Steroidogenic Enzyme AKR1C3 Is a Novel Androgen Receptor-Selective Coactivator that Promotes Prostate Cancer Growth

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Abstract

**Purpose:** Castration-resistant prostate cancer (CRPC) may occur by several mechanisms including the upregulation of androgen receptor (AR), coactivators, and steroidogenic enzymes, including aldo keto reductase 1C3 (AKR1C3). AKR1C3 converts weaker 17-keto androgenic precursors to more potent 17-hydroxy androgens and is consistently the major upregulated gene in CRPC. The studies in the manuscript were undertaken to examine the role of AKR1C3 in AR function and CRPC.

**Experimental Design:** LNCaP cells stably transfected with AKR1C3 and VCaP cells endogenously expressing AKR1C3 were used to understand the effect of AKR1C3 on prostate cancer cell and tumor growth in nude mice. Chromatin immunoprecipitation, confocal microscopy, and co-immunoprecipitation studies were used to understand the recruitment of AKR1C3, intracellular localization of AKR1C3 and its interaction with AR in cells, tumor xenograft, and in Gleason sum 7 CRPC tissues. Cells were transiently transfected for AR transactivation. Novel small-molecule AKR1C3-selective inhibitors were synthesized and characterized in androgen-dependent prostate cancer and CRPC models.

**Results:** We identified unique AR-selective coactivator- and prostate cancer growth-promoting roles for AKR1C3. AKR1C3 overexpression promotes the growth of both androgen-dependent prostate cancer and CRPC xenografts, with concomitant reactivation of androgen signaling. AKR1C3 interacted with AR in prostate cancer cells, xenografts, and in human CRPC samples and was recruited to the promoter of an androgen-responsive gene. The coactivator and growth-promoting functions of AKR1C3 were inhibited by an AKR1C3-selective competitive inhibitor.

**Conclusions:** AKR1C3 is a novel AR-selective enzymatic coactivator and may represent the first of more than 200 known nuclear hormone receptor coactivators that can be pharmacologically targeted.

Introduction

Prostate cancer is the most frequently diagnosed cancer in men with more than 200,000 new prostate cancer cases estimated for 2012 (American Cancer Society, Facts and Figures). The mainstay of therapy for advanced prostate cancer is the reduction of peripheral androgens to castrate levels. Androgen deprivation therapy creates an androgen-deficient state in which prostate cancer evolves into castration-resistant prostate cancer (CRPC) and tumor progression occurs (1). Newer therapies that target androgen metabolizing enzymes and/or androgen receptor (AR) have shown clinical efficacy, indicating the continued importance of the androgen signaling axis in advanced prostate cancer (2).

A variety of mechanisms are thought to contribute to the emergence of the androgen hypersensitivity that is observed in CRPC. Overexpression of AR and coactivators drives the growth and metastasis of CRPC even in response to low levels of androgens (3–5). Despite having castrate levels of serum testosterone, tumor samples obtained from men with CRPC have only a 60% reduction in intratumoral dihydrotestosterone (DHT). This suggests that intratumoral conversion of weak adrenal androgens or androgen precursors produced de novo in the tumor fuel the growth of CRPC (6).

Androgen biosynthesis enzyme inhibitors have been used in the treatment of advanced prostate cancer. Drugs such as ketoconazole and abiraterone acetate to treat...
advanced prostate cancer have emphasized the importance of conversion of androgen precursors either de novo or from adrenal androgens in maintaining CRPC growth (7). Because these inhibitors inhibit early steps in steroidogenesis, they not only inhibit androgen biosynthesis, but also affect the levels of other physiologically relevant steroids such as glucocorticoids and mineralocorticoids.

Aldo keto reductase 1C3 (AKR1C3) is an enzyme with 17β-hydroxysteroid dehydrogenase (17β-HSD) activity and important for the biosynthesis of testosterone and estradiol, is speculated to play a pivotal role in the emergence of CRPC (8–10). AKR1C3, downstream in the steroidogenic enzyme cascade, facilitates the conversion of weak androgens androstenedione (Androne) and 5α-androstane-3β,17β-dione (5α-dione) to the more active androgens, testosterone, and DHT, respectively (Fig. 1A). Gene expression studies showed a significant increase in AKR1C3 expression in CRPC over normal prostate (9, 10). Although the overexpression of AKR1C3 in CRPC is well documented, its role in prostate cancer progression remains unclear, and AKR1C3 is thought to be limited to its role as an enzyme in androgen biosynthesis. The interactions of AKR1C3 with AR and its role in AR function have not been explored. If AKR1C3 plays a role in CRPC progression, tumor confined expression will make it an ideal tissue-selective therapeutic target for prostate cancer.

