

Inhibition of AKR1C3 Activation Overcomes Resistance to Abiraterone in Advanced Prostate Cancer

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Abstract

Abiraterone suppresses intracrine androgen synthesis via inhibition of CYP17A1. However, clinical evidence suggests that androgen synthesis is not fully inhibited by abiraterone and the sustained androgen production may lead to disease relapse. In the present study, we identified AKR1C3, an important enzyme in the steroidogenesis pathway, as a critical mechanism driving resistance to abiraterone through increasing intracrine androgen synthesis and enhancing androgen signaling. We found that overexpression of AKR1C3 confers resistance to abiraterone while downregulation of AKR1C3 resensitizes resistant cells to abiraterone treatment. In abiraterone-resistant prostate cancer cells, AKR1C3 is overexpressed and the levels of intracrine androgens are elevated. In addition, AKR1C3 activation increases intracrine

androgen synthesis and enhances androgen receptor (AR) signaling via activating AR transcriptional activity. Treatment of abiraterone-resistant cells with indomethacin, an AKR1C3 inhibitor, overcomes resistance and enhances abiraterone therapy both *in vitro* and *in vivo* by reducing the levels of intracrine androgens and diminishing AR transcriptional activity. These results demonstrate that AKR1C3 activation is a critical mechanism of resistance to abiraterone through increasing intracrine androgen synthesis and enhancing androgen signaling. Furthermore, this study provides a preclinical proof-of-principle for clinical trials investigating the combination of targeting AKR1C3 using indomethacin with abiraterone for advanced prostate cancer. *Mol Cancer Ther*; 16(1): 35–44. ©2016 AACR.

Introduction

For patients with late-stage prostate cancer, the current standards of care are chemotherapeutic drugs such as docetaxel and cabazitaxel (1, 2), and/or next-generation anti-androgens such as enzalutamide and abiraterone (3, 4). Abiraterone inhibits CYP17A1 (5), resulting in the reduction of testosterone levels in advanced prostate cancer patients. Although initially effective, most patients receiving abiraterone will progress in their disease by 15 months of treatment (4). The elucidation of adaptive resistance mechanisms and targeting against it to overcome the resistance are urgently needed.

Abiraterone resistance has been linked to reactivation of androgen receptor (AR) signaling in prostate cancer cells. This may be partially due to the presence of AR splice variants and mutation of the AR. Clinically, detection of AR-V7 in prostate cancer patients may indicate abiraterone resistance (6). Furthermore, the progesterone-activated T878A AR mutant is associated with resistance to abiraterone (7), and circulating tumor DNA extracted from plasma samples revealed that the L702H mutation in the ligand-binding domain of AR, which results in activation of the AR by

glucocorticoids, might also induce abiraterone resistance (8). In addition to AR modification, increased expression of steroidogenic enzymes is another likely contributor to both prostate cancer progression and abiraterone resistance by increasing androgen levels and inducing AR activation. Mostaghel and colleagues detected a 1.3- to 4.5-fold increase in enzymes involved in steroidogenesis in abiraterone-treated LuCaP cell lines, including CYP17A1, AKR1C3, HSD17B3, and SDR5A2 (9). In addition, Chang and colleagues observed the 1245C mutation in HSD3B1 in abiraterone-resistant xenograft models leading to aberrant androgen production (10). AKR1C3 in particular is an important enzyme in the steroidogenesis pathway. Its activation contributes to Castration Resistant Prostate Cancer (CRPC) drug resistance in patients treated with both abiraterone and enzalutamide, and it has been suggested as a biomarker for assessing prostate cancer progression (11, 12). AKR1C3 facilitates the conversion of the weak androgens androstenedione (A' dione) and 5 α -androstenedione (5 α -dione) to the more active androgens, testosterone and DHT, respectively and cannot be inhibited by abiraterone (13, 14). AKR1C3 is the major AKR1C isozyme in the human prostate (15), and elevated expression of this enzyme is associated with prostate cancer progression and aggressiveness (16, 17). AKR1C3 has also been identified as an AR coactivator (18). AKR1C3 is not detectable in normal prostatic epithelium; however, it is highly elevated in metastasized prostate cancer (16, 17). In two independent prostate datasets in the OncoPrint data base, AKR1C3 expression was correlated with Gleason score and recurrence status in prostate cancer patients (12).

Indomethacin (Indocin) is a NSAID that is commonly prescribed to reduce fever, pain, stiffness, and/or swelling due to a myriad of different causes. In addition to its function as a

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nonselective inhibitor of cyclooxygenase (COX) 1 and 2, studies have demonstrated that Indocin can inhibit AKR1C3 activity (19, 20). Other studies have shown that Indocin can increase the sensitivity of cancer cells to chemotherapeutics. Specifically, it was observed to increase the response of colon cancer cells to cisplatin and the response of melanoma cells to TRAIL-induced apoptosis (21, 22). Overexpression of AKR1C3 in prostate cancer cells confers resistance to enzalutamide, and combination of Indocin and enzalutamide results in inhibition of enzalutamide-resistant tumor growth in xenografts (12). These data suggest that AKR1C3 activity is integral to the development of enzalutamide resistance in prostate cancer and that cotreatment with Indocin and enzalutamide is a viable method to overcome enzalutamide resistance. To date, however, the effects of Indocin on abiraterone resistance remain unknown.

