Cooperative Interactions between Androgen Receptor (AR) and Heat-Shock Protein 27 Facilitate AR Transcriptional Activity

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Abstract

Androgen receptor (AR) transactivation is known to enhance prostate cancer cell survival. However, the precise effectors by which the prosurvival effects of androgen and AR drive prostate cancer progression are poorly defined. Here, we identify a novel feed-forward loop involving cooperative interactions between ligand-activated AR and heat-shock protein 27 (Hsp27) phospho-activation that enhance AR stability, shuttling, and transcriptional activity, thereby increasing prostate cancer cell survival. Androgen-bound AR induces rapid Hsp27 phosphorylation on Ser^78 and Ser^82 residues in an AR- and p38 kinase-dependent manner. After this androgen-induced, non-nuclear phospho-activation, Hsp27 displaces Hsp90 from a complex with AR to chaperone AR into the nucleus and interact with its response elements to enhance its genomic activity. Inhibition of Hsp27 phosphorylation, or knockdown using the antisense drug OGX-427, shifted the association of AR with Hsp90 to MDM2, increased proteasome-mediated AR degradation, decreased AR transcriptional activity, and increased prostate cancer LNCaP cell apoptotic rates. OGX-427 treatment of mice bearing LNCaP xenografts transfected with an androgen-regulated, probasin-luciferase reporter construct resulted in decreased bioluminescence and serum PSA levels as pharmacodynamic readouts of AR activity, as well as AR, Hsp27, and Hsp90 protein levels in LNCaP tumor tissue. These data identify novel nongenomic mechanisms involving androgen, AR, and Hsp27 activation that cooperatively interact to regulate the genomic activity of AR and justify further investigation of Hsp27 knockdown as an AR disrupting therapeutic strategy in prostate cancer. [Cancer Res 2007;67(21):10455–65]

Introduction

The androgen receptor (AR), a ligand-dependent transcription factor and member of the class I subgroup of the nuclear receptor superfamily, plays a key role in prostate carcinogenesis and progression. The classic model of androgen-regulated AR transcriptional activity has not fully defined the many diverse effects of androgens on prostate cancer cell survival and growth. In response to androgen, cytoplasmic AR rapidly translocates to the nucleus and interacts with sequence-specific androgen response elements (ARE) in the transcriptional regulatory regions of target genes (1, 2). In addition to this transcriptional genomic action, androgens and other steroid hormones like progesterone and estrogen can exert rapid nongenomic effects that are not mediated through nuclear receptors, but rather initiated at the plasma membrane, presumably through surface receptors (3, 4).

Like progesterone (PR) and estrogen (ER) receptors, AR can interact with the intracellular tyrosine kinase c-Src to activate the mitogen-activated protein kinase (MAPK) pathway (5, 6). In androgen-sensitive LNCaP prostate cancer cells, AR interacts with the SH3 domain of Src within minutes of androgen treatment to promote cell survival, proliferation, and differentiation (5, 6). Androgens also rapidly stimulate Raf-1 and Erk-2, both components of the MAPK signaling cascade (5), suggesting that androgen-stimulated MAPK activation occurs via nongenomic mechanisms. This nongenomic action of androgen can also influence classic genomic AR activity, including modulation of AR by coactivators (7). Phosphoinositide-3-kinase (PI3K)/Akt is another pathway involved in nongenomic activity of ER and PR (8, 9), as well as AR (3, 10). In fact, androgen-activated PI3K and Akt enhance cell growth and survival in AR-positive cells, which can be inhibited by PI3K inhibitors or dominant-negative Akt. AR is found in a large protein complex with p85α-PI3K and Src, both required for androgen-stimulated PI3K/Akt activation (3, 10).

