Steroidogenic Enzyme AKR1C3 is a Novel Androgen Receptor-Selective Coactivator That Promotes Prostate Cancer Growth

Muralimohan Yepuru, Zhongzhi Wu, Anand Kulkarni, Feng Yin, Christina M. Barrett, Juhyun Kim, Mitchell S. Steiner, Duane D. Miller, James T. Dalton and Ramesh Narayanan

Preclinical Research and Development, GTx Inc., Memphis, TN

1Department of Pathology, University of Tennessee Health Science Center, Memphis, TN.

#RN & JTD share senior authorship.

*Address correspondence to

James T. Dalton (jdalton@gtxinc.com) or Ramesh Narayanan (rnarayanan@gtxinc.com)

Preclinical Research and Development
GTx Inc., 3 North Dunlap, Memphis, TN-38163
Ph. 901-523-9700
Fax. 901-523-9772

Conflict of interest: MY, ZW, FY, CMB, JK, MSS, DDM, JTD, and RN are employees of GTx, Inc.
Translational Relevance

Castration resistant prostate cancer (CRPC) is characterized by the emergence of a hypersensitive androgen signaling axis after orchiectomy or medical castration. The revival of androgen signaling is believed to be due to high intra-tumoral androgen synthesis, fueled by up-regulation of steroidogenic enzymes, including AKR1C3. Here, for the first time, using molecular and in vivo preclinical models, and human CRPC tissues, we demonstrate 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 demonstrating the importance of AKR1C3 and the utility of its inhibitors in prostate cancer.
Purpose: Castration resistant prostate cancer (CRPC) may occur by several mechanisms including the up-regulation of androgen receptor (AR), coactivators, and steroidogenic enzymes, including AKR1C3. AKR1C3 converts weaker 17-keto androgenic precursors to more potent 17-hydroxy androgens and is consistently the major up-regulated 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. ChIP, confocal microscopy, and co-IP 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 over-expression 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 over 200 known nuclear hormone receptor coactivators that can be pharmacologically targeted.

Key Words: Androgen Receptor, Coactivator, Steroidogenic Enzyme, Prostate Cancer, CRPC, AKR1C3.

Running Title: AKR1C3 is an AR coactivator
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 CRPC and tumor progression occurs (1). Newer therapies that target androgen metabolizing enzymes and/or androgen receptor (AR) have demonstrated 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. Over-expression 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). Since 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 aka 17βHSD5), an enzyme with 17β-hydroxysteroid dehydrogenase (17βHSD) 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 (A’dione) and 5α-androstanedione (5α-dione) to the more active androgens, testosterone and DHT, respectively (Figure 1A). Gene expression studies demonstrated a significant increase in AKR1C3 expression in CRPC over normal prostate (9, 10). Although the over-expression 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 of four members (1C1-1C4) (11, 12). While 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 greater than 83% identity and their crystal structures demonstrate conservation of key residues. 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 over-expressed 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 demonstrate 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 (Billerica, MA), AR antibody, AR N-20, was obtained from Santa Cruz biotechnology (Santa Cruz, CA), AKR1C3 mouse monoclonal antibody was obtained from Sigma (St. Louis, MO) and AKR1C3 rabbit polyclonal antibody was obtained from Life Technologies (Carlsbad,
CA). Actin antibody was procured from Chemicon international (Temecula, CA). Human PSA ELISA was procured from R&D systems (Minneapolis, MN). Accell siRNA was procured from Dharmacon. All other reagents used were analytical grade from Fisher.

**Cell culture:** All cells were obtained from ATCC (Manassas, VA) 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 ChIP and co-immunoprecipitation 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., Gaithersburg, MD) were performed according to the manufacturer’s protocol. Stable LNCaP and NIH3T3 cell lines were generated by lentiviral infection of AKR1C3 cloned into pLenti U6 Ptg-puro vector as described earlier (16, 17). LacZ and AR adenovirus were made at Seven Hills Bioreagents (Cincinnati, OH).

**Chromatin immunoprecipitation (ChIP) assay:** ChIP assays were performed with AR N-20 and AKR1C3 rabbit polyclonal antibodies as described earlier (18). PSA enhancer and non-specific region primers and fluorescent probe sequences are described earlier.
**Duolink PLA:** Duolink kit (O’link, Uppsala, Sweden) 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, Institutional Review Board (IRB) 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 PLA with AR and AKR1C3 antibodies. For negative control, the samples were probed with AKR1C3 antibody and IgG.

