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

Ludwig Rinnab[*,3], Stefanie V. Schütz[*,†,3], Jeannine Diesch[†], Evi Schmid[†], Rainer Küfer[*], Richard E. Hautmann[*], Klaus-Dieter Spindler[†] and Marcus V. Cronauer[†]

*Department of Urology, University of Ulm, Ulm, Germany; †Institute of General Zoology and Endocrinology, University of Ulm, Ulm, Germany

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

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 pFop-Flash (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 se-

rum (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.
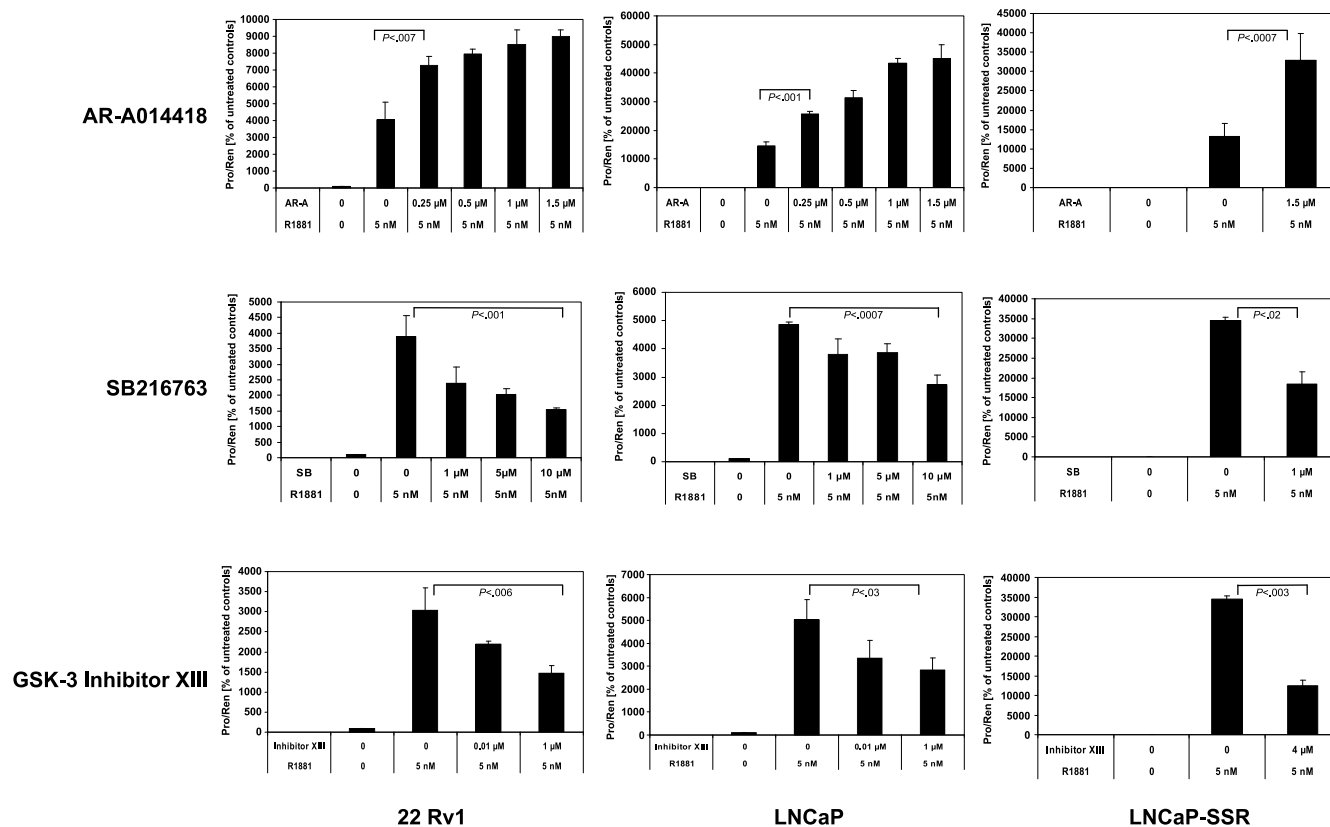
Figure 2. Modulation of AR transactivation by pharmacological GSK-3 inhibitors. AR-receptor–positive cell lines 22Rv1, LNCaP, and LNCaP-SSR were incubated with different pharmacological GSK-3 inhibitors in the presence/absence of 5-nM R1881 for 96 hours. AR transcriptional activity was measured using 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