Molecular chaperones are involved in processes of folding, activation, trafficking, and transcriptional activity of most steroid receptors, including AR. In the absence of ligand, AR is predominantly cytoplasmic, maintained in an inactive but highly responsive state by a large dynamic heterocomplex composed of heat-shock proteins (Hsp), co-chaperones, and tetratricopeptide repeat (TPR)–containing proteins. Ligand binding leads to a conformational change in the AR and dissociation from the large Hsp complex. Subsequently, the AR translocates to the nucleus, interacts with coactivators, dimerizes, and binds to its ARE to transactivate target gene expression (11). Dissociation of the AR-chaperone complex after ligand binding is viewed as a general regulatory mechanism of AR signaling (11). Molecular chaperones remain important players in the events downstream of receptor activation and throughout the life cycle of the AR. For example, the Hsp90 inhibitor, geldanamycin, destabilizes AR and increases its proteasomal degradation, thereby decreasing the expression of AR-regulated genes (12). Recent reports further highlight the important roles of other co-chaperones on AR activation. Bag-1L is overexpressed in hormone refractory prostate cancer (13) and enhances the transactivation of the AR by using its NH2 and COOH-terminal domains to bind to COOH- and NH2-terminal sequences of AR (14). Another co-chaperone, FKBP52, also increases AR transactivation activity (15, 16), and FKBP52 knock-out mice exhibit defects in male reproductive tissues, including ambiguous external genitalia and defects in prostate and seminal vesicle development.
Another class of Hsp70s that complex with ER and glucocorticoid receptors (GR) are ATP independent and include Hsp27 (17). Hsp27 is a cytoprotective chaperone expressed in response to many stress signals to regulate key effectors of the apoptotic machinery, including the apoptosome, the caspase activation complex (18, 19), and proteasome-mediated degradation of apoptosis-regulatory proteins (20, 21). Antisense knockdown of Hsp27 delays prostate cancer xenograft growth and androgen-independent progression (22, 23). Although Hsp27 is induced by estrogens and glucocorticoids (24, 25), its relationship with AR and androgens is undefined. Here, we identify a feed-forward loop whereby androgen-bound AR induces rapid Hsp27 phosphorylation that, in turn, cooperatively facilitates genomic activity of the AR, thereby enhancing prostate cancer cell survival.

Materials and Methods

Cell culture and antisense oligonucleotide transfection. LNCaP cells are maintained in RPMI with 5% fetal bovine serum (FBS; ref. 26) and treated with Hsp27 antisense oligonucleotide (ASO) designated OGX-427 (Invitrogen-Life Technologies, Inc.) for 20 min. Four hours later, 5% FBS was added. Cells were treated once daily for 2 successive days and harvested 48 h after the second treatment.

Plasmids, reagents, and antibodies. An Hsp27 wild type (wt) was subcloned into pcDNA3.1-GFP (Invitrogen-Life Technologies). The Hsp27 triple mutant (TM) was generated by introducing direct mutagenesis, replacing Ser15, Ser29, and Ser32 with alanine using QuikChange® II XL, site-directed mutagenesis kit according to the manufacturer’s instructions (Stratagene). R1881 (Perkin-Elmer) was dissolved in 100% ethanol. Cycloheximide, MG-132, SB 203580, and LY 294002 were purchased from Calbiochem; Hsp27, anti-AR, or immunoglobulin G (IgG) as a control overnight at 4°C. Cells were washed with radioimmunoprecipitation assay buffer (RIPA) at least thrice, and immune complexes were recovered with protein-G sepharose for 2 h and then incubated for 20 min at room temperature and then incubated for 20 min at room temperature with double-stranded DNA of (pGEM) labeled with 32P (with specific activity of about 300,000 cpm) PSA-ARE oligonucleotide (27).

Chromatin immunoprecipitation. LNCaP cells were treated with 10 nmol/L of R1881 for 4 h and paraformaldehyde cross-linked and sonicated. Chromatin immunoprecipitation (ChIP) assay was done using EZ ChIP kit according to the manufacturer (Upstate) on the PSA gene regions ARE I and ARE III as described in ref. 28.

Reverse transcription-PCR. About 4 µg of total RNA were reverse transcribed in cDNA using 100 pmol of random hexamer primers (Pharmacia) and Moloney murine leukemia virus reverse transcriptase (Life Technologies). The cDNA was successively amplified with two pairs of AR-specific primers. About 5 µL of cDNA were used as the starting DNA template in the PCR assay. To verify RNA quality, each sample was amplified with a set of primers specific to β-actin (5′-TGATCCA-CATCTGTCGGAAGTGGA-3′ sense and 5′-GGACCTGACTGACCTCCT-3′ antisense). Analysis of PCR products was done by electrophoresis in 2% agarose gel and visualized by ethidium bromide staining.

Protein stability. LNCaP cells were plated and treated with OGX-427 or mismatch control or transfected with Hsp27 wt or TM, and treated with SB203580 before R1881 (10 nmol/L) treatment for 24 h using TRIzol/chloroform extraction (Invitrogen Life Technologies, Inc.). A 10-µg aliquot from each sample was subjected to horizontal electrophoresis and followed by hybridization with Hsp27 (700 pb) or prostate-specific antigen (PSA; 2 kb) cDNA probe as described (23) and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe for normalization of loading levels.