The AKR1C family comprises four members (1C1–1C4; ref. 11, 12). Although all the four isoforms are expressed in liver, their expression varies in other tissues. AKR1C3 is expressed in many endocrine organs such as prostate, adrenals, breast, and uterus. The four isoforms have high sequence homology sharing more than 83% identity, and their crystal structures show conservation of key residues.

Translational Relevance

Castration-resistant prostate cancer (CRPC) is characterized by the emergence of a hypersensitive androgen signaling axis after orchietomy or medical castration. The revival of androgen signaling is believed to be due to high intratumoral androgen synthesis, fueled by upregulation of steroidogenic enzymes, including aldo keto reductase 1C3 (AKR1C3). Here, for the first time, using molecular and in vivo preclinical models, and human CRPC tissues, we show that the steroidogenic enzyme AKR1C3 also acts as a selective coactivator for androgen receptor to promote CRPC growth. Moreover, novel small-molecule inhibitors inhibit both the enzymatic and coactivator functions of AKR1C3 resulting in androgen-dependent prostate cancer and CRPC regression. These observations identify AKR1C3 as the first novel receptor- and tissue-selective pharmacologically targetable coactivator that promotes prostate cancer growth. This is also the first in vivo evidence showing the importance of AKR1C3 and the use of its inhibitors in prostate cancer.

Considering that all isoforms, other than AKR1C3, convert potent to weak androgens, it is important to develop inhibitors of AKR1C3 that do not cross react with other isoforms (11).

The functions of AR are highly dependent on its coactivators. More than 200 coactivators have been identified and many of them are overexpressed in prostate cancer (13). Several groups successfully used peptides and siRNAs to block coactivator interaction with AR, thereby restricting AR function and subsequently prostate cancer growth (14). However, the lack of known binding pockets nullifies the coactivators’ potential to be small-molecule drug targets.

In this study, we show that AKR1C3 is an AR-selective coactivator that facilitates the growth of both androgen-dependent prostate cancer and CRPC. AKR1C3 interacts with AR in cells and in advanced prostate cancer samples and gets recruited to the promoter of an AR-responsive gene. We further show that a novel small molecule that competitively binds to the AKR1C3 substrate-binding pocket can inhibit its role as a biosynthetic enzyme and AR coactivator, suggesting that AKR1C3 inhibitors may have utility in targeted therapy of both androgen-dependent prostate cancer and CRPC.

Materials and Methods

Reagents

AR antibody, PG-21, were obtained from Millipore, AR antibody, AR N-20, was obtained from Santa Cruz biotechnology, AKR1C3 mouse monoclonal antibody was obtained from Sigma, and AKR1C3 rabbit polyclonal antibody was obtained from Life Technologies. Actin antibody was procured from Chemicon international. Human PSA ELISA was procured from R&D systems. Accell siRNA was procured from Dharmacon. All other reagents used were analytical grade from Fisher.

Cell culture

All cells were obtained from American Type Culture Collection and were grown according to the instructions provided. The cell lines were authenticated by the provider and were cultured for less than 6 months after resuscitation in the laboratory. For the chromatin immunoprecipitation (ChiP) and co-immunoprecipitation (co-IP) assays, cells were plated in 10 cm dishes at 5 million cells per dish in medium supplemented with 1% charcoal-stripped FBS (csFBS).

Transfection and transactivation assay

Plasmids and transfection assays were described earlier (15). GRE-LUC and SRC-2 coactivator plasmids were kindly provided by Dr. Nancy L. Weigel and Dr. Bert W O’Malley (Baylor College of Medicine, Houston, TX). Transfections of LNCaP cells with Amaxa electroporator (Amaxa Inc.,) were conducted according to the manufacturer’s protocol. Stable LNCaP and NIH3T3 cell lines were generated by lentiviral infection of AKR1C3 cloned into pLenti U6 Pgk-puro vector as described earlier (16, 17). LacZ and AR adenovirus were made at Seven Hills Bioreagents.
Chromatin immunoprecipitation assay

ChIP assays were conducted with AR N-20 and AKR1C3 rabbit polyclonal antibodies as described earlier (18). Pros-
Figure 2. AKR1C3 physically interacts with AR. A, left, co-IP of AR and AKR1C3. LNCaP-AKR1C3 cells were serum starved for 2 days and treated with 10 nmol/L R1881 for 6 hours. Cells were harvested, protein extracted, and immunoprecipitated with AKR1C3 antibody and Western blotted for AR. A, right, AR-AKR1C3 colocalization showed by laser confocal microscopy. (Continued on the following page.)
Duolink PLA
Duolink kit (O’link) was purchased and was used to determine the interaction between AR and AKR1C3 (19). Images were obtained using deconvolution fluorescent microscopy.