**RNA isolation and gene expression:** RNA was isolated using cell to ct kit (Applied Biosystems, Carlsbad, CA) and realtime PCR was performed 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 performed as previously published (15). Briefly, a mixture of $2 \times 10^6$ LNCaP or VCaP cells were suspended in 0.0375ml RPMI+10% FBS and 0.0625ml Matrigel/animal and
were injected s.c. Once the tumor size reached 200mm$^3$-300mm$^3$, 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.

**Results**

*AKR1C3 over-expression 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 (Figure 1B left panel) or R1881 (Figure 1B middle panel). AKR1C3 over-expression (Figure 1B right panel) increased PSA gene expression in response to both A’dione and R1881.

*AKR1C3 over-expression 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 sub-optimal concentration of DHT (Figure 1C) or left intact (Figure S1). AKR1C3 transfected tumors had a higher incidence of tumor implantation (Figure 1C right panel) and better rate of tumor growth (Figure 1C left panel) 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 (Figure 1C lower panel)
panels) increased significantly and their protein levels were higher in LNCaP-AKR1C3 tumors (Figure 1C lower panels), 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 (Figure S2).

**AKR1C3 siRNA reduces AR signaling in VCaP cells**

To determine if endogenous AKR1C3 is important for androgen signaling, VCaP cells were transfected with non-specific or AKR1C3 siRNA, treated with R1881 and expression of AR target genes was measured. Reduction in AKR1C3 expression by 70-80% reduced R1881-induced PSA and TMPRSS2 gene expression by 30-40% (Figure 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 expression, and attenuate R1881 response by AKR1C3 siRNA establish and support a non-enzymatic function for AKR1C3.

**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-immunoprecipitation 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 (Figure 2A left panel).
Immunofluorescence studies in LNCaP-AKR1C3 cells were performed using laser confocal microscopy (Figure 2A right panel). 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.

In order to ensure that AKR1C3’s translocation is dependent on AR, NIH3T3 cells stably transfected with AKR1C3 were infected with adenovirus expressing Lac Z (Figure S3 top panel) or AR (Figure S3 bottom panel) and were treated with 10 nM 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 demonstrated 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 co-translocated with AR. However, when cells were treated with 10 μM SNARE-1, AR only partially translocated into the nucleus and predominantly remained in the cytoplasm. AKR1C3 followed the same pattern (Figure S4).
The Duolink proximity ligation assay (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. While AR and AKR1C3 interaction (represented by red fluorescence) was detected in LNCaP-AKR1C3 cells (Figure S5A), interaction was undetected in LNCaP-mock cells or when an antibody was replaced with IgG (Figure S5A). These results were reproduced in VCaP cells, which contain endogenous AR and AKR1C3 (Figure 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 non-specific region, both in response to its substrate, A’dione, as well as in response to R1881 (Figure 2B). Although AR was recruited to the PSA enhancer in LNCaP-vector cells (Figure 2B), recruitment of AKR1C3 to the PSA enhancer could not be detected in LNCaP-vector cells due to its limited expression.

To demonstrate that AR and AKR1C3 interact in a system where both proteins are endogenously expressed, co-immunoprecipitation studies were performed in VCaP cells and VCaP CRPC xenograft tumors. AR and AKR1C3 interact robustly in VCaP cells (Figure 2C left panel) in response to R1881 and in CRPC tumor xenograft tissues (Figure 2C right panel).
AKR1C3 and AR interacts in advanced prostate cancer

To determine if 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. While interaction between the two proteins (as visualized by red fluorescent spots) was clearly observed in the tumors (Figure 2D), the interaction was not detected when one antibody was replaced with IgG (Figure 2D bottom panel). The intensity and the extent of interaction varied between samples. The fluorescent spots were quantified using automated software. The graphs below Figure 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 (Figure 3A). AKR1C3 facilitated AR activation as evidenced by the lower EC\textsubscript{50} and higher Emax of A’dione in AKR1C3 transfected cells. Over-expression of AKR1C3 significantly reduced the EC\textsubscript{50} of A’dione to transactivate AR from 415 nM to 175 nM (Table ST1). These results support the hypothesis that AKR1C3 over-expression 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) (21). Consistent with gene expression results, AKR1C3 increased AR transactivation in response to all ligands that bind to the AR (Figure 3B). AKR1C3 not only reduced the EC$_{50}$ of these ligands, but also increased the maximum level of AR transactivation (Emax) (Table ST1).