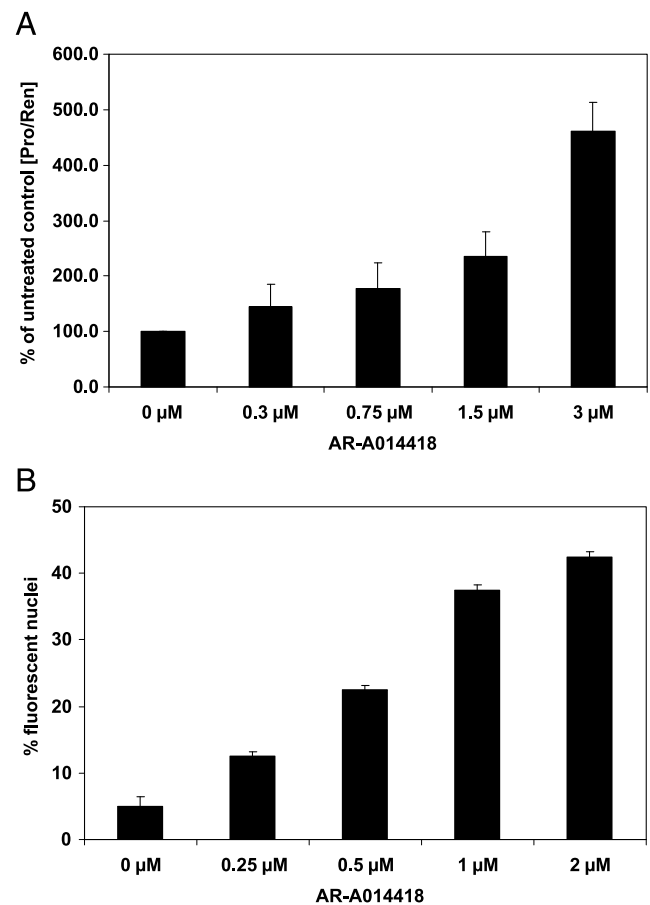
Figure 3. Androgenic properties of the GSK-3 inhibitor AR-A014418. (A) AR-A014418 activates the AR in absence of androgens: 22Rv1 cells were treated for 30 hours with AR-A014418 in the absence of androgens. AR-activity was determined using an AR-reporter gene assay as described in the Materials and Methods 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.
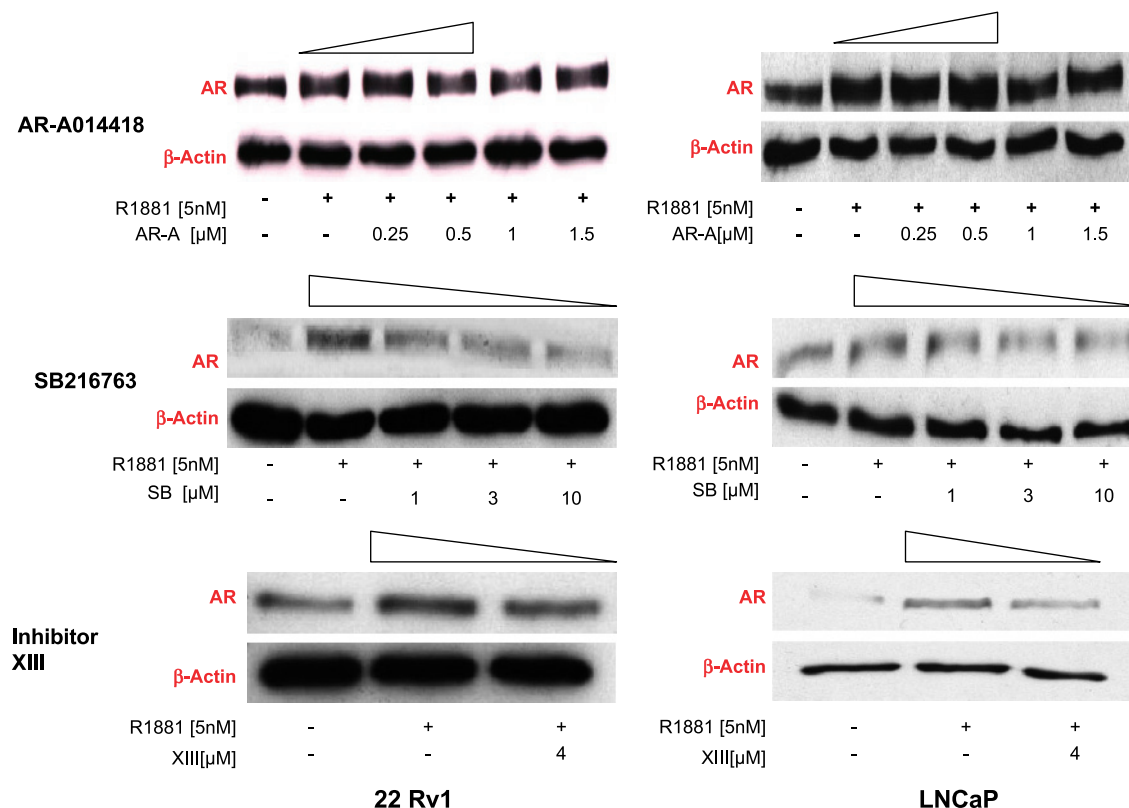
Figure 4. Effects of GSK-3 inhibitors on AR-protein expression. Cell extracts isolated from AR-reporter gene assays were analyzed by Western blot analysis as described in the Materials and Methods section. Inhibition of AR-transactivation by pharmacological inhibitors of GSK-3 was paralleled by a decrease 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
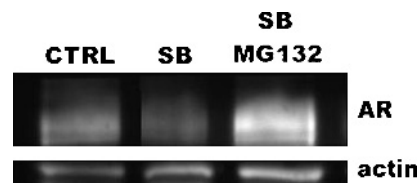