In the present study, we utilized drug-resistant prostate cancer cell lines to investigate the effects of AKR1C3 and Indocin on abiraterone resistance. We found that overexpression of AKR1C3 induces abiraterone resistance and that higher expression levels of AKR1C3 in drug-resistant cells result in modification of AR signaling and regulated AR transcriptional activity. Chronic abiraterone acetate treatment in prostate cancer cells induced high levels of AKR1C3 and AR. Knockdown of AKR1C3 expression resensitized abiraterone-resistant prostate cancer cells to abiraterone treatment. Furthermore, treatment of resistant cells with Indocin enhances abiraterone response both *in vitro* and *in vivo*. Data from this study further the understanding of abiraterone resistance in prostate cancer and provide the groundwork for the development of meaningful treatment strategies by targeting AKR1C3 using Indocin in combination with abiraterone in advanced prostate cancer patients.

Materials and Methods

Reagents and cell culture

LNCaP, VCaP, and CWR22Rv1 cells were obtained from the American Type Culture Collection (ATCC). All experiments with cell lines were performed within 6 months of receipt from the ATCC or resuscitation after cryopreservation. The ATCC uses short tandem repeat profiling for testing and authentication of cell lines. C4-2B cells were kindly provided and authenticated by Dr. Leland Chung, Cedars-Sinai Medical Center, Los Angeles, CA. VCaP cells were maintained in DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 0.1 mg/mL streptomycin. Other cell lines were maintained in RPMI 1640 supplemented with 10% FBS, 100 units/mL penicillin, and 0.1 mg/mL streptomycin. LNCaP-neo and LNCaP-AKR1C3 cells were generated by stable transfection of LNCaP cells with either empty vector pcDNA3.1 or pcDNA3.1 encoding AKR1C3 and were maintained in RPMI1640 medium containing 300 µg/mL G418. Cells resistant to enzalutamide were referred to as C4-2B MDVR (C4-2B enzalutamide resistant) as described before (12, 23). C4-2B cells were incubated with increasing concentrations of abiraterone acetate (1 µmol/L ~ 20 µmol/L) in RPMI1640 plus 10% FBS and stored for further analysis. The resistant cells were isolated and referred to as C4-2B AbiR (C4-2B abiraterone resistant; ref. 24). Parental C4-2B cells were passaged alongside the abiraterone acetate-treated cells as an appropriate control. C4-2B AbiR cells were maintained in 10 µmol/L abiraterone acetate-containing medium. All cells were maintained at 37°C in a humidified incubator with 5% carbon

dioxide. Indocin was purchased from Sigma, Abiraterone was purchased from LKT Laboratories, Inc., and Abiraterone acetate was purchased from AK Scientific Inc. All drugs were dissolved in DMSO and stored at -20°C.

Cell transfection and luciferase assay

AKR1C3 shRNA (TRCN0000026561 and TRCN0000025694) was purchased from Sigma. For luciferase assays, C4-2B MDVR cells (1×10^5 cells per well of 12-well plate) were transfected with 0.5 µg of pGL3-PSA6.0-Luc reporter plasmid or the control plasmid and subsequently treated with 20 µmol/L Indocin. The luciferase activity was determined 48 hours after transfection using a dual-luciferase reporter assay system (Promega) as described previously (23).

Sample preparation and steroid analysis

The steroid extraction and analysis has been described previously (12, 25). Briefly, 100 million LNCaP-neo and LNCaP-AKR1C3 cells were cultured in serum and phenol red-free RPMI1640 medium for 5 days. Fifty million C4-2B MDVR cells were cultured in serum and phenol red-free RPMI1640 medium for 5 days and then treated with 20 µmol/L Indocin for another 3 days. Subsequently, cells were suspended in 4 mL of a 1:1 water/methanol mixture. The suspension was homogenized, and the resulting homogenate was cooled on ice. The precipitated material was removed by centrifuging at high speed for 5 minutes, and the supernatant was removed and evaporated in a SpeedVac (Labconco Inc.) followed by lyophilizer (Labconco Inc.). The residue was suspended in 150 µL of CH₃OH/H₂O (1:1), filtered through a 0.2-µm ultracentrifuge filter (Millipore Inc.), and subjected to UPLC/MS-MS analysis. Samples were run in duplicate during UPLC-MS/MS analysis. Samples were placed in an Acquity sample manager which was cooled to 8°C to preserve the analytes. Pure standards were used to optimize the UPLC-MS/MS conditions prior to sample analysis. The standard mixture was run before the first sample to prevent errors due to matrix effect and day-to-day instrument variations. In addition, immediately after the initial standard and before the first sample, two spiked samples were run to calibrate for the drift in the retention time of all analytes due to the matrix effect. After standard and spiked sample runs, a blank was injected to wash the injector and remove carry over effect.