Results

Androgens and Hsp27 protect LNCaP cells from apoptotic stress. Androgen is assumed to have an important role as a survival factor in prostate epithelial cells. The synthetic androgen
R1881 enhances LNCaP cell survival in the presence of cytotoxic stress, including etoposide (30), cyclohexamide (10), and PI3K inhibitors (31). We recently reported that Hsp27 conferred resistance to androgen ablation in LNCaP (23) and next set out to explore whether androgen- and Hsp27-induced prosurvival activities were interrelated. Before beginning to study relationships between androgen and Hsp27, we first confirmed that R1881 added to androgen-depleted CSS increased LNCaP cell growth (Fig. 1A, left) and decreased apoptotic rates (Fig. 1A, right) compared with CSS alone. R1881 protected LNCaP cells to paclitaxel treatment, lowering apoptotic rates and increasing cell growth (Supplementary Data S1). R1881 also protected LNCaP cells from apoptosis induced by OGX-427, a second-generation ASO that potently decreases Hsp27 levels (23). OGX-427–induced knockdown of Hsp27 was associated with decreased apoptotic rates in R1881-treated cells, as measured by fluorescence-activated cell sorting (FACS) and PARP cleavage (Fig. 1B and C). Collectively, these results indicate that androgens suppress apoptosis induced by Hsp27 knockdown or paclitaxel.

**Androgens lead to rapid Hsp27 phosphorylation via p38 MAPK pathway.** Androgens stimulate growth and survival via both genomic and nongenomic pathways. Recently identified nongenomic effects include activation of Src, PI3K, and Akt (3, 10). Because Akt has been reported to phosphorylate Hsp27 in

![Figure 1. Androgen enhances LNCaP cell survival. A, left, R1881 enhances LNCaP cell growth. Cells were treated with 1 nmol/L R1881 for 2, 4, and 6 d, and cell growth rates were determined by MTS assay and compared with control (day of treatment defined as 100%). Right, R1881 protects LNCaP cells from apoptosis. Cells were treated with 1 nmol/L R1881 for 4 d, and proportion of cells in sub-G₀, G₀-G₁, S, G₂-M was determined by propidium iodide staining. B and C, LNCaP cells were pretreated with 10 nmol/L R1881 or CSS for 48 h before treatment with OGX-427 or mismatch control. PARP cleavage and Hsp27 expression levels were measured by Western blot. All experiments were repeated at least thrice.](image-url)
intact neutrophils (32), we tested whether androgen leads to Hsp27 phosphorylation. Interestingly, androgen induced rapid phosphorylation of Hsp27 on both Ser78 and Ser82 residues in a dose- and time-dependent manner (Fig. 2A). Within 15 min after incubation with 10 nmol/L R1881, Ser78 and Ser82 phosphorylation levels increased 3.2- and 5.3-fold, respectively (Fig. 2A, right).

To identify upstream effectors of androgen-induced Hsp27 phosphorylation, we analyzed R1881-induced changes in p38 kinase and Akt phosphorylation levels, both previously associated with Hsp27 activation (32, 33). R1881 enhances p38 kinase and Akt phosphorylation in a time-dependent manner, with maximum stimulation at 15 and 5 min, respectively (Fig. 2B, left and right). These results suggest that Hsp27 may be phosphorylated via p38 and/or Akt pathways. Preincubation of LNCaP cells with the p38 kinase inhibitor, SB 203580, abolished R1881-induced Hsp27 phosphorylation (Fig. 2C, left), whereas the Akt inhibitor, LY 294002, did not alter androgen-induced Hsp27 phosphorylation (Fig. 2C, right), indicating that androgen-induced Hsp27 phosphorylation occurs via the p38 kinase pathway.

**Androgen-induced Hsp27 phosphorylation is AR dependent.**

To determine whether AR is required for androgen-induced phosphorylation of Hsp27, changes in Ser78 and Ser82 phosphorylation levels were analyzed after treatment with R1881 ± the antiandrogen bicalutamide. Bicalutamide inhibited R1881-induced Hsp27 phosphorylation at both sites (Fig. 3A), suggesting that ligand binding to AR is required for Hsp27 phosphorylation by R1881. Next, PC3 cells, which do not express endogenous AR, were transfected with increasing amounts of AR or empty vector with or without R1881. As shown in Fig. 3A, Hsp27 phosphorylation levels at both Ser78 and Ser82 sites increased with increasing AR levels after R1881 treatment. In AR-negative PC3 cells, Hsp27 phosphorylation is insensitive to androgens, but is enhanced when AR is transiently overexpressed in PC3 cells. These results confirm that AR is required for androgen-induced Hsp27 phosphorylation.