Figure 5. Downmodulation of AR by GSK-3 is due to proteasomal degradation. LNCaP cells were incubated with/without 5 μM of the proteasome inhibitor MG132 for 60 minutes, followed by a SB216763 treatment (final concentration, 1 μM) for another 60 minutes. Subsequently, R1881 (5 nM) was added to the medium, and the cells were grown for another 6 hours. Subsequently, cell extracts were analyzed by Western blot analysis.
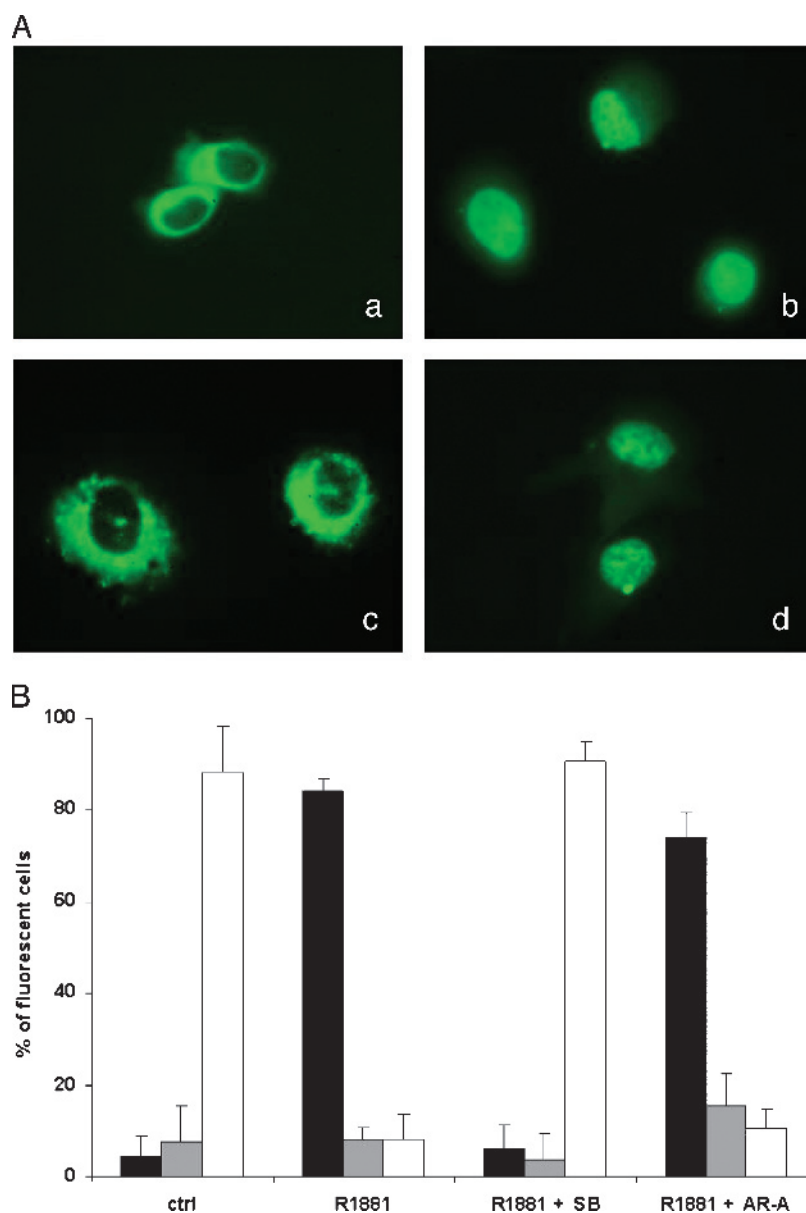
Figure 6. Effects of GSK-3 inhibitors on nuclear translocation of the EosAR. (A) Fluorescence microscopy of pAR-t1EosFP–transfected PC3 cells: (a) absence of androgens, (b) presence of androgens, (c) preincubation with SB216763 before androgen treatment, and (d) pretreatment with AR-A014418 before androgen 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 downregulation 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