Chromatin immunoprecipitation assay

C4-2B MDVR cells were transiently transfected with control shRNA or AKR1C3 shRNA (# 561 and # 694) for 3 days or treated with DMSO or Indocin for 2 days. DNA-AR protein complexes were cross-linked inside the cells by the addition of 1% formaldehyde. Whole-cell extracts were prepared by sonication, and an aliquot of the cross-linked DNA-protein complexes was immunoprecipitated by incubation with the AR-specific antibody (AR-441; Santa Cruz Biotechnology) overnight at 4°C with rotation. Chromatin-antibody complexes were isolated from solution by incubation with protein A/G agarose beads for 1 hour at 4°C with rotation. The bound DNA-protein complexes were washed and eluted from beads with elution buffer (1% SDS and 0.1 mol/L NaHCO₃), crosslinking was reversed, and DNA was extracted. The resulting chromatin preparations were analyzed by PCR using primers spanning Androgen Responsive Elements (ARE) of the PSA promoter as described previously (26). Iso-type-matched IgG was used as control.

Western blot analysis

Cellular protein extracts were resolved on SDS-PAGE and proteins were transferred to nitrocellulose membranes. After blocking for 1 hour at room temperature in 5% milk in PBS/0.1% Tween-20, membranes were incubated overnight at 4°C with the indicated primary antibodies [AR (441, SC-7305, Santa Cruz Biotechnology); AR (N20, SC-816, Santa Cruz Biotechnology) AKR1C3 (A6229, Sigma); HSD3B (SC-28206, Santa Cruz Biotechnology); Tubulin (T5168, Sigma-Aldrich)]. Tubulin was used as loading control. Following secondary antibody incubation, immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (Millipore).

Cell growth assay

C4-2B AbiR, C4-2B MDVR, and CWR22Rv1 cells were seeded on 12-well plates at a density of 0.5×10^5 cells/well in RPMI 1640 media containing 10% FBS and transiently transfected with AKR1C3 shRNA or control shRNA and then treated with 10 $\mu\text{mol/L}$ abiraterone. Total cell numbers were counted after 3 days, and the cell survival rate was normalized to the DMSO treatment for each group. LNCaP-neo and LNCaP-AKR1C3 cells were treated with different concentrations of abiraterone for 48 hours. C4-2B parental and C4-2B AbiR cells were treated with different concentrations of abiraterone acetate, and total cell numbers were counted on day 3. CWR22Rv1 cells were seeded on 12-well plates at a density of 0.5×10^5 cells/well in RPMI 1640 media containing 10% FBS and treated with DMSO, 20 $\mu\text{mol/L}$ Indocin, 10 $\mu\text{mol/L}$ abiraterone, or their combination for 3 days. Total cell numbers were counted and the cell survival rate (%) was calculated. Cell survival rate (%) = (Treatment group cell number/Control group cell number) \times 100.

Clonogenic assay

C4-2 parental or C4-2B AbiR cells were treated with DMSO, 2.5 $\mu\text{mol/L}$, 5 $\mu\text{mol/L}$, or 10 $\mu\text{mol/L}$ abiraterone acetate in media containing 10% FBS. Cells were plated at equal density (2,000 cells/dish) in 100 mm dishes for 14 days. CWR22Rv1 cells were treated with 20 $\mu\text{mol/L}$ indocin with or without 10 $\mu\text{mol/L}$ abiraterone, and were plated at equal density (1,500 cells/dish) in 100 mm dishes for 14 days. The medium was changed every 3 days. Colonies were rinsed with PBS before staining with 0.5% crystal violet/4% formaldehyde for 30 minutes and the numbers of colonies were counted.

Real-time quantitative RT-PCR

Total RNAs were extracted using TriZOL reagent (Invitrogen). cDNAs were prepared after digestion with RNase-free RQ1 DNase (Promega). The cDNAs were subjected to real-time reverse transcription-PCR (RT-PCR) using Sso Fast Eva Green Supermix (Bio-Rad) according to the manufacturer's instructions and as described previously (27). Each reaction was normalized by amplification of actin. Triplicates of samples were run on default settings of Bio-Rad CFX-96 real-time cyclers. Primers used for real-time PCR are: AKR1C3, 5'-gagaagtaaagcttgagggtcaca-3' (forward) and 5'-caactgctctctattgtataatga-3' (reverse); HSD3B, 5'-cgggccaactcctacaag-3' (forward) and 5'-ttttcagaggctctctctgt-3' (reverse); PSA, 5'-gccctgccgaaagg-3' (forward) and 5'-gatcaccctccggaatgca-3' (reverse); AR, 5'-cctggcttcgcaactcac-3' (forward) and 5'-ggactgtgcatcggtactca-3' (reverse); NKX3.1, 5'-ccgagacgctggcagagacc-3' (forward) and 5'-

gcttaggggttgggaag-3' (reverse); and Actin, 5'-agaactggccctcttgagg-3' (forward) and 5'-gttttatgtctctatggg-3' (reverse).