To further define mechanisms of androgen-induced Hsp27 phosphorylation, we determined whether Hsp27 and AR interact using coimmunoprecipitation for Hsp27 and AR in LNCaP cells. Hsp27 is detected in AR immunoprecipitated complexes, and
conversely, AR is detected in Hsp27 immunoprecipitated complexes (Fig. 3C, left). The domain of AR required for the interaction with Hsp27 was next analyzed using glutathione S-transferase (GST)–pulldown assays. GST pulldown after initial equimolar normalization of GST-AR domain fusion as previously described (34) indicates that Hsp27 binds with NH2-terminal and ligand binding (COOH-terminal) domains, but not the DNA-binding domain of AR (Supplementary Data S2). Furthermore, immunofluorescence illustrates that Hsp27 and AR colocalize in the cytoplasm of LNCaP cells cultured in the absence of androgens. Importantly, and of potential functional relevance, both proteins translocate and colocalize in the nucleus after R1881 treatment (Fig. 3C, right).

Before ligand binding, AR exists in a complex with Hsp90 and other co-chaperones. The AR-Hsp90 interaction maintains AR in a ligand-binding conformation necessary for efficient response to hormone (35). Upon ligand binding, AR is released from Hsp90 and is translocated into the nucleus. Interestingly, AR immunoprecipitation blots show that shortly after androgen treatment, Hsp27 levels increase in complex with AR as Hsp90 levels decrease (Fig. 3D), suggesting that upon ligand binding, phospho-activated Hsp27 replaces Hsp90 to chaperone AR into the nucleus.

**Effect of Hsp27 on genomic activity of AR.** Many proteins participate in the activation of AR, either by direct binding or as part of a tertiary complex regulating the activity of other transactivators. Because Hsp27 colocalizes with and shuttles ligand-activated AR during nuclear translocation, we next investigated the effects of phospho-Hsp27 levels on AR transcriptional activity. This was done by gain-of-function strategies using overexpressing wt Hsp27, as well as loss-of-function strategies using an Hsp27 ASO (OGX-427), dominant-negative Hsp27 phosphorylation TM, or an Hsp27 phosphorylation inhibitor (p38 kinase inhibitor, SB 203580). PSA transactivation assays were done using LNCaP cells transiently transfected with the luciferase reporter plasmid regulated by the PSA enhancer-promoter region (6.1 kb) in the presence or absence of an increasing amount of exogenous wt Hsp27 (0, 0.25, 0.5, and 1 μg). R1881 treatment increased AR reporter gene expression 34-fold, whereas wt Hsp27 overexpression increases androgen-stimulated transcriptional activity of PSA a further 3-fold (Fig. 4A, left). In contrast, Hsp27 knockdown using OGX-427 decreased the transactivation of the androgen-regulated PSA reporter in a dose-dependent manner, further supporting a role for Hsp27 in AR transactivation (Fig. 4A, right).

To determine whether Hsp27 phosphorylation is required for R1881-induced AR-mediated gene activation, we overexpressed an Hsp27 TM (Ser15, Ser78, and Ser82 substituted by alanine) in a dose-dependent manner and tested its ability to affect R1881-stimulated PSA transactivation. As shown in Fig. 4B (left), Hsp27 TM transfection inhibited luciferase transactivation from the PSA enhancer in response to R1881. Similarly, inhibition of androgen-induced, p38 kinase–mediated Hsp27 phosphorylation using the p38 kinase inhibitor, SB 203580, suppressed R1881-mediated transactivation of PSA enhancer–driven luciferase activity (Fig. 4B,
Figure 4. Effect of Hsp27 on AR transactivation. A, Hsp27 overexpression increases AR transactivation. LNCaP cells were transiently cotransfected with 1 μg of PSA-luciferase and indicated concentrations of wt Hsp27, followed by R1881 or vehicle for 24 h. Total amount of plasmid DNA transfected was normalized to 2 μg per well by the addition of an empty vector (left). Hsp27 knockdown decreases AR transactivation. LNCaP cells were treated with the indicated dose of OGX-427 or mismatch control (right). Cells were harvested, and luciferase activity was determined. Columns, means of at least three independent experiments done in triplicate. Fold is measured relative to PSA activation with no treatment. B, effect of Hsp27 phosphorylation on AR transactivation. LNCaP cells were transiently cotransfected with 1 μg of PSA-luciferase with indicated concentrations of Hsp27 TM (Ser15, Ser78, and Ser82 were mutated to alanine) followed by R1881 or vehicle for 24 h. The total amount of plasmid DNA transfected was normalized to 2 μg per well by the addition of an empty vector (left). LNCaP cells were transfected with 1 μg per well of PSA-luciferase before the indicated concentration of SB 203580 for 1 h before R1881 treatment (right). Columns, means of at least three independent experiments done in triplicate. Fold is measured relative to PSA activation with no treatment. C, Hsp27 knockdown decreases PSA mRNA expression (left). LNCaP cells were treated as indicated and RNA extracted for Northern blot analysis of PSA and Hsp27, using GAPDH as a loading control. Hsp27 TM decreases PSA mRNA expression (middle). LNCaP cells were transfected with Hsp27 TM for 24 h after transfection, cells were treated with R1881 for 16 h, and RNA was extracted for Northern blot analysis. p38 kinase inhibition decreases PSA mRNA expression (right). LNCaP cells were treated for 2 h with SB203580 (10 μmol/L) before R1881, and RNA were extracted for Northern blot analysis. D, Hsp27 knockdown decreases AR binding to its response elements. EMSA was done using radiolabeled PSA oligonucleotides with nuclear cell extracts isolated from LNCaP treated with different concentrations of Hsp27 ASO. For controls, 10 μg of nuclear protein were incubated 20 min with cold PSA oligonucleotide. E, Hsp27 recruitment to the promoter of PSA gene. LNCaP cells were treated with 10 nmol/L R1881 for 4 h. Soluble chromatin was prepared from formaldehyde cross-linked and sonicated cell culture. Immunoprecipitation was done using AR or Hsp27 antibodies, along with IgG as control. The final DNA extractions were amplified using the primers for ARE I and ARE III.
Collectively, these results indicate that androgen-induced p38 kinase-mediated phosphorylation of Hsp27 is important for AR-mediated PSA expression.

