored by their ability to increase TCF-signaling. Inhibitors of GSK3 like the maleimide SB216763 or the aminopyrazole GSK-inhibitor XIII inhibited AR-dependent reporter gene activity and AR-expression in PCa cells. Nuclear translocation of the AR was analyzed in PC3 cells transfected with a green fluorescent EosAR-fusion protein. Treatment of PCa-cells with SB216763 prior to an androgenic stimulus almost completely inhibited nuclear import of EosAR. When PCa cells were preincubated with methyltrienolone, subsequent treatment with SB216763 decreased nuclear localization of EosAR from 90% to 30% within 30 minutes. In contrast, the thiazole-containing urea AR-A014418 increased AR-activity/expression and nuclear import of the AR.

Conclusion: Although not all GSK-inhibitors had an effect on AR-stability/function our observations suggest a potential therapeutic application for these compounds in PCa.

ANDROGENS 2008
5th biennial metting on androgen receptor function
October 1-3, 2008, Rotterdam, the Netherlands

Inhibition of Glycogen Synthase Kinase-3β modulates Androgen Receptor-Signalling in Prostate Cancer Cell Lines

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The glycogen-synthase-kinase-3 (GSK-3) is a serine/threonine kinase widely expressed in mammalian tissues. In humans two isoforms GSK-3α and GSK-3β have been identified, which share a 97% sequence homology within their kinase domains. GSK-3 was initially identified by its ability to modulate glycogen synthesis, but it turned out to be a multifunctional enzyme, able to phosphorylate many proteins (β-catenin, p53, TCF, Cyclin D1), including members of the steroid receptor superfamily. Although GSK-3 has been recently shown to phosphorylate the androgen receptor (AR) its effects on AR-mediated transcriptional activity are still discussed controversially.

Silencing of GSK-3β by shRNA in 22Rv1 prostate cancer cells (PCa) lead to a 50 % decrease in AR transcriptional activity and AR protein levels. Therefore we tested pharmacological GSK-3β-inhibitors for their ability to modulate AR-signalling in the AR-positive PCa cell lines LNCaP and 22Rv1. The efficiency of the compounds to inhibit GSK-3β was measured *in vitro* by a kinase assay and *in vivo* by their ability to increase TCF-signalling. As shown by immunoprecipitation-analysis of LNCaP cells, GSK-3β is bound to the AR in the cytoplasm as well as in the nucleus. Long term treatment (> 24 h) of AR-positive PCa cells with GSK-3β-inhibitors like the maleimide SB216763 and the aminopyrazole GSK3-inhibitor XIII almost completely inhibited AR-dependent reporter gene activity. The reduction of transcriptional activity was paralleled by proteasomal degradation of AR-protein. In short term experiments (< 300 min) pre-treatment of PCa-cells with SB216763 and GSK-3 inhibitor XIII almost completely inhibited androgen stimulated nuclear import of AR-Eos, a green fluorescent AR-fusion protein. Most interestingly, in PCa cells pre-treated with the synthetic androgen R1881, SB216763 and GSK-3 inhibitor XIII triggered a rapid nuclear export of AR-Eos. In contrast to SB216763 and GSK-3 inhibitor XIII the thiazole-containing urea compound AR-A014418, another potent GSK-3 inhibitor, increased AR-expression and AR-functions in the presence/absence of androgens suggesting that this compound exhibits androgenic properties. This assumption is furthermore supported by the observation that AR-A014418 supports the proliferation of AR-positive cells whereas SB216763 inhibits it.

In summary, there is experimental evidence that GSK-3β modulates AR-stability as well as different AR-functions. Although not all GSK-3 inhibitors inhibited AR-signalling our observations suggest a potential therapeutical application for some GSK-3 inhibitors in the treatment of PCa.