Measurement of PSA

C4-2B MDVR cells were transiently transfected with AKR1C3 shRNA or control shRNA for 3 days, LNCaP-neo and LNCaP-AKR1C3 cells were cultured in CS-FBS conditions for 3 days, and PSA level was measured using the PSA ELISA Kit (KA0208, Abnova, Inc.) according to the manufacturer's instructions as described previously (28).

In vivo tumorigenesis assay

CWR22Rv1 cells (4×10^6) were mixed with Matrigel (1:1) and injected subcutaneously into the flanks of 6- to 7-week male SCID mice. Tumor-bearing mice (tumor volume around 50–100 mm^3) were randomized into four groups (5 mice in each group) and treated as follows: (1) vehicle control (5% Tween 80 and 5% ethanol in PBS, i.p.), (2) abiraterone acetate (200 mg/kg, p.o.), (3) indocin (3 mg/kg, i.p.), and (4) abiraterone acetate (200 mg/kg, p.o.) + indocin (3 mg/kg, i.p.). Tumors were measured using calipers twice a week, and tumor volumes were calculated using length \times width²/2. Tumor tissues were harvested after 3 weeks of treatment.

Immunohistochemistry

Tumors were fixed by formalin. Paraffin-embedded tissue blocks were dewaxed, rehydrated, and blocked for endogenous peroxidase activity. Antigen retrieval was performed in sodium citrate buffer (0.01 mol/L, pH 6.0) in a microwave oven at 1,000 W for 3 minutes and then at 100 W for 20 minutes. Nonspecific antibody binding was blocked by incubating with 10% FBS in PBS for 30 minutes at room temperature. Slides were then incubated with anti-Ki-67 (at 1:500; NeoMarker) at 4°C overnight. Slides were then washed and incubated with biotin-conjugated secondary antibodies for 30 minutes, followed by incubation with avidin DH-biotinylated horseradish peroxidase complex for 30 minutes (Vectastain ABC Elite Kit, Vector Laboratories). The sections were developed with the diaminobenzidine substrate Kit (Vector Laboratories) and counterstained with hematoxylin. Nuclear staining of cells was scored and counted in five different vision fields. Images were taken with an Olympus BX51 microscope equipped with DP72 camera.

Statistical analysis

All data are presented as mean \pm standard deviation of the mean (SD). Statistical analyses were performed with Microsoft Excel analysis tools. Differences between individual groups were analyzed by one-way ANOVA followed by the Scheffé procedure for comparison of means. $P < 0.05$ was considered statistically significant.

Results

Overexpression of AKR1C3 confers resistance to abiraterone

Abiraterone inhibits CYP17A1, resulting in the reduction of testosterone levels in advanced prostate cancer patients. However, the serum level of DHEA-S concentration is still in the high range, and may serve as an ample pool for intracrine androgen synthesis through AKR1C3 (29). AKR1C3 is one of the most important enzymes catalyzing androstenedione conversion to testosterone classically, and it also facilitates the 5' dione and back door

synthesis pathway by catalyzing 5 α ' androstenedione and androsterone to 5' androstenediol respectively which are then converted to DHT. Both testosterone and DHT activate the AR (Fig. 1A). To determine if AKR1C3 is able to induce abiraterone resistance in prostate cancer cells, LNCaP-neo and AKR1C3-overexpressing LNCaP-AKR1C3 cells were treated with different concentrations of abiraterone for 2 days, and cell numbers were counted and cell survival rates were calculated. As shown in Fig. 1B, overexpression of AKR1C3 in LNCaP cells significantly induced abiraterone resistance in a dose-dependent manner. Interestingly, overexpression of AKR1C3 in LNCaP cells also increased AR protein expression. To further confirm that AKR1C3 drives abiraterone resistance, CWR22Rv1, C4-2B MDVR, and VCaP cells were transiently transfected with control shRNA or AKR1C3 shRNA (#561 and #694) and were subsequently treated with abiraterone for 3 days. Both C4-2B MDVR and CWR22Rv1 cells showed high resistance to abiraterone and VCaP cells had a moderate response to abiraterone treatment. Knockdown of AKR1C3 expression significantly enhanced the response to abiraterone in all cell lines (Fig. 1C). Knockdown of AKR1C3 expression also significantly decreased AR and AR variants expression (Fig. 1D). Taken together, these

results demonstrated that overexpression of AKR1C3 in prostate cancer cells confers resistance to abiraterone.