The preceding data identified a role for phospho-Hsp27 in the transactivation of PSA. To provide further evidence to substantiate this hypothesis, Northern blot analyses were done (Fig. 4C) and indicated that levels of AR-regulated endogenous PSA mRNA decreases significantly after Hsp27 knockdown (OGX-427; Fig. 4C, left), p38 kinase inhibition (SB 203580; Fig. 4C, middle), or Hsp27 phosphorylation inhibition (Hsp27 TM; Fig. 4C, right). These results confirm that Hsp27 levels and phosphorylation status enhance AR-mediated PSA mRNA expression.

As a transcription factor, AR translocates to the nucleus after androgen binding, where it interacts with ARE to transactivate its target genes. Suppression of Hsp27 levels or phosphorylation negatively regulates androgen-stimulated transcriptional activity of PSA. To determine whether AR interaction with its response elements is affected by Hsp27 levels, we next analyzed the effects of Hsp27 knockdown on AR binding to PSA-ARE using electrophoretic mobility shift assay (EMSA) with nuclear lysates of LNCaP cells treated with OGX-427. As shown in Fig. 4D, 10 nmol/L R1881 increased AR binding to ARE, and this effect was specifically blocked by excess cold PSA oligonucleotide. Hsp27 knockdown using OGX-427 decreased AR binding to its ARE, confirming that Hsp27 knockdown decreases AR binding to its ARE. Collectively, the preceding data indicate that phospho-activated Hsp27 plays a critical role in androgen-induced nuclear translocation and transcriptional activity of the AR.

We next explored whether Hsp27 forms a complex with AR on the ARE of the PSA promoter using ChIP assay in LNCaP cells ± R1881. β-Actin fragment was amplified as control. As shown in Fig. 4E, Hsp27 is present, along with AR (which serves as a positive control) on the AREs. In the presence of R1881, higher levels of AR and Hsp27 were recruited to the promoter proximal region (ARE I) and also to the enhancer region (ARE III) of the PSA promoter. These data indicate that Hsp27 continues to interact with the AR transcriptional complex at the level of the ARE and seems to be regulated by androgen.

Hsp27 knockdown induces AR degradation via the proteasome-mediated pathway. Hsp27 knockdown or inhibition of phosphorylation inhibits androgen-stimulated nuclear translocation of the AR with subsequent suppression of AR-regulated gene expression. To investigate the fate of AR after Hsp27 knockdown, changes in AR mRNA and protein levels after treatment with OGX-427 were evaluated. AR mRNA levels did not change after Hsp27 knockdown (Fig. 5A, left). In contrast, Hsp27 knockdown decreased AR (Fig. 5A, middle) and Hsp90 (Fig. 5A, right) protein levels in a dose-dependent manner. The effect of Hsp27 knockdown on AR protein stability was next evaluated using cycloheximide, which inhibits protein synthesis. As shown in Fig. 5C, AR protein levels decrease significantly with rapid degradation after OGX-427–induced knockdown of Hsp27. In contrast, Hsp27 overexpression prolonged AR half-life compared with empty vector-transfected controls (Fig. 5C). AR degradation after Hsp27 knockdown occurs via the proteasome pathway because treatment with the proteasome inhibitor MG-132 suppressed Hsp27 knockdown-induced AR degradation (Fig. 5C). Taken together, these findings indicate that Hsp27 knockdown induces AR degradation via a proteasome-mediated pathway.