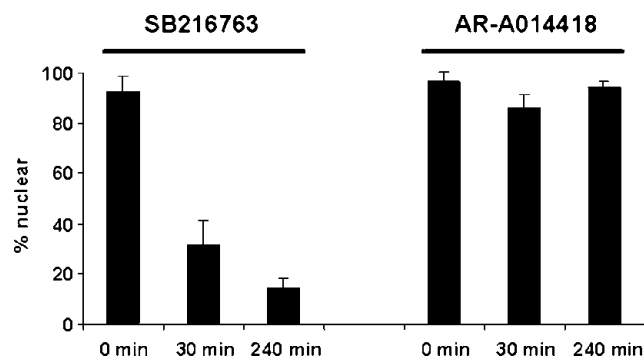
Figure 7. Inhibition of GSK-3 by SB216763 induces a rapid export of EosAR from the nucleus. PC3 cells grown in a 24-well plate were transfected with pAR-t1EosFP. Subsequently, cells were treated with 5-nM R1881 for 30 minutes resulting in a >90% of nuclear EosAR. Subsequently, cells were treated with/without 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.
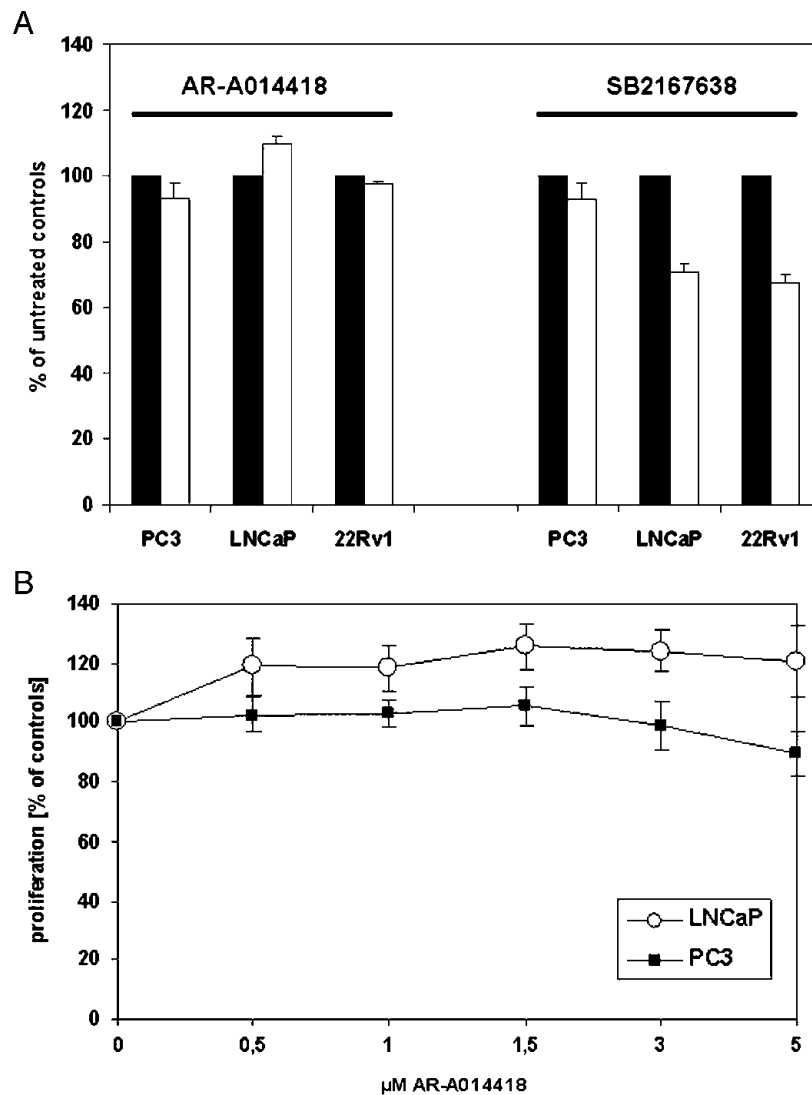
Figure 8. Effects of GSK-3 inhibitors on prostate cancer cell growth. (A) Modulation of cell growth by AR-A014418 and SB216763 in the AR-positive cell lines 22Rv1 and LNCaP: 22Rv1, LNCaP, and PC3 prostate cancer cells were seeded in 96-well plates and allowed to adhere overnight. Subsequently, medium 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.

Acknowledgments

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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%.