AKR1C3 regulates AR transcriptional activity in abiraterone-resistant prostate cancer cells

AKR1C3 is not only an important enzyme of androgen synthesis but also acts as an AR coactivator (18). To examine the influence of AKR1C3 on AR activity in C4-2B MDVR cells, AKR1C3 shRNA was transiently transfected into C4-2B MDVR cells. Knockdown of AKR1C3 expression significantly inhibited the expression of the AR target genes such as PSA and NKX3.1 (Fig. 2A and B). To further understand the mechanisms of AR transcriptional activity influenced by AKR1C3, a luciferase assay and ChIP assay were performed. As shown in Fig. 2C, knockdown of AKR1C3 significantly inhibited PSA luciferase activity and reduced the recruitment of AR to the AREs. The results were confirmed by use of the AKR1C3 enzyme inhibitor Indocin (12). Treatment with Indocin significantly inhibited PSA luciferase activity and reduced the recruitment of AR to the AREs in C4-2B MDVR cells (Fig. 2D). We also examined testosterone levels in C4-2B MDVR cells treated with Indocin by LC-MS. As shown

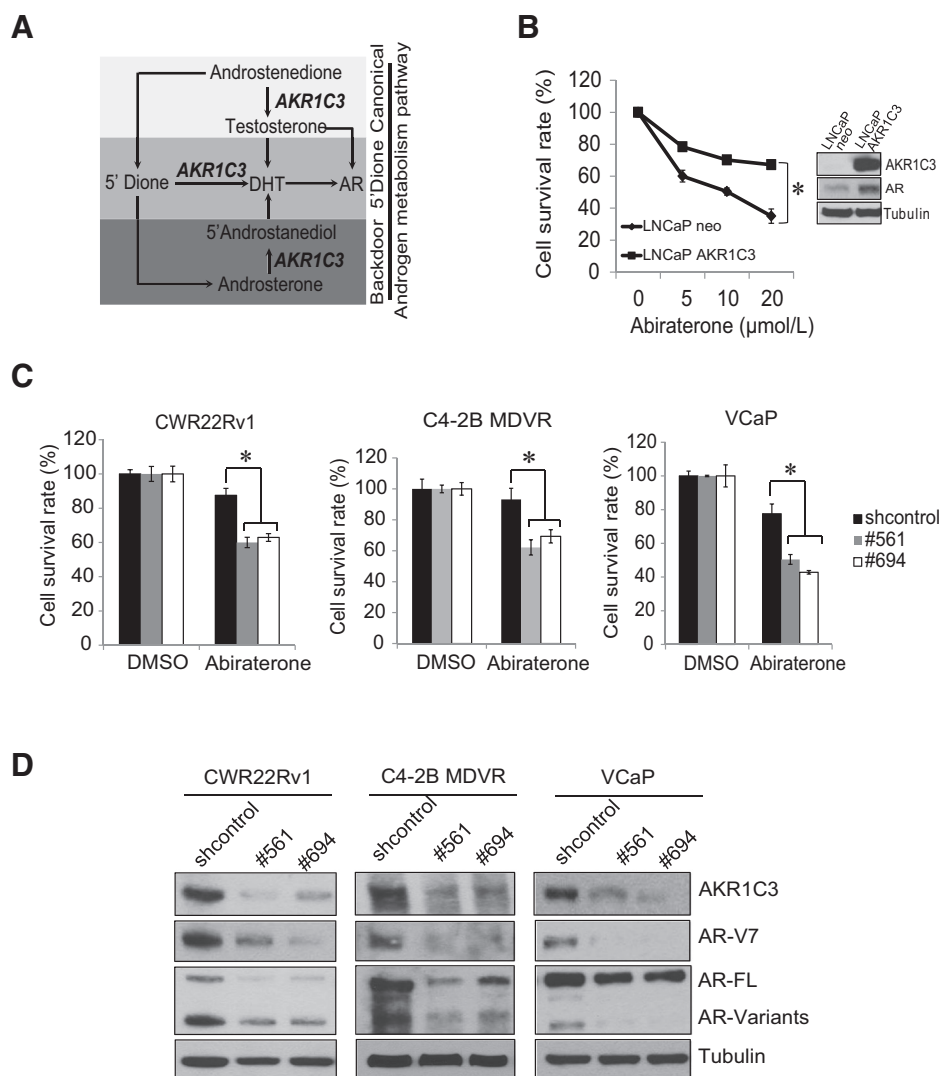
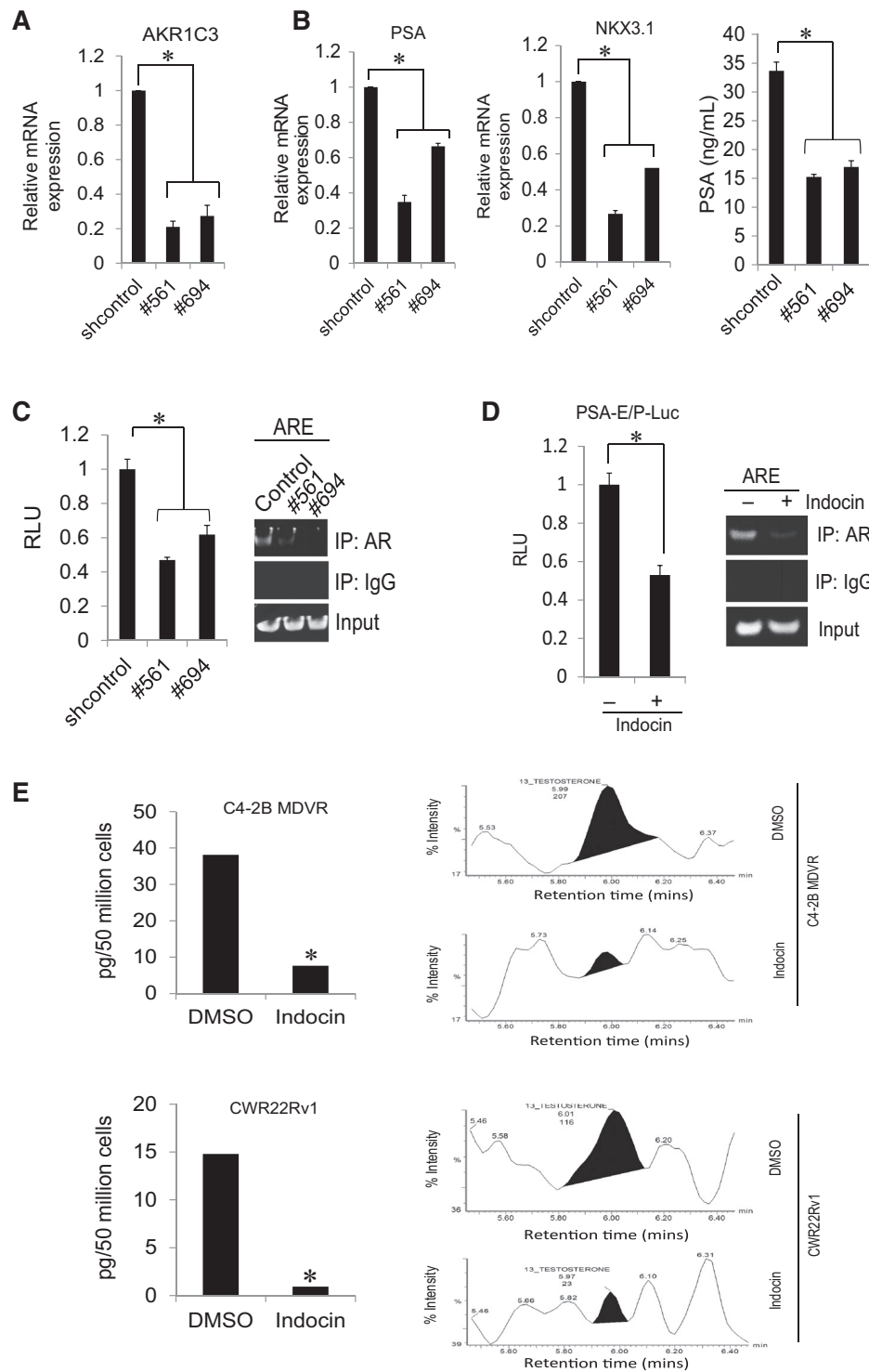