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

Steroid receptors share sequence homology in many of their functional domains, facilitating their interaction with the same coactivator. Transactivation experiments performed with glucocorticoid receptor (GR), mineralocorticoid receptor (MR),
progesterone receptor (PR), estrogen receptor (ERα), and peroxisome proliferator and receptor γ (PPARγ) established that AKR1C3 is a selective activator of AR function (Figure S8).

**AR augmentation effect is selective to AKR1C3**

Since members of AKR1C family share high sequence homology, the effect of AKR1C isoforms 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 (Figure S9).

**AKR1C3 and SRC-2 synergize to increase AR transactivation**

To determine if AKR1C3 and a bonafide coactivator, SRC-2, that is over-expressed in prostate cancer (22), synergistically increase AR transactivation, plasmids encoding both proteins were transfected and AR transactivation studies were performed. While SRC-2 (Figure S6 top left panel) and AKR1C3 (Figure S6 top right panel) concentration-dependently increased AR transactivation, co-transfection of sub-optimal concentrations of the two plasmids synergistically increased AR transactivation (Figure S6 bottom panel), suggesting different interaction sites with AR.

**Different regions of AKR1C3 mediate the enzymatic and activation function**

In order to determine the regions responsible for the enzymatic and activation functions of AKR1C3, truncated and mutated AKR1C3 constructs were generated in pCR3.1
vector (Figure S10A) and AR transactivation studies in response to A’dione and R1881 were performed. Amino acids 1-182 failed to mediate the effects of A’dione or R1881. While 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 (Figure 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 (Figure S10D). The enzymatic functions of these constructs were confirmed by TLC (Figure 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 (Figure 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 (Figure 4B left panel) in purified enzyme assays, GTx-560 alone inhibited AKR1C3 enzyme activity in cells (Figures 4B right panel and 4C), without cross reacting with a highly homologous AKR1C1 (25) (Figure S11). The difference in the IC₅₀s between purified enzyme-based system (~2-3 μM) and cell-based system (10-50
nM) is due to the difference in concentration of substrate A’dione used (12 μM vs. 100 nM).

Since 5α-reductase converts testosterone to DHT (26), we speculated that finasteride, a 5α-reductase inhibitor, would increase testosterone levels in LNCaP-AKR1C3 cells (Figure 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 AKR1C3-dependent A’dione-induced AR transactivation (Figure 5A), but not the 17β-HSD3- (Figure 5B) and AKR1C1-dependent (27) transactivation (Figure S13). This inhibition was observed at all AKR1C3 levels, indicating the potency of GTx-560 to block AKR1C3 enzyme activity (Figure S14).

To understand if 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 (Figure 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 (Figure 5D).

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

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

**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 (Figure 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 (Figure S15). While finasteride increased PSA gene expression under serum-starved conditions, GTx-560 significantly inhibited PSA gene expression (Figure 6B). The ability of finasteride to increase PSA gene expression could be due to the higher expression of AKR1C1 (Figure S15) as finasteride significantly increased testosterone levels (Table ST2).

**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 200-300 mm³, the animals were
treated with vehicle or GTx-560 at 30 mg/kg/day s.c. for 2 weeks. The growth of AKR1C3-dependent tumors was slowed by treatment with GTx-560 (Figure 6C top left panel). Comparable inhibition was also observed in another CRPC xenograft model utilizing VCaP cells, where GTx-560 effectively reduced the tumor growth by 50% (Figure 6C top right panel), serum PSA to one-third of that observed in vehicle treated group (Figure 6C bottom left panel), and tumor PSA completely (Figure 6C bottom middle panel). 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 Figure 6C (bottom right panel), 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 performed 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 androgenic precursors from blood or to synthesize de novo testosterone by up-regulating 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 post-chemotherapy 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 (Figure 1A filled arrows) in spite of 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 demonstrate 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, due to 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 over-expressed in advanced prostate cancer and specifically coactivates AR. Human prostate cancer samples used in our experiment had up-regulation of AKR1C3 (Figure 2D), validating other publications. All these make AKR1C3 a potential drug target to treat advanced prostate cancer. We also demonstrate 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.