Prostate cancer specimens
Section of prostate cancer specimens \(n = 6\) with Gleason score 7 \((4 + 3)\) were obtained under The University of Tennessee (Memphis, TN), Institutional Review Board approval. Pathologist report indicates a minimum of 60% and maximum of 85% of the samples to be positive for prostate cancer. The specimens were subjected to Duolink’s proximity ligation assay (PLA) with AR and AKR1C3 antibodies. For negative control, the samples were probed with AKR1C3 antibody and immunoglobulin G (IgG).

RNA isolation and gene expression
RNA was isolated using Cells-to-CT Kit (Applied Biosystems) and real-time PCR was conducted using TaqMan primers and probes from Applied Biosystems on ABI 7900 (Applied Biosystems).

Growth assay
LNCaP cells were plated at 10,000 cells per well of a 96-well plate in RPMI supplemented with 1% csFBS. The cells were treated as indicated in the figures. Cell viability was measured using sulforhodamine (SRB) reagent.

Tumor xenograft experiments
All animal protocols were approved by The University of Tennessee Animal Care and Use Research Committee. Xenograft experiments were carried out as previously published (15). Briefly, a mixture of 2 x 10³ LNCaP or VCaP cells were suspended in 0.0375 mL RPMI + 10% FBS and 0.0625 mL Matrigel/animal and were injected subcutaneously. Once the tumor size reached 200 to 300 mm³, the animals were castrated or sham operated, randomized, DHT pellets were implanted subcutaneously or not supplemented, and treated as indicated in the figures. Tumor volume and body weight were measured.

(Continued.) LNCaP-AKR1C3 cells plated on coverslips and serum starved for 2 days were treated with vehicle, 100 nmol/L A’dione, or 10 nmol/L R1881 for 6 hours. Cells were fixed and immunostained with primary antibodies specific to AR and ARK1C3 and fluorescent-tagged secondary antibodies. The immunofluorescent signals were captured by laser confocal microscopy. B, AKR1C3 is recruited to PSA enhancer. LNCaP-AKR1C3 or LNCaP-Vector cells were maintained in serum-free conditions for 3 days and were treated with 100 nmol/L A’dione or 10 nmol/L R1881 for 2 hours and CHIP assay was conducted with AR (left) or AKR1C3 (right) antibodies. DNA was purified and real-time PCR was conducted for PSA enhancer or a nonspecific region (N.S.) and normalized to input. Representative data from triplicate experiments is given. C, endogenous AR and AKR1C3 interact in VCaP cells (left) and VCaP xenograft (right). VCaP cells were serum starved for 2 days and treated with vehicle or 10 nmol/L R1881 and immunoprecipitation conducted as described in panel A. Right, protein was extracted from VCaP CRPC xenografts \(n = 3\) and was subjected to immunoprecipitation with AR antibody or IgG. The Iped samples and 20% input samples were fractionated on an SDS-PAGE and Western blotted with AKR1C3 antibody and AR antibody. D, AR and AKR1C3 interact in advanced prostate cancer specimens. Tissue sections from Gleason score 7 prostate cancer \(n = 6\) were subjected to Duolink PLA with AR and AKR1C3 antibodies (interaction) or AR antibody replaced with IgG (negative control bottom). Nucleus was counterstained with 4’, 6-diamidino-2-phenylindole (DAPI) and images captured using fluorescent microscopy. Representative images from each sample are displayed. Bar graph of pixel intensity of scanned slides is represented. Figures are representative of \(n = 3\). IB, immunoblotting.

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Results

AKR1C3 overexpression increases AR-dependent gene expression
Although AKR1C3 is increased in CRPC, its role on gene expression and growth of prostate cancer cells is not known. PSA gene expression was measured in LNCaP cells transfected with AKR1C3 using Amaxa electroporator and treated with increasing concentrations of A’dione (Fig. 1B, left) or R1881 (Fig. 1B, middle). AKR1C3 overexpression (Fig. 1B, right) increased PSA gene expression in response to both A’dione and R1881.