Figure 1.

Overexpression of AKR1C3 confers resistance to abiraterone. **A**, AKR1C3 is involved in the canonical, 5 α -diol, and backdoor pathways of androgen synthesis and activates AR. **B**, LNCaP-neo and LNCaP-AKR1C3 cells were treated with different concentrations of abiraterone for 2 days. Total cell numbers were counted and cell survival rate (%) was calculated. Whole-cell lysates from LNCaP-neo and LNCaP-AKR1C3 cells were subjected to Western blot (inside panel). **C**, CWR22Rv1, C4-2B MDVR, and VCaP cells were transiently transfected with AKR1C3 shRNA (#561 and #694). Following treatment with 10 μ mol/L abiraterone for 3 days, total cell numbers were counted and cell survival rate (%) was calculated. **D**, CWR22Rv1 cells, C4-2B MDVR, and VCaP cells were transiently transfected with AKR1C3 shRNA (#561 and #694) for 3 days, and AKR1C3, AR-V7, and AR expression were examined by Western blot. *, $P < 0.05$.

**Figure 2.**

AKRIC3 inhibition suppressed testosterone level and AR activity in prostate cancer. **A**, C4-2B MDVR cells were transiently transfected with control shRNA or AKRIC3 shRNA (#561 and #694) for 2 days. Total RNA was extracted and AKRIC3 mRNA level was examined by qRT-PCR. **B**, C4-2B MDVR cells were transiently transfected with control shRNA or AKRIC3 shRNA (#561 and #694). Total RNA was extracted, PSA and NKX3.1 mRNA levels were examined by qRT-PCR, and the supernatants were collected and subjected to PSA ELISA. **C**, C4-2B MDVR cells were transiently transfected with control shRNA or AKRIC3 shRNA (#561 and #694) with PSA-E/P-luc reporter. The luciferase activity was detected by dual luciferase reporter system (left), and whole-cell lysates were subjected to ChIP assay (right). **D**, C4-2B MDVR cells were transiently transfected with PSA-E/P-luc reporter, followed by treatment with DMSO or 20 $\mu\text{mol/L}$ Indocin for 3 days. Luciferase activity was detected by dual luciferase reporter system (left), and whole-cell lysates were subjected to ChIP assay (right). **E**, C4-2B MDVR (top) and CWR22Rv1 (bottom) cells were treated with DMSO or 20 $\mu\text{mol/L}$ Indocin in serum-free, phenol red-free RPMI1640 medium for 3 days. Fifty million cells were collected per treatment and testosterone level was examined by LC-MS. *, $P < 0.05$. Indocin, Indomethacin.

in Fig. 2E, Indocin significantly reduced testosterone levels in C4-2B MDVR and CWR22Rv1 cells.