Additionally, AKR1C3 also possesses prostaglandin F2 (PGF2) synthase activity responsible for the synthesis of proliferative and anti-differentiating PGF2. Human myeloid leukemia cells over-expressing 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 PPARγ. These effects were overcome by treating cells with a 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.

Since 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. Since immunohistochemistry and immunofluorescence techniques gave rise to high autofluorescence, to overcome this issue, we utilized PLA, which gave fluorescent signals specific to areas of interaction only (Figure 2D). Although over 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 non-canonical signaling pathways.
such as Src, MAPK and others. Identifying this function could further enhance the therapeutic utility 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 over-expressing 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 to LNCaP-mock xenograft samples (Figure 1C) provided compelling data that AR signaling is robust in tissues expressing AKR1C3 even in the presence of DHT, a response likely magnified due to coactivation.

The evidence in this manuscript clearly suggests a new mechanism for AKR1C3 action in CRPC (Figure 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 non-canonical pathways.
Future clinical trials with AKR1C3 inhibitors will be needed to demonstrate 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.

Acknowledgement

The authors thank Mr. Terrence Costello for assistance with the animal studies and Ms. Ashley Ezekiel for her assistance with pathology slide preparation and scanning.

References


Figure Legend

**Figure-1: AKR1C3 enhances androgen signaling and prostate cancer xenograft growth.** A. Steroidogenic enzyme synthesis pathways. Bold arrows indicate back-door pathway. B. AKR1C3 transfection increases androgen-induced PSA gene expression. LNCaP cells were transfected with 10 μg vector (solid line) or AKR1C3 (broken line) using Amaxa electroporator. Cells were maintained in serum free medium for 3 days, treated for 16 hrs with indicated concentrations of A’dione (left panel) or R1881 (middle panel) and expression of PSA was measured and normalized to GAPDH by realtime PCR. Right panel shows AKR1C3 expression in cells transfected with AKR1C3 (closed bars) or vector (open bars; not visible in the figure). EC$_{50}$ of R1881-induced PSA gene expression is given above the middle panel. C. AKR1C3 increases DHT-induced LNCaP tumor xenograft implantation and growth. Nude mice (n=11/group) bearing LNCaP-vector (solid line, diamond) or LNCaP-AKR1C3 (broken line, square) were castrated and a 90 day sustained release DHT pellet (12.5 mg) implanted under the skin. Tumor volume was measured biweekly. Tumor uptake of LNCaP-vector (solid line) or LNCaP-AKR1C3 (broken line) cells is shown as Kaplan-Meier plot (right panel). Expression of indicated genes and proteins was measured in tumors using realtime PCR or Western blotting. Open bars are LNCaP-vector tumors (n=3) and filled bars are LNCaP-AKR1C3 tumors (n=6). D. AKR1C3 siRNA reduces androgen signaling. VCaP prostate cancer cells were transfected with non-specific siRNA (N.S.) or AKR1C3 siRNA and the expression of androgen responsive genes was measured in response to 0.1 nM R1881 (filled bars). * indicates significance at P<0.05 from vector tumors or vehicle-treated cells. In vitro experiment figures are representative of n=3 experiments each conducted in triplicates;
cyclo-cyclophilin; A’dione-androstenedione; LNCaP-AKR1C3-LNCaP cells stably transfected with AKR1C3.