18th Meeting of the European Society for Urological Research (ESUR)

16-18 October 2008, Barcelona, Spain

Modulation of Androgen Receptor-Signalling by Inhibition of Glycogen Synthase Kinase-3β in Prostate Cancer Cell Lines

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The glycogen-synthase-kinase-3 (GSK-3) is a serine/threonine kinase widely expressed in mammalian tissues. Two isoforms GSK-3α and GSK-3β have been identified in humans, which share a 97% sequence homology within their kinase domains. Initially identified by its ability to modulate glycogen synthesis, GSK-3 turned out to be a multifunctional enzyme, able to phosphorylate many proteins including members of the steroid receptor superfamily. GSK-3 has been recently shown to phosphorylate the androgen receptor (AR), but its effects on AR-mediated transcriptional activity are still discussed controversially.

As shown by immunoprecipitation-analysis of cellular extracts GSK-3β is bound to the AR in the cytoplasm as well as in the nucleus of prostate cancer cells (PCa). shRNA-mediated downmodulation of GSK-3β in 22Rv1 prostate cancer cells lead to a 50 % decrease in AR transcriptional activity and AR protein levels. In consequence we tested pharmacological inhibitors of GSK-3β function for their ability to modulate AR-signalling in AR-positive PCa cell lines. GSK-3β-inhibitors like the maleimide SB216763 and the aminopyrazole GSK3-inhibitor XIII almost completely inhibited AR-dependent reporter gene activity as well as intracellular PSA-levels. These effects were paralleled by a proteasomal degradation of the AR-protein. Moreover, short-term treatment of PCa-cells with SB216763 and GSK-3 inhibitor XIII almost completely inhibited androgen stimulated nuclear import of AR-Eos, a green fluorescent AR-fusion protein. Most interestingly, in PCa cells pre-treated with the synthetic androgen R1881, SB216763 and GSK-3 inhibitor XIII triggered a rapid nuclear export of AR-Eos. This reduction of nuclear AR-protein was verified by Western blot analysis of nuclear extracts. In contrast, another potent GSK-3 inhibitor, the thiazole-containing urea compound AR-A014418 was able to increase AR-expression and AR-functions in the presence/absence of androgens suggesting that this compound exhibits androgenic properties. This assumption was furthermore supported by the observation that AR-A014418 supports the proliferation of AR-positive cells.

In summary, there is experimental evidence that GSK-3β modulates AR-stability as well as different AR-functions. Although not all GSK-3 inhibitors were able to downregulate AR-signalling our observations suggest a potential therapeutical application for some of these compounds in the treatment of PCa.

61. Kongress der Deutschen Gesellschaft für Urologie e.V.

16. -19. September 2009, Messe Dresden

Inhibition of Glycogen Synthase Kinase-3 promotes Crm1-dependent nuclear export of the Androgen Receptor

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Objectives: There is experimental evidence that in hormone refractory prostate cancer (PCa) cell lines the androgen receptor (AR) is predominantly located in the nucleus, enabling these cells to grow in the absence or very low levels of androgens. Besides its ability to modulate glycogen synthesis, glycogen-synthase-kinase-3 (GSK-3) has been shown to phosphorylate the androgen receptor (AR) thereby modulating its stability and nuclear localization. Consequently, we analyzed the effects of pharmacological GSK-3 inhibitors on the nuclear localization of the AR.

Material and Methods: AR-protein as well as AR-protein complexes were examined by western blotting. AR translocation was analyzed using a green fluorescent Eos-AR fusion protein or immunohistochemistry. Receptor-activity was measured by reporter gene assay.

Results: Immunofluorescent staining and immunoprecipitation analysis revealed that GSK-3 and AR form a functional complex. GSK-3 binding to the AR could be restrained to the N-terminus of the receptor. Addition of pharmacological GSK-3 inhibitors to PCa cells grown in presence of DHT triggered a rapid nuclear export of the AR in these cells. This nuclear export most likely operates via a Crm1/exportin1 dependent mechanism as inhibition of Crm1 by Leptomycin B prevented the AR export.

Conclusion: Inhibition of GSK-3 function leads to a Crm1 mediated nuclear export of the AR. This mechanism may be of therapeutical use for the treatment of hormone refractory PCa.