To examine if exogenous expression of AKR1C3 increases testosterone level and upregulates AR transcriptional activity, LNCaP-neo and LNCaP-AKR1C3 cells were cultured in phenol red-free and serum-free conditions for 3 days and testosterone levels were measured by LC-MS. LNCaP-AKR1C3 cells express significantly higher levels of testosterone than parental LNCaP cells (Fig. 3A). AR transcriptional activity was examined in LNCaP-neo and LNCaP-AKR1C3 cells by real-time PCR, Western blot, and ChIP assay. As shown in Fig. 3B–D, LNCaP-AKR1C3 cells express higher PSA at both the mRNA and protein level and exhibit enhanced AR recruitment to the PSA promoter compared with LNCaP-neo cells. These data suggest that AKR1C3 not only functions as a steroidogenesis enzyme that induces androgen production but also influences AR transcriptional activity, possibly through AR overexpression and AR coactivation, and thus conferring resistance to abiraterone.

Abiraterone-resistant prostate cancer cells express higher levels of AKR1C3

To further confirm our hypothesis that AKR1C3 plays a pivotal role in abiraterone resistance, we generated an abiraterone acetate-resistant prostate cancer cell line by continuous culture of C4-2B cells in media containing abiraterone acetate. As shown in Fig. 4A, after 12 months of being cultured in media containing abiraterone acetate, C4-2B AbiR cells exhibited more resistance to abiraterone acetate treatment than C4-2B parental cells. Abiraterone

acetate significantly suppressed cell growth in C4-2B parental cells in a dose-dependent manner, while abiraterone acetate had moderate effect on C4-2B AbiR cells. This result was also confirmed by clonogenic assay. Abiraterone acetate significantly inhibited the number of colonies in C4-2B parental cells in a dose-dependent manner compared with C4-2B AbiR cells (Fig. 4B). C4-2B AbiR cells express higher levels of AKR1C3 mRNA and protein than C4-2B parental cells (Fig. 4C). C4-2B AbiR cells also express higher levels of AR protein than C4-2B parental cells. Next, we examined if knockdown of AKR1C3 expression in C4-2B AbiR cells could resensitize these cells to abiraterone. Knockdown of AKR1C3 expression using two different AKR1C3 shRNA significantly resensitized C4-2B AbiR cells to abiraterone treatment (Fig. 4D). These data suggest chronic abiraterone treatment induces AKR1C3 overexpression, which in turn confers resistance to abiraterone.

Targeting AKR1C3 with Indocin enhances abiraterone treatment *in vitro* and *in vivo*

To examine if targeting AKR1C3 enhances abiraterone treatment, we treated CWR22Rv1 cells with Indocin, an AKR1C3 inhibitor (12), in conjunction with abiraterone. CWR22Rv1 cells were treated with 20 $\mu\text{mol/L}$ Indocin with or without 10 $\mu\text{mol/L}$ abiraterone for 3 days, and total cell numbers were counted. As shown in Fig. 5A, 10 $\mu\text{mol/L}$ abiraterone had limited effects on cell growth, 20 $\mu\text{mol/L}$ Indocin inhibited cell growth, and the combination treatment further inhibited cell growth. These results were also confirmed by clonogenic assay: Indocin