AKR1C3 overexpression promotes prostate cancer xenograft implantation and growth
LNCaP-vector or LNCaP-AKR1C3 cells (LNCaP cells stably infected with lentivirus carrying AKR1C3) were injected subcutaneously in nude mice. The animals were castrated and supplemented with suboptimal concentration of DHT (Fig. 1C) or left intact (Supplementary Fig. S1). AKR1C3-transfected tumors had a higher incidence of tumor implantation (Fig. 1C, right) and better rate of tumor growth (Fig. 1C, left) than LNCaP-vector tumors. Consistently, the tumor incidence was two times higher in LNCaP-AKR1C3–bearing animals than LNCaP-vector–bearing animals. Expression of AR-dependent genes such as FKBP51 and TMPRSS2 (Fig. 1C, lower) increased significantly and their protein levels were higher in LNCaP-AKR1C3 tumors (Fig. 1C, lower), indicating the role for AKR1C3 to enhance AR signaling in androgen-dependent prostate cancer and CRPC progression. A significant increase in the androgen-dependent FKBP51 protein expression was observed in LNCaP-AKR1C3 tumors without concomitant increase in AR protein levels (Supplementary Fig. S2).

AKR1C3 siRNA reduces AR signaling in VCaP cells
To determine whether endogenous AKR1C3 is important for androgen signaling, VCaP cells were transfected with nonspecific or AKR1C3 siRNA, treated with R1881, and expression of AR target genes was measured. Reduction in AKR1C3 expression by 70% to 80% reduced R1881-induced PSA and TMPRSS2 gene expression by 30% to 40% (Fig. 1D), indicating the important role played by AKR1C3 in AR function. The ability of AKR1C3 to augment R1881-induced gene expression, DHT-dependent tumor growth and gene


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AKR1C3 interacts with AR and is recruited to PSA enhancer

The ability of AKR1C3 to potentiate AR function suggested that it might possibly function as a coactivator. Co-IP studies conducted in LNCaP-AKR1C3 or LNCaP-vector cells treated with R1881 indicate that AR and AKR1C3 interacted in a ligand-dependent manner in LNCaP-AKR1C3 cells, but not in LNCaP-vector cells (Fig. 2A, left).

Immunofluorescence studies in LNCaP-AKR1C3 cells were conducted using laser confocal microscopy (Fig. 2A, right). Both AR and AKR1C3 were cytoplasmic in the absence of AR ligands, but translocated into the nucleus upon binding of an AR ligand (R1881) or A’dione. The migratory patterns for AR and AKR1C3 overlapped substantially, supporting the idea that the two proteins interact with each other.

To ensure that AKR1C3’s translocation is dependent on AR, NIH3T3 cells stably transfected with AKR1C3 were infected with adenovirus expressing Lac Z (Supplementary Fig. S3, top) or AR (Supplementary Fig. S3, bottom) and were treated with 10 nmol/L R1881. Although cells were treated with R1881, AKR1C3 was cytoplasmic in the absence of AR and translocated into the nucleus only in the presence of AR, indicating the requirement for the AR presence for AKR1C3 to translocate into nucleus.

Our earlier publication showed that an AR antagonist, SNARE-1, inhibited ligand-dependent AR nuclear translocation (15). We tested the translocation of AR and AKR1C3 in response to R1881 in the presence or absence of SNARE-1. R1881 efficiently translocated AR into the nucleus and AKR1C3 cotranslocated with AR. However, when cells were treated with 10 μmol/L SNARE-1, AR only partially translocated into the nucleus and predominantly remained in the cytoplasm.

The Duolink PLA detects protein–protein interaction by fluorescent visualization (20). DNA attached to the secondary antibodies is ligated and amplified, and the amplified DNA fluoresces red only if the two proteins are in proximity. LNCaP-AKR1C3 cells were treated with R1881 and subjected to PLA. Although AR and AKR1C3 interaction (represented by red fluorescence) was detected in LNCaP-AKR1C3 cells (Supplementary Fig. S5A), interaction was undetected in LNCaP-mock cells or when an antibody was replaced with IgG (Supplementary Fig. S5A). These results were reproduced in VCaP cells, which contain endogenous AR and AKR1C3 (Supplementary Fig. S5B).

If AKR1C3 interacts with AR, it could also be recruited to the response element (ARE) of an AR target gene. LNCaP-AKR1C3 or LNCaP-vector cells were treated with R1881 or A’dione, and the recruitment of AKR1C3 to PSA enhancer ARE was examined using ChIP assay. AKR1C3 was recruited to the PSA enhancer, but not to a nonspecific region, both in response to its substrate, A’dione, as well as in response to R1881 (Fig. 2B). Although AR was recruited to the PSA enhancer in LNCaP-vector cells (Fig. 2B), recruitment of AKR1C3 to the PSA enhancer could not be detected in LNCaP-vector cells due to its limited expression.