9. Deutschsprachige Zusammenfassung

Das Prostatakarzinom (PCa) zählt zu den häufigsten Tumorerkrankungen in den westlichen Industrienationen. Die gesunde Prostata entwickelt sich unter dem Einfluß von Androgenen und benötigt im adulten Zustand für ihre Funktion einen kontinuierlichen Androgenstimulus. Auch die meisten Prostatakarzinome wachsen initial abhängig von Androgenen. Beim fortgeschrittenen Prostatakarzinom wird diese Hormonabhängigkeit durch Androgenentzug therapeutisch genutzt. Hierbei führt die Androgenablation zur Induktion der Apoptose in hormonabhängigen Zellen. Obwohl die Mehrzahl der PCas auf eine antiandrogene Therapie anfänglich sehr gut anspricht, kommt es nach einem Zeitraum von 18 – 24 Monaten zur Tumorprogression mit der Bildung hormonrefraktärer PCa (HRPCa) Zellen. Die Proliferation von HRPCa Zellen während einer Hormonablationstherapie stellt eine der Hauptursachen für die hohe Mortalitätsrate beim fortgeschrittenen PCa dar. Die Ursachen für dieses Phänomen bleiben weitgehend ungeklärt. Während *in vitro* der Verlust des Androgenrezeptors (AR) der vorherrschende Mechanismus für die Entstehung des HRPCa zu sein scheint, bleibt die Expression des AR *in vivo* nicht nur weitgehend erhalten, sondern ist sogar oftmals verstärkt. Somit ist eine umfassende Analyse von Faktoren, die sowohl die Aktivität als auch die Stabilität und Lokalisation des AR modulieren die Vorraussetzung für die Identifikation neuer Marker/Zielmoleküle, welche zu verbesserter Diagnostik und Therapie von HRPCa beitragen.

In der vorliegenden Arbeit wurde der Einfluss zweier Kinasen auf die androgene Signalkette untersucht. Hierbei handelt es sich einerseits um die Glykogen Synthase Kinase-3 (GSK-3), einem Schlüsselelement des WNT-Signalweges. Zum anderen wurde die in den NF-κB Signalweg involvierte IκB Kinase (IKK) näher auf ihre Wechselwirkungen mit dem AR untersucht.

Die GSK-3 ist eine ubiquitär vorkommende Serin/Threonin Kinase, welche in zwei Isoformen – GSK-3α und GSK-3β – exprimiert wird. Ursprünglich wurde die GSK-3 als wichtige Schaltstelle im Glykogen-Metabolismus identifiziert. Außerdem ist die GSK-3 wesentlich am proteasomalen Abbau von β-Catenin im WNT-Signalweg beteiligt. In den letzten Jahren stellte sich allerdings heraus, dass die GSK-3 eine multifunktionelle Kinase ist, welche viele Proteine phosphorylieren und somit deren Funktion beeinflussen kann. Zu diesen Zielproteinen der GSK-3 gehören unter anderem auch Steroidrezeptoren, wie der AR. Erste Untersuchungen bezüglich des Einflusses der GSK-3 auf die Funktion des AR verliefen bisher widersprüchlich. In der vorliegenden Arbeit konnte festgestellt werden, dass die GSK-3 und der AR unter denselben physiologischen Bedingungen (Anwesenheit/Abwesenheit von androgenen Stimuli) überwiegend co-lokalisiert vorliegen und einen Komplex bilden. Des Weiteren ist die GSK-3 nicht nur in der Lage – wie bisher beschrieben – den AR direkt an

Serin-Resten, sondern auch an Threonin-Resten zu phosphorylieren. Eine langzeitige Inhibition der GSK-3 durch siRNA oder pharmakologische GSK-3 Inhibitoren führt in den meisten Fällen zu verminderter Transaktivierung und proteasomalem Abbau des AR. Zu den Ausnahmen zählt der GSK-3 Inhibitor AR-A014418, welcher, wie in dieser Arbeit erstmalig beschrieben, den AR auf Grund seiner androgenen Wirkung stabilisiert. Durch ihren negativen Einfluss auf die AR-Signalkette, sind pharmakologische GSK-3 Inhibitoren zudem in der Lage das Wachstum AR-positiver PCa Zellen zu hemmen. Eine kurzzeitige pharmakologische GSK-3 Inhibition führt interessanterweise zu einem schnellen, nukleären Export des AR. Bemerkenswerterweise wird dieser schnelle AR-Export über einen CRM1-abhängigen Mechanismus reguliert. Obwohl Steroidrezeptoren, wie bisher angenommen, keine typischen, für einen CRM1-vermittelten Export notwendigen, leucinreichen nukleären Exportsequenzen (NES) besitzen, konnte dennoch ein funktionelles CRM1-NES (Aminosäuren 803-815) am C-Terminus des AR in dessen Ligandenbindungsdomäne identifiziert werden. Zusammenfassend könnte eine gezielte Inhibition der GSK-3 künftig von therapeutischem Nutzen bei der Behandlung des fortgeschrittenen PCa sein, da zeitgleich Lokalisation und Stabilität des AR reguliert werden können.