Hsp27 knockdown disrupts AR-Hsp90 association and increases AR-MDM2 association and ubiquitination. AR forms a heterodimer complex with Hsp90 to provide stability for ligand-unbound AR. Indeed, without Hsp90 binding, the unfolded protein will be recognized and degraded by the ubiquitin-proteasome system (12, 35). Hsp27 knockdown by OGX-427 significantly disrupts the association between AR and Hsp90 (Fig. 5D, left). This indicates that Hsp27 knockdown–induced dissociation between AR and Hsp90 may render the AR-Hsp90 heterocomplex vulnerable to degradation by the proteasome-mediated pathway. Because AR has been reported to be ubiquitinated by the E3 ligase, MDM2 (36, 37), we next set out to determine whether interaction between MDM2 and AR increased after OGX-427–induced Hsp27 knockdown. As predicted, Hsp27 knockdown increased the association between endogenous MDM2 and AR as shown by co-immunoprecipitation experiments in Fig. 5D (middle). Furthermore, Hsp27 knockdown increased levels of ubiquitinated AR (Fig. 5D, right). These results indicate that OGX-427–induced Hsp27 knockdown disrupts the Hsp90-AR heterocomplex and increases MDM2-mediated AR ubiquitination and degradation.

In vivo Hsp27 knockdown by OGX-427 decreases LNCaP proliferation rates, serum PSA levels, and AR client protein expression levels. Our results establish a novel mechanism whereby ligand-activated AR phospho-activates Hsp27 to cooperatively enhance AR nuclear translocation and transcriptional activity. Hsp27 knockdown leads to AR degradation and reduced PSA transcription and LNCaP cell growth in vitro. We sought to determine whether in vivo Hsp27 knockdown with OGX-427 decreased AR activity in LNCaP xenografts. Male nude mice were injected s.c. with 1 × 10⁶ LNCaP-Probasin–driven luciferase-transfected cells (Supplementary Data S3), and once tumors were palpable with serum PSA levels ~25 ng/mL and bioluminescence was detectable using the IVIS Xenogen monitor, mice were treated with 20 mg/kg of OGX-427 or mismatch control. A bioluminescent signal first became detectable when serum PSA levels reached ~5 ng/mL and was easily detected at serum PSA levels above 20 ng/mL. Mice were subjected to three different types of measurements: bioluminescence of probasin-promoter–driven luciferase activity (a measure of in vivo AR activity in LNCaP xenografts), serum PSA levels, and tumor volume. Measurements were done at baseline before treatment (day 0), during treatment (day 4), and at the end of treatment (day 8). Beginning day 4, OGX-427 decreased bioluminescence and reduced circulating PSA levels by 60% (P < 0.01), with a slight 15% decrease in tumor volume (Fig. 6A and B); individual data are presented in Supplementary Data S4. In contrast, mice treated with mismatch control ODN showed an anticipated increasing trend on bioluminescence and PSA levels, coincident with increasing tumor volume during the 8-day treatment period. Western blot of snap-frozen xenograft samples (Fig. 6C) and immunostaining data (Supplementary Data S5) indicate that OGX-427 decreased LNCaP xenograft levels of AR, Hsp27, and Hsp90. In addition, OGX-427 also decreased Ki67 staining (Supplementary Data). Collectively, these data suggest that the in vivo anticancer activity of OGX-427 results, in part, from AR disruption, and that serum PSA may prove a useful surrogate of pharmacodynamic activity as OGX-427 moves into human trials.

Discussion

Hsp27 is an ATP-independent chaperone that is phospho-activated by cell stress to prevent aggregation and/or regulate activity/degradation of certain client proteins. The chaperone activity
of Hsp27 is regulated by stress-induced changes in phosphorylation and oligomerization (38). As a cytoprotective chaperone situated as a Hub at the center of many pathways regulating cellular response to therapeutic stress, targeted inhibition of Hsp27 would inhibit many pathways implicated in cancer progression and resistance. The cytoprotective effects of Hsp27 result from its ubiquitin binding and degradation of IκB (20), direct interference of caspase activation, modulation of oxidative stress, and regulation of the cytoskeleton (19, 39). Higher levels of Hsp27 are commonly detected in many cancers including prostate (22, 23, 40) and is associated with metastasis, poor prognosis, and resistance to chemotherapy or radiation (41, 42). We recently reported that overexpression of Hsp27 in LNCaP cells suppressed castration-induced apoptosis and confers androgen resistance (23), whereas Hsp27 knockdown using ASO potently decreases Hsp27 levels, increases caspase-3 cleavage and apoptosis, enhances paclitaxel chemosensitivity, and delays tumor progression in vivo (22, 23).