To show that AR and AKR1C3 interact in a system where both proteins are endogenously expressed, co-IP studies were conducted in VCaP cells and VCaP CRPC xenograft tumors. AR and AKR1C3 interact robustly in VCaP cells (Fig. 2C, left) in response to R1881 and in CRPC tumor xenograft tissues (Fig. 2C, right).

AKR1C3 and AR interact in advanced prostate cancer

To determine whether the interaction between AR and AKR1C3 observed in prostate cancer cells and xenografts is also observed in human prostate cancer, prostate cancer specimens (n = 6; Gleason sum 4 + 3 = 7) were subjected to PLA with AR and/or AKR1C3 antibody. Although interaction between the two proteins (as visualized by red fluorescence spots) was clearly observed in the tumors (Fig. 2D), the interaction was not detected when one antibody was replaced with IgG (Fig. 2D, bottom). The intensity and the extent of interaction varied between samples. The fluorescent spots were quantified using automated software. The graphs below Fig. 2D clearly indicates a robust increase in pixel intensity, a representation of interaction between the two proteins, in advanced prostate cancer samples.

AR transactivation as AKR1C3 functional assay

AKR1C3 converts A’dione to testosterone resulting in a ligand with stronger AR activity. We captured this principle in an AR transactivation assay. AR activity in response to A’dione in AKR1C3-transfected cells was higher than the AR activity in vector-transfected cells (Fig. 3A). AKR1C3 facilitated AR activation as evidenced by the lower half maximal effective concentration (EC50) and higher Emax of A’dione in AKR1C3-transfected cells. Overexpression of AKR1C3 significantly reduced the EC50 of A’dione to transactivate AR from 415 to 175 nmol/L (Supplementary Table S1). These results support the hypothesis that AKR1C3 overexpression in prostate cancer amplifies or hypersensitizes the AR signaling pathway.

AKR1C3 activates AR in response to active androgens

The results observed in LNCaP-AKR1C3 cells, VCaP cells, and CRPC samples indicated the ability of AKR1C3 to coactivate AR. We conducted AR transactivation studies in AKR1C3-transfected cells using A’dione and two 17-hydroxy AR agonists (testosterone and DHT; ref. 21). Consistent with gene expression results, AKR1C3 increased AR transactivation in response to all ligands that bind to the AR (Fig. 3B). AKR1C3 not only reduced the EC50 of these ligands, but also increased the maximum level of AR transactivation (Emax; Supplementary Table S1).

Varying the level of AKR1C3 expression showed that starting from 0.5 μg AKR1C3 plasmid DNA increased AR transactivation in a concentration-dependent manner (Fig. 3C) without altering the expression of AR (Fig. 3D). The increase in AR transactivation facilitated by AKR1C3 is comparable with that observed with an established
coactivator, SRC-2 (Supplementary Fig. S6), indicative of the robustness in the ability of AKR1C3 to augment AR function. AKR1C3 overexpression was confirmed by real-time rtPCR (Fig. 3C, lower). Transactivation experiments in different cell types (COS-1 and NIH3T3), various transfection conditions (lipofectamine, fugene, and Amaxa electroporator), and cells stably expressing AKR1C3 showed that the AR activation effect of AKR1C3 was not unique to a cell type or transfection condition (Supplementary Fig. S7).

Steroid receptors share sequence homology in many of their functional domains, facilitating their interaction with the same coactivator. Transactivation experiments carried out with glucocorticoid receptor, mineralocorticoid receptor, progesterone receptor, estrogen receptor-α, and peroxisome proliferator-activated receptor γ (PPARγ) established that AKR1C3 is a selective activator of AR function (Supplementary Fig. S8).

AR augmentation effect is selective to AKR1C3

Because members of AKR1C family share high sequence homology, the effect of AKR1C isofoms on AR transactivation in response to R1881 was tested. Interestingly, we found that R1881-induced AR transactivation was augmented only by AKR1C3, but not by AKR1C1, 1C2, or 1C4, indicating the selective ability of AKR1C3 to function as an AR activator (Supplementary Fig. S9).
AKR1C3 and SRC-2 synergize to increase AR transactivation

To determine whether AKR1C3 and a bona fide coactivator, SRC-2, that is overexpressed in prostate cancer (22), synergistically increase AR transactivation, plasmids encoding both proteins were transfected and AR transactivation studies were conducted. Although SRC-2 (Supplementary Fig. S6, top left) and AKR1C3 (Supplementary Fig. S6, top right) concentration dependently increased AR transactivation, cotransfection of suboptimal concentrations of the two plasmids synergistically increased AR transactivation (Supplementary Fig. S6, bottom), suggesting different interaction sites with AR.