**Figure-2: AKR1C3 physically interacts with AR.** A (left panel). Co-immunoprecipitation of AR and AKR1C3. LNCaP-AKR1C3 cells were serum starved for 2 days and treated with 10 nM R1881 for 6 hrs. Cells were harvested, protein extracted, and immunoprecipitated with AKR1C3 antibody and Western-blotted for AR. A (right panel). AR-AKR1C3 colocalization demonstrated by laser confocal microscopy. LNCaP-AKR1C3 cells plated on coverslips and serum starved for 2 days were treated with vehicle, 100 nM A’dione, or 10 nM R1881 for 6 hrs. Cells were fixed and immunostained with primary antibodies specific to AR and AKR1C3 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 nM A’dione or 10 nM R1881 for 2 hrs and ChIP assay was performed with AR (left panel) or AKR1C3 (right panel) antibodies. DNA was purified and realtime PCR was performed for PSA enhancer or a non-specific region (N.S.) and normalized to input. Representative data from triplicate experiments is given. C. Endogenous AR and AKR1C3 interact in VCaP cells (left panel) and VCaP xenograft (right panel). VCaP cells were serum starved for 2 days and treated with vehicle or 10 nM R1881 and immunoprecipitation performed as described in panel A. Right panel. Protein was extracted from VCaP CRPC xenografts (n=3) and was subjected to immunoprecipitation (IP) with AR antibody or IgG. The Iped samples and 20% input samples were fractionated on a SDS PAGE and Western blotted.
with AKR1C3 antibody and AR antibody. D. AR and AKR1C3 interact in advanced prostate cancer specimens. Tissue section from Gleason score 7 prostate cancer (n=6) were subjected to Duolink proximity ligation assay with AR and AKR1C3 antibodies (interaction) or AR antibody replaced with IgG (negative control bottom panel). Nucleus was counterstained with 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; A’dione-androstenedione; IP-immunoprecipitation; IB-Immunoblotting; ChIP-chromatin immunoprecipitation.

**Figure-3: AKR1C3 augments AR transactivation in response to active androgens.** A. AKR1C3 augments A’dione-dependent-AR transactivation. HEK-293 cells were transfected with 0.25 µg GRE-LUC, 50 ng CMV hAR, 10 ng CMV-renilla luciferase and 1 µg vector or AKR1C3. Twenty-four hours after transfection the cells were treated with a titration of A’dione. Cells were harvested forty-eight hours after transfection and firefly luciferase levels were measured and normalized to renilla luciferase. B. AKR1C3 increases active androgen-induced AR transactivation. HEK-293 cells were transfected as mentioned in panel A, treated with a titration of the indicated androgens, and luciferase assay was performed. C. AKR1C3 concentration-dependently increases R1881-induced AR transactivation. HEK-293 cells were transfected with indicated concentration of AKR1C3. Total amount of transfected plasmids were normalized to 1 µg with vector pCR3.1. The cells were treated with a titration of R1881 and luciferase assay performed. Bottom panel shows the AKR1C3 RNA levels. D. AKR1C3 does not increase AR
expression. Cells transfected with vector or AKR1C3 and treated with 10 nM R1881 were fractionated by SDS-PAGE and Western blotted for AR. All figures are representative of n=3 experiments; AR-Androgen Receptor; A’dione-Androstenedione; RLU-relative light units; solid lines are vector transfected and broken lines are AKR1C3 transfected cells.

**Figure-4: GTx-560 inhibits AKR1C3 enzyme activity in vitro.** A. Structure of GTx-560. B (left panel). GTx-560 and indomethacin inhibit AKR1C3 enzyme activity. Purified AKR1C3 enzyme was incubated with ATP and [14C]-A’dione (12 μM) in the presence of a titration of GTx-560 or indomethacin for 60 min. A’dione and testosterone were fractionated using TLC and quantified using phosphorimager. The amount of testosterone synthesized is represented as a line graph (lower panel). B (right panel). GTx-560, but not indomethacin, inhibits AKR1C3 enzyme activity in HEK-293 cells. HEK-293 cells were transiently transfected with 1 μg AKR1C3 and treated with a titration of GTx-560 or indomethacin in the presence of [14C]-A’dione (1 μM). After over-night incubation, A’dione and testosterone in the medium were fractionated using TLC and were quantified using phosphorimager. The amount of testosterone synthesized is represented in the line graph (lower panel). C. GTx-560, but not indomethacin, inhibits AKR1C3 enzyme activity in adrenal (left panel) and prostate cancer (right panel) cell lines. H295R adrenal cells and LNCaP-AKR1C3 cells were treated with [14C]-A’dione (12 μM) in the presence or absence of 10 μM GTx-560 or indomethacin. Medium was collected and the testosterone synthesized was detected by TLC and quantified using phosphorimager. Western blot showing AKR1C3 over-expression in LNCaP cells is
shown in the bottom panel. * indicates significance at P<0.05 of n=3. Representative experiment shown in the figure. D. GTx-560 inhibits finasteride-dependent testosterone formation. LNCaP-Vector or LNCaP-AKR1C3 were treated with [14C]-A’dione (1 μM) in the presence or absence of 10 μM finasteride, GTx-560, GTx-594 or indomethacin (indo). Percent testosterone formed was calculated and represented as numbers below the TLC image. Representative of n=3 experiments is depicted here. A’dione-Androstenedione; T-Testosterone; TLC-Thin Layer Chromatography; LN-LNCaP-mock; LN-AKR-LNCaP-AKR1C3; veh-Vehicle; 560-GTx-560; indo-indomethacin.