Figure 5. Effect of Hsp27 knockdown on AR expression and stability. A, AR mRNA levels are not affected by Hsp27 knockdown (left). LNCaP cells were treated in a dose-dependent manner with OGX-427 or mismatch control oligos, and AR Hsp27 mRNA levels in LNCaP cells were analyzed by reverse transcription-PCR, and actin was monitored as a control. AR and Hsp90 protein levels are decreased by Hsp27 knockdown. LNCaP cells were treated with the indicated concentration of OGX-427 or mismatch control, and protein levels of AR (middle), Hsp90 (right), and Hsp27 were determined by Western blot analysis. AR protein levels were measured by Western blot analysis. B, Hsp27 levels affect AR stability. LNCaP cells were treated with 70 nmol/L OGX-427 or mismatch control (left) or transfected with empty vector or Hsp27 wt and then treated with 10 μmol/L cycloheximide for the indicated time period. DMSO was used as control. AR and Hsp27 protein levels were measured by Western blot analysis. C, Hsp27 knockdown accelerates proteasomal degradation of AR. LNCaP cells were treated with OGX-427 or mismatch control and 10 μmol/L MG-132 for 6 h. DMSO was used as control. AR protein level was measured by Western blot analysis. D, effect of Hsp27 knockdown on AR/MDM2 association: LNCaP cells were treated with 70 nmol/L OGX-427 in the presence of MG-132 (10 μmol/L) for 6 h. AR immunoprecipitation and Western blot was done with anti-AR antibody (left). Effect of Hsp27 knockdown on AR/MDM2 association: LNCaP cells were treated with 70 nmol/L OGX-427 in the presence of MG-132 (10 μmol/L) for 6 h. AR immunoprecipitation and Western blot was done with anti-AR antibody (right). Membrane was stripped and blotted with anti-AR antibody as control immunoprecipitation. Input was blotted with Hsp27 antibody.
interleukin-6 (IL-6), IL-1, heat shock and tumor necrosis factor-α (44). Using LNCaP cells, which express AR and are sensitive to androgens, we show that androgens increase phospho-Hsp27 levels within minutes in a dose-, time-, and p38 kinase pathway-dependent manner. This rapid androgen/AR-mediated Hsp27 phosphorylation identifies a nongenomic mechanism for AR in LNCaP cells. These findings are in agreement with previous studies indicating that steroids can act via classic steroid receptors or

Figure 6. OGX-427 suppresses probasin luciferase (Pb-Luc) bioluminescence as well as AR, Hsp90, and Hsp27 levels in vivo. A, in vivo imaging of LNCaP–Pb-Luc xenografts after OGX-427 treatment by IVIS imaging system. Intact male mice were injected s.c. with 1 × 10^6 LNCaP–Pb-Luc cells and, once tumors formed with serum PSA levels >25 ng/mL, were treated with 20 mg/kg of OGX-427 or mismatch for 7 d. Bioluminescence indicates probasin luciferase activity before (day 0), during (day 4), and after (day 8) treatment. B, effect of OGX-427 treatment on serum PSA levels. Changes in serum PSA levels from mice treated with OGX-427 or mismatch control were measured using IMX immunoassays (left). Effect of OGX-427 treatment on LNCaP tumor volume. Xenograft volume was measured by caliper at indicated time (right). Points, average of five mice per group; bars, SE. C, total LNCaP xenograft proteins were extracted in RIPA buffer after mismatch or OGX-427 treatment (five mice per group) and Western blots done with AR, Hsp90, and Hsp27 antibodies; vinculin was used as a loading control. D, schema illustrating cooperative interactions between ligand-activated AR and Hsp27 phosphorylation: Androgen binding to AR leads to rapid Hsp27 phosphorylation via p38 kinase pathway, which displaces Hsp90 and chaperones AR to the nucleus to enhance activation of AR-regulated genes (left). OGX-427 induced Hsp27 knockdown destabilizes AR/Hsp90 heterocomplex and leads to MDM2-mediated ubiquitination and AR proteasomal degradation (right).
through apical membrane receptors, and that AR mediates nongenomic activation in response to androgens (6, 10). Ligand binding to AR induces its association with Src via Src SH3 domain and AR proline-rich domain, triggering Src-dependent pathway activation (6, 10). In AR-negative COS-1 and PC3 cells, transfection of AR is necessary for R1881 induction of c-Src/Raf/extracellular signal-regulated kinase and Akt, respectively. In AR-positive LNCaP cells, the anti-androgen bicalutamide suppresses R1881-stimulated Hsp27 phosphorylation. In AR-negative PC3 cells, Hsp27 phosphorylation is insensitive to androgens, but is enhanced when AR is transiently overexpressed in PC3 cells. This nongenomic stimulation is supported further by the interaction and colocalization of Hsp27 with AR. Hsp27 binds with AR via its Neh2-terminal domain and may directly or indirectly involve other AR coregulators, including signal transducers and activators of transcription 3 (STAT3) and ARA55, both of which have been reported to associate with Hsp27 (45, 46).