Im zweiten Teil dieser Arbeit wurden die Auswirkungen einer pharmakologischen Inhibition der IKK auf die androgene Signalkette untersucht. Die IKK ist eine Serin/Threonin-Kinase, deren katalytische Aktivität v.a. wichtig für die Aktivierung des NF-κB Signalweges ist. Aufgrund ihrer meist antiapoptotischen und proliferationsfördernden Wirkung, wird konstitutive NF-κB Aktivität oft mit der Entstehung verschiedenster Tumore in Verbindung gebracht, zu denen auch das PCa zählt. Eine permanente Aktivierung des NF-κB Signalweges wird einer Deregulation der beiden katalytischen IKK-Untereinheiten – IKKα und IKKβ – zugeschrieben. Somit zielen die meisten Therapieansätze, welche auf NF-κB ausgerichtet sind auf Repression der IKK ab. Obwohl die IKK bisher nicht mit dem AR in Verbindung gebracht wurde, konnte gezeigt werden, dass diese Kinase in der Lage ist, den Östrogenrezeptor α (ERα) in Brustkrebszellen an Serin-Resten zu phosphorylieren. Deshalb wurden mögliche Auswirkungen einer pharmakologischen Inhibition der IKK auf die androgene Signalkette untersucht.

Wie in dieser Arbeit erstmals gezeigt werden konnte, sind pharmakologische IKK-Inhibitoren in der Lage, die transkriptionelle Aktivität des AR herunter zu regulieren. Dieser negative Effekt auf die AR-Transaktivierung war jedoch – im Vergleich zu GSK-3 Inhibition – nicht auf eine Destabilisierung des AR zurückzuführen. Des Weiteren wurden weder Dimerisierung noch Lokalisation des AR durch pharmakologische IKK Inhibitoren beeinflusst. Jedoch ging die verminderte AR-Transaktivierung nach IKK Inhibition mit einer reduzierten

Phosphorylierung der Aminosäure S308 in der Transaktivierungsdomäne des AR einher. Zudem konnten IKK Inhibitoren v.a. in den HRPCa Zellen (22Rv1 und PC3) eine deutliche Verminderung der Zell-Proliferation bewirkten, weswegen diesen Inhibitoren therapeutisches Potential bei der Behandlung des HRPCa zugeschrieben werden kann.

Zusammenfassend lässt sich sagen, dass die Aktivität zweier Serin/Threonin-Kinasen – GSK-3 und IKK – für die volle Funktionsfähigkeit des AR notwendig zu sein scheint. Sowohl eine pharmakologische Inhibition der GSK-3 als auch der IKK wirkte sich negativ auf den Signalweg des AR aus. Dies ist besonders interessant im Hinblick darauf, dass beide Kinasen unterschiedliche Ebenen AR-induzierter Signale beeinflussen. Erste pharmakologische GSK-3 Inhibitoren haben zur Behandlung von Alzheimer und Typ II Diabetes bereits die erste klinische Prüfphase erfolgreich durchlaufen. Somit könnten einige dieser Kinase-Inhibitoren in naher Zukunft viel versprechende Substanzen bei der Behandlung des PCa sein.

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Publikationen

Begutachtete Veröffentlichungen

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Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, sowie die wörtlich und inhaltlich übernommenen Stellen als solche kenntlich gemacht habe.

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