**Figure-5: GTx-560 inhibits AKR1C3 enzyme- and coactivator-activities in cells.** A. GTx-560, but not indomethacin, inhibits AKR1C3-mediated A’dione-induced AR transactivation. HEK-293 cells transfected as indicated above were treated with 10 nM A’dione alone or in combination with a titration of GTx-560 or indomethacin and luciferase assay performed. A (right panel). GTx-560 increases the EC$_{50}$ of A’dione. HEK-293 cells transfected as indicated above were treated with a titration of A’dione alone or in combination with 10 μM GTx-560 and luciferase assay performed. EC$_{50}$ values are given on top of the figure. B. GTx-560 inhibition of AR transactivation is selective to AKR1C3. HEK-293 cells transfected with 1 µg pCR3.1, pCR3.1-AKR1C3 or pCR3.1 17βHSD3 were treated with 10 nM A’dione in the presence or absence of a titration of GTx-560 and transactivation assay performed. C. GTx-560 inhibits AKR1C3-dependent R1881-induced AR transactivation. HEK-293 cells transfected as indicated above were treated with a titration of R1881 alone or in combination with 10 μM GTx-560 and luciferase assay was performed. Solid line is vector transfected cells, broken line
is AKR1C3 transfected cells, and dotted line is AKR1C3 transfected cells treated with GTx-560. EC$_{50}$ of R1881 under various conditions is described. D. GTx-560 requires full length AKR1C3 to inhibit its coactivation effect. HEK-293 cells transfected as indicated in the figure were treated with a titration of R1881 alone or in combination with 10 μM GTx-560 and luciferase assay performed. Results shown in the figures are representative of three experiments. A’dione-androstenedione; RLU-relative light units; 560-GTx-560.

**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 μM GTx-560 in the presence of R1881 or A’dione. PSA gene expression was measured and normalized to GAPDH (left panel). Right panel. LNCaP-vector (open bars) or LNCaP-AKR1C3 (filled bars) cells were treated with vehicle or 10 μM 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 μM GTx-560 (upper panel), or 10 μM finasteride (lower panel). RNA was extracted and PSA gene expression was measured and normalized to GAPDH. C. GTx-560 reduces the growth of LNCaP and VCaP prostate cancer xenograft growth and androgen-dependent gene expression. Top left panel: LNCaP-AKR1C3 cells (2 million cells/mouse) were subcutaneously implanted in nude mice (n=7). Once tumors reach 100 mm$^3$, the animals were castrated and A’dione pellets (5.25 mg 21 day release pellets) were subcutaneously implanted. Once the tumors re-emerged, the animals were
randomized and treated with vehicle or 30 mg/kg GTx-560 subcutaneously. Tumor volumes were measured biweekly. * p<0.05. Top right panel. GTx-560 reduces VCaP castration resistant prostate cancer (CRPC) xenograft growth. VCaP cells (2 million cells/mouse) were subcutaneously implanted in nude mice. Once tumors reached 200-300 mm$^3$, the animals were castrated and the tumors were allowed to develop as CRPC. Once the tumors re-emerged, the animals were randomized and treated with vehicle (n=6) or 40 mg/kg GTx-560 (n=8) subcutaneously for four weeks. Tumor volumes were measured biweekly. Bottom left panel. GTx-560 suppresses serum PSA of animals bearing VCaP CRPC xenograft. PSA was measured in the serum of animals shown in panel B top right panel. The numbers about the bars represent the mean of each group (n=5). C bottom middle panel. GTx-560 suppresses tumor PSA. Protein was extracted from tumors shown in panel E, fractionated by SDS-PAGE and Western blotted for PSA and actin. C bottom right panel. Serum GTx-560 inversely correlates with tumor volume. GTx-560 concentrations in serum was measured and correlated with tumor volume shown in panel B top right. D. Model depicting AKR1C3’s mechanism of action. The in vitro figures are representative of n=3 and each experiment was performed with triplicate samples; finasteride; 560-GTx-560; cyclo-cyclophilin; A’dione-androstenedione; LNCaP-AKR1C3-LNCaP cells stably transfected with AKR1C3. In panels A-C, * indicates significance at p<0.05 from vehicle treated samples.
**FIGURE-1**