Different regions of AKR1C3 mediate the enzymatic and activation function

To determine the regions responsible for the enzymatic and activation functions of AKR1C3, truncated and mutated AKR1C3 constructs were generated in pCR3.1 vector (Supplementary Fig. S10A), and AR transactivation studies in response to A’dione and R1881 were conducted. Amino acids 1-182 failed to mediate the effects of A’dione or finasteride (Supplementary Fig. S6, bottom), suggesting different interaction sites with AR.
R1881. Although full length AKR1C3 (construct A) was required for the effects of A’dione, amino acids 1-282 were sufficient to mediate the effects of R1881 (Supplementary Fig. S10B). The region of AKR1C3 spanning amino acids 171-237 (construct G) was sufficient to mediate the effects of R1881. Point mutation F306A that eliminates the binding of A’dione to AKR1C3 also eliminated its effect on AR transactivation in response to A’dione, but not to R1881 (23). These results were confirmed with PSA gene expression in LNCaP cells expressing construct G or H (Supplementary Fig. S10D). The enzymatic functions of these constructs were confirmed by thin layer chromatography (TLC; Supplementary Fig. S10C). These results suggest that the full-length protein is required to mediate AKR1C3’s enzymatic functions, but that amino acids 171-237 were sufficient to mediate the AR activation.

**Novel small molecules inhibit AKR1C3 activity**

We designed and synthesized a novel series of AKR1C3 inhibitors and compared the activity of the most potent inhibitor, GTx-560 (Fig. 4A), to a known AKR1C3 inhibitor, indomethacin, in *in vitro* purified and cell based-enzyme assays (24). Though GTx-560 and indomethacin comparably inhibited AKR1C3-dependent conversion of A’dione to testosterone (Fig. 4B, left) in purified enzyme assays, GTx-560 alone inhibited AKR1C3 enzyme activity in cells (Figs. 4B, right and C), without cross-reacting with a highly homologous AKR1C1 (ref. 25; Supplementary Fig. S11). The difference in the IC_{50} values between purified enzyme-based system (1-2 μmol/L) and cell-based system (10-50 nmol/L) is due to the difference in concentration of substrate A’dione used (12 μmol/L vs. 100 nmol/L).

Because 5α-reductase converts testosterone to DHT (26), we speculated that finasteride, a 5α-reductase inhibitor, would increase testosterone levels in LNCaP-AKR1C3 cells (Fig. 4D). As expected, finasteride blocked the conversion of testosterone to DHT in LNCaP-AKR1C3 cells, thereby significantly increasing the testosterone levels. By acting upstream of 5α-reductase in the steroidogenic pathway, GTx-560 completely blocked the formation of testosterone from A’dione, indicating the ability of AKR1C3 inhibitors, unlike 5α-reductase inhibitors, to reduce testosterone levels.

**GTx-560 selectively inhibits AKR1C3-dependent AR transactivation**

In agreement with the enzyme inhibition results, GTx-560, but not indomethacin, effectively inhibited the
Figure 6. GTx-560 inhibits androgen signaling and prostate cancer cell and tumor growth. A, GTx-560 inhibits AKR1C3-dependent PSA gene expression and LNCaP-AKR1C3 cell growth. LNCaP-AKR1C3 cells were treated with vehicle or 10 µmol/L GTx-560 in the presence of R1881 or A'dione. PSA gene expression was measured and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH, left). Right, LNCaP-vector (open bars) or LNCaP-AKR1C3 (filled bars) cells were treated with vehicle or 10 µmol/L GTx-560 for 3 days. Cells were fixed and stained with sulforhodamine Blue (SRB) and the optical density (OD) was measured at OD 535 nm. B, GTx-560 inhibits PSA gene expression in VCaP cells. VCaP prostate cancer cells were maintained in serum-free medium for 3 days and treated with vehicle, 10 µmol/L GTx-560 (top), or 10 µmol/L finasteride (bottom). (Continued on the following page.)
AKR1C3-dependent A’dione-induced AR transactivation (Fig. 5A), but not the 17β-HSD3- (Fig. 5B) and AKR1C1-dependent (27) transactivation (Supplementary Fig. S13). This inhibition was observed at all AKR1C3 levels, indicating the potency of GTx-560 to block AKR1C3 enzyme activity (Supplementary Fig. S14).