In the classic model of androgen action, in response to androgens, AR dissociates from Hsp90 (11). Interestingly, we found that following androgen treatment, Hsp27 becomes more abundantly associated with AR as AR dissociates from Hsp90, suggesting a dynamic role for molecular chaperones in AR shuttling. This nongenomic action of AR ultimately influences its classic genomic effects. Once in the nucleus, ligand-bound nuclear receptors like AR are recruited to target gene promoters either through direct binding to hormone response elements or association with other promoter-bound transcription factors (11). Many proteins participate in the activation of AR, some directly binding to AR, and others via a tertiary complex with other transactivators. Hsp27 has now been identified as a chaperone interacting with the AR transcriptional complex at the level of its ARE. Recent reports indicate that the AR coactivators STAT3 and ARA55 also interact with Hsp27 (23, 45). Hence, Hsp27 and AR may complex with either ARA55 or STAT3 to promote AR translocation and transcription. Interestingly, PSA transactivation in LNCaP cells is enhanced by Hsp27 overexpression and suppressed by Hsp27 knockdown or inhibition of Hsp27 phosphorylation. Similarly, in ER+ MCF-7 breast cancer cells, inhibition of p38 kinase signaling also blocks ER-mediated transcription by inhibiting nuclear translocation of ERα (47). Furthermore, Hsp27 knockdown decreases AR nuclear translocation and binding to its ARE.

Previous studies emphasize the importance of Hsps in steroid receptor stability. For example, Hsp90 inhibitors such as geldanamycin induce steroid receptor degradation by directly binding to the ATP-binding pocket of Hsp90 and thereby inhibiting its function (12, 35). Results shown in Figs. 5 and 6 indicate that Hsp27 knockdown destabilizes AR by inducing the dissociation of the AR/ Hsp90 heterocomplex and increasing AR association with the E3 ligase MDM2, with subsequent ubiquitin-proteasome–mediated AR degradation. Although both OGX-427 and geldanamycin share the ability to abrogate the interaction between AR and Hsp90, they do so by different mechanisms. Interestingly, OGX–427 induces the degradation of Hsp27, AR, and Hsp90, whereas geldanamycin binding to the ATP-binding pocket of Hsp90 inhibits its chaperone activity (48), induces degradation of client proteins (49), and is accompanied by stress-activated increases in Hsp70 and Hsp27 (50).

The effects of OGX-427 on AR expression and activity in vitro were recapitulated in vivo. OGX-427 induced rapid decreases in probasin-luciferase reporter–driven bioluminescence that correlated with early decreases in serum PSA and decreased LNCaP xenograft levels of AR and its chaperones, Hsp90 and Hsp27. In prostate cancer where the AR is critically important, ligand-activated AR leads to rapid p38 kinase–mediated phosphorylation of Hsp27, which, in turn, complexes with and chaperones the AR to enhance its stability, shuttling, and transcriptional activity. OGX-427–induced knockdown of Hsp27 destabilizes the AR and enhances its ubiquitination and degradation. Collectively, these data identify a novel mechanism for Hsp27 as an AR chaperone (as summarized in model illustrated in Fig. 6D) and justifies further investigation targeting Hsp27 as therapeutic for prostate cancer.

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The University of British Columbia has submitted patent applications, listing Dr. Gleave as inventor, on the antisense sequence described in this paper. This IP has been licensed to OncoGenex Technologies, a Vancouver-based biotechnology company that Dr. Gleave has founding shares in.

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Cooperative Interactions between Androgen Receptor (AR) and Heat-Shock Protein 27 Facilitate AR Transcriptional Activity

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