A

Cholesterol

\[ \text{Pregnenolone} \rightarrow \text{Progesterone} \rightarrow 20\alpha\text{(OH)Proges} \]

\[ \text{DHEA} \rightarrow \text{Androstenedione} \rightarrow \text{ Estrone} \]

\[ \text{Androstenediol} \rightarrow \text{ Testosterone} \rightarrow \text{ Estradiol} \]

\[ 17\beta\text{-HSD6} \rightarrow \text{AKR1C3} \rightarrow \text{DHT} \rightarrow \text{Androsterone} \]

**B**

**EC**\(_{50}\)

Vector = 43.51 pM

AKR1C3 = 13.71 pM

**C**

Tumor Volume (mm\(^3\))

Weeks

**D**

\[ \text{PSA/GAPDH} \rightarrow \text{AKR1C3} \rightarrow \text{TMPRSS2/GAPDH} \rightarrow \text{AKR1C3} \]

siRNA

N.S. AKR1C3 siRNA

N.S. AKR1C3

**Research.**

Downloaded from clincancerres.aacrjournals.org on October 16, 2017. © 2013 American Association for Cancer Research.
FIGURE-2

A

<table>
<thead>
<tr>
<th>R1881</th>
<th>Vector</th>
<th>AKR1C3</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

IP:AKR1C3
IB:AR
AR: 10% Input
AKR1C3 10% input

B

Graph showing AR and AKR1C3 levels with different treatments.

C

Vehicle 10 nM R1881

IP:AKR1C3
IB:AR
AR: 10% Input

D

Case

DAPI

Interaction

Negative (AKR1C3+IgG)

Graph showing pixel intensity in different conditions.
FIGURE-3

A

B

C

D

Research.

on October 16, 2017. © 2013 American Association for Cancer clincancerres.aacrjournals.org Downloaded from
FIGURE-4

A B

\[
\begin{align*}
&\text{GTx-560} & \text{Indomethacin} \\
&\text{GTx-560} & \text{GTx-560} \\
&\text{Indomethacin} & \text{Indomethacin}
\end{align*}
\]

C

\[
\begin{align*}
&\text{LNCaP} & \text{LNCaP-AKR} \\
&\text{GTx-560} & \text{Indo} \\
&\text{GTx-560} & \text{Indo} \\
&\text{GTx-560} & \text{Indo}
\end{align*}
\]

D

\[
\begin{align*}
&\text{LNCaP} & \text{LNCaP-AKR} \\
&\text{Veh} & \text{Veh} \\
&\text{Veh} & \text{Veh} \\
&\text{Veh} & \text{Veh}
\end{align*}
\]
**FIGURE-5**

A  

![Graph showing RLU vs. µM with data points for Vector, AKR1C3, and AKR+560 at different concentrations of 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.6, 0.8, 1.0, 1.2, and 1.4.](image)

B  

![Graph showing EC50 values with data points for Vector, AKR1C3, and AKR+560 at different concentrations of GTx-560 and A'dione.](image)

C  

![Graph showing EC50 values with data points for Vector, AKR1C3, and AKR+560 at different concentrations of R1881.](image)

D  

![Graph showing RLU vs. µM with data points for Vector, AKR1C3, and AKR+560 at different concentrations of 0.01, 0.1, and 1 nM.](image)
Steroidogenic Enzyme AKR1C3 is a Novel Androgen Receptor-Selective Coactivator That Promotes Prostate Cancer Growth

Muralimohan Yepuru, Zhongzhi Wu, Anand Kulkarni, et al.

Clin Cancer Res  Published OnlineFirst August 30, 2013.

Updated version  Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-13-1151

Supplementary Material  Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2013/09/09/1078-0432.CCR-13-1151.DC1

Author Manuscript  Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.