To understand whether a competitive inhibitor of AKR1C3 such as GTx-560 has the potential to inhibit R1881-induced AKR1C3-dependent AR transactivation, HEK-293 cells transfected with vector or AKR1C3 were treated with increasing concentrations of R1881 alone or in combination with GTx-560. GTx-560 completely inhibited the AKR1C3-dependent transactivation induced by R1881 (Fig. 5C). Though R1881 required amino acids 1-282 of AKR1C3 to elicit its coactivation, GTx-560 required the full-length AKR1C3 to bind and inhibit R1881-induced activity (Fig. 5D).

**GTx-560 inhibits the AKR1C3-induced PSA gene expression and cell growth**

In concordance with AR transactivation, GTx-560, at 10 μmol/L concentration, was very effective in inhibiting the AKR1C3-dependent A’dione- and R1881-induced PSA gene expression (Fig. 6A, left) and A’dione-induced cell growth (Fig. 6A, right).

**Testosterone levels are reduced by AKR1C3 inhibition and not by 5α-reductase inhibition**

As intratumoral levels of testosterone can drive prostate cancer progression, we tested the activity of GTx-560 and finasteride (Fig. 6B) to reduce AR signaling and testosterone production in VCaP, which are CRPC cells that express endogenously high levels of AKR1C1, AKR1C3, and 5α-reductase type-1 (Supplementary Fig. S15). Although finasteride increased PSA gene expression under serum-starved conditions, GTx-560 significantly inhibited PSA gene expression (Fig. 6B). The ability of finasteride to increase PSA gene expression could be due to the higher expression of AKR1C1 (Supplementary Fig. S15) as finasteride significantly decreased these levels (Supplementary Table S2).

**GTx-560 inhibits prostate cancer xenograft growth**

Animals bearing LNCaP-AKR1C3 tumors were castrated when tumors reached 100 mm³ and were allowed to progress. Once the tumors reached 200 to 300 mm³, the animals were treated with vehicle or GTx-560 at 30 mg/kg/d s.c. for 2 weeks. The growth of AKR1C3-dependent tumors was slowed by treatment with GTx-560 (Fig. 6C, top left). Comparable inhibition was also observed in another CRPC xenograft model using VCaP cells, where GTx-560 effectively reduced the tumor growth by 50% (Fig. 6C, top right), serum PSA to one-third of that observed in vehicle-treated group (Fig. 6C, bottom left), and tumor PSA completely (Fig. 6C, bottom middle). To ensure that the tumor volume reduction is due to the exposure to GTx-560, drug concentration was measured in the serum of animals bearing VCaP xenograft and correlated with final tumor volume. As shown in Fig. 6C (bottom right), concentration of GTx-560 in serum inversely correlated with tumor volume, indicating that increased exposure to an AKR1C3 inhibitor such as GTx-560 will impede the growth of CRPC tumors. Statistical analysis of this inverse correlation slope was conducted using regression Bivariate fit (JMP 9.0 from SAS institute). The results indicate a high level of significance with P value for intercept being 0.0022 and for the drug concentration being 0.0351.

**Discussion**

Numerous treatment options for men who develop CRPC are evolving with many new therapies in development. Most of these agents target the AR signaling axis, including abiraterone and prednisone, MDV-3100, TOK-001, TAK-700, and Capesaris (28). The primary mechanism of CRPC progression is the ability of these cancer cells to use androgen precursors from blood or to synthesize de novo testosterone by upregulating steroidogenic enzymes including Cyp17A1 (17, 20 lyase, and 17α-hydroxylase), AKR1C3, and 5α-reductase. Abiraterone, by inhibiting Cyp17A1, has been shown to treat CRPC in a postchemotherapy setting by reducing serum PSA, increasing progression-free survival, and improving overall survival. Interestingly, animal models have indicated that resistance to abiraterone treatment may be due to increase in the expression of its target, Cyp17A1, as well as higher expression of AR and AKR1C3 (8). Moreover, the back door pathways (29–31) to synthesize DHT (Fig. 1A, filled arrows) despite treatment with abiraterone (29) require 17β-HSDs for biosynthesis of potent androgens. With AKR1C3 being the major 17β-HSD...
expressed in CRPC, blocking any enzyme other than AKR1C3 might not be beneficial in CRPC.

The studies presented herein clearly show the dual role for AKR1C3 as an enzyme that converts androstenedione to testosterone and as a coactivator of AR. Both of the actions could be inhibited by a competitive AKR1C3 inhibitor, GTx-560. We were surprised that a receptor-type selective coactivator had a drug-binding pocket that could serve as a therapeutic target. Although p160 coactivators have histone acetyl transferase enzyme activity, they are challenging drug targets due to their lack of a ligand-binding pocket. Even if a drug that targets their interaction surface with the steroid receptor is designed, because of their interaction with multiple receptors, it will likely not be selective to AR. On the other hand, AKR1C3 is an example of a coactivator that is overexpressed in advanced prostate cancer and specifically coactivates AR. Human prostate cancer samples used in our experiment had upregulation of AKR1C3 (Fig. 2D), validating other publications. All these make AKR1C3 a potential drug target to treat advanced prostate cancer. We also show for the first time an AKR1C3 inhibitor, GTx-560, is effective in prostate cancer both in vitro and in vivo, which provides validation of AKR1C3 as a therapeutic target for CRPC.

In addition, AKR1C3 also possesses prostaglandin F2 (PGF2) synthase activity responsible for the synthesis of proliferative and anti-differentiating PGF2. Human myeloid leukemia cells overexpressing AKR1C3 were more proliferative and resistant to the growth inhibitory effects of all-trans retinoic acid and 1, 25 (OH)2 vitamin D3. This was due to the deprivation of PGJ2, a ligand for pro-differentiating PPARy. These effects were overcome by treating cells with a nonsteroidal anti-inflammatory drug (NSAID) AKR1C3 inhibitor, indomethacin (32). Although we speculate that the proliferative effects of AKR1C3 in our studies were due to its 17β-HSD activity, we cannot rule out its 11-ketoreductase or PGF2 synthase activity as one of the contributors to its activity in prostate cancer cells.

Because this is the first study depicting a steroidogenic enzyme as a coactivator of AR, we confirmed the interaction using multiple tools and in different systems. The interaction was convincing in both cells and in human prostate cancer tissue. Because immunohistochemistry and immunofluorescence techniques gave rise to high autofluorescence, to overcome this issue, we used PLA, which gave fluorescent signals specific to areas of interaction only (Fig. 2D). Although more than 200 coactivators for nuclear receptors have been discovered to date, AKR1C3 is distinct in being the first to be pharmacologically targetable. Several questions remain to be answered for this coactivator function of AKR1C3. The most important being whether AKR1C3 could function as AR coactivator when AR is activated by noncanonical signaling pathways such as Src, mitogen-activated protein kinase, and others. Identifying this function could further enhance the therapeutic use of AKR1C3 as an AR-selective coactivator. Others such as the AR domain responsible for AKR1C3 interaction and correlation between AR:AKR1C3 interaction and patient survival data in AKR1C3 overexpressing prostate cancer remain to be addressed.

Interestingly, AKR1C3 interaction with AR in cells is very strong enabling it to migrate with AR from cytoplasm to nucleus and this migration is purely dependent on AR. Furthermore, the FKBP51 protein expression in LNCaP-AKR1C3 xenograft samples compared with LNCaP-mock xenograft samples (Fig. 1C) provided compelling data that AR signaling is robust in tissues expressing AKR1C3 even in the presence of DHT, a response likely magnified because of coactivation.

The evidence in this manuscript clearly suggests a new mechanism for AKR1C3 action in CRPC (Fig. 6D). AKR1C3 converts intratumorally the adrenal androgens into testosterone, which binds to AR or get converted to DHT, resulting in ligand occupancy of AR. AR then interacts with AKR1C3 and gets recruited to the ARE on the promoter of androgen-responsive genes. This recruits several other cofactors leading to magnified transcription and translation of target genes. Blocking AKR1C3 will not only eliminate the enzymatic conversion, but also will block its AR coactivation potential, that could have been activated by noncanonical pathways.

Future clinical trials with AKR1C3 inhibitors will be needed to show their potential to be the next generation of tissue-specific therapeutics for CRPC. However, the unique roles of AKR1C3 as coactivator and androgen biosynthetic enzyme involved in prostate cancer progression identify it as a high priority target for study, and indicate that the interactions between steroid biosynthetic enzymes and steroid receptors may be exceedingly complex and involved in a variety of hormone-dependent cancers.

Disclosure of Potential Conflicts of Interest
C.M. Barrett has ownership interest (including patents) in GTx, Inc. M.S. Steiner is employed (other than primary affiliation; e.g., consulting) as a CEO and CMO, and has ownership interest (including patents) in GTx, Inc. D.D. Miller has commercial research grant and is a consultant/advisory board member of GTx, Inc. No potential conflicts of interest were disclosed by the other authors.

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