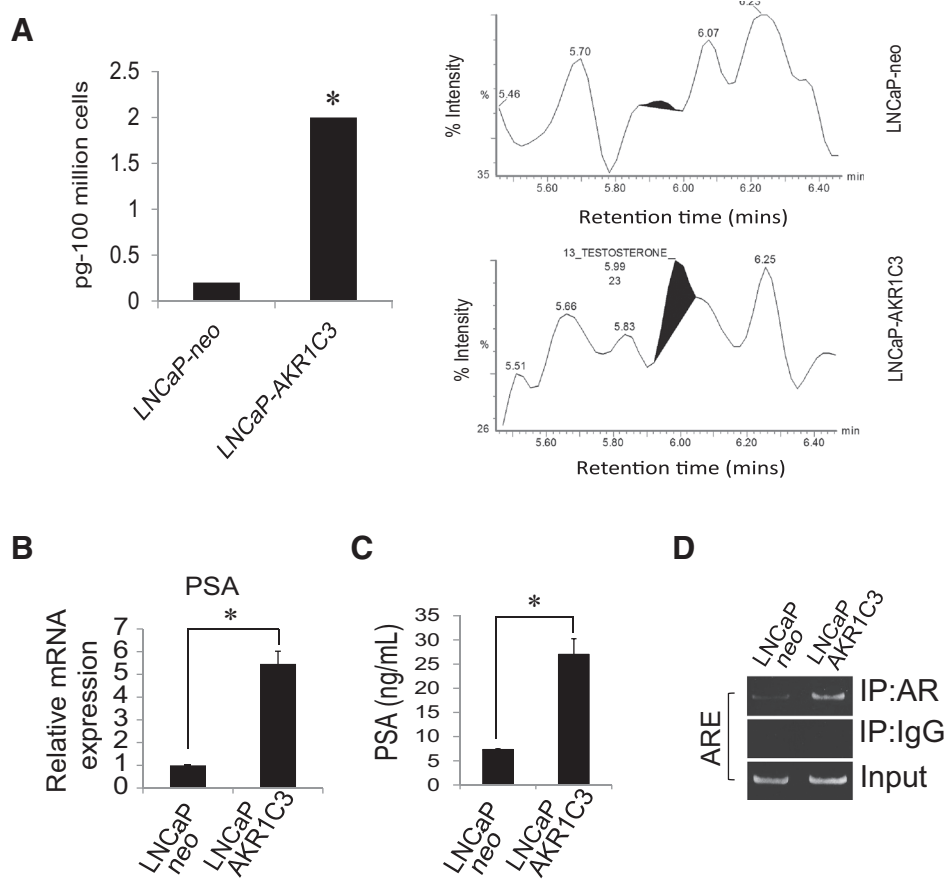


Figure 3.

Exogenous AKR1C3 promotes testosterone production and regulates AR transcriptional activity in prostate cancer. **A**, LNCaP-neo and LNCaP-AKR1C3 cells were cultured in serum-free, phenol red-free RPMI1640 medium for 3 days. One hundred million cells were collected per group, and testosterone level was examined by LC-MS. **B**, LNCaP-neo and LNCaP-AKR1C3 cells were cultured in CS-FBS conditions for 3 days, total RNA was extracted, and PSA mRNA levels were examined by qRT-PCR. **C**, The supernatants were collected and PSA level was examined by PSA ELISA. **D**, LNCaP-neo and LNCaP-AKR1C3 cells were cultured in CS-FBS conditions for 3 days, and whole-cell lysates were subjected to ChIP assay. *, $P < 0.05$.

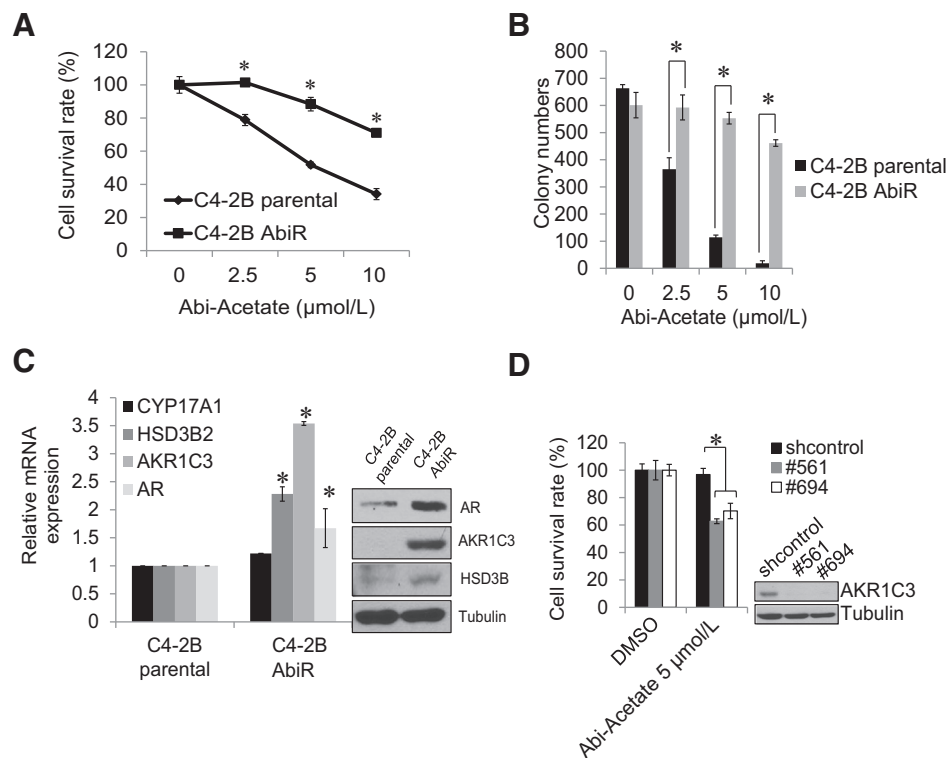


Figure 4.

Abiraterone-resistant prostate cancer cells express higher levels of AKR1C3. **A**, C4-2B parental cells and C4-2B AbiR cells were treated with different concentrations of abiraterone acetate in RPMI 1640 media containing 10% FBS, total cell numbers were counted, and cell survival rate was calculated on day 3. **B**, The clonogenic ability of C4-2B parental and C4-2B AbiR cells treated with 2.5 $\mu\text{mol/L}$, 5 $\mu\text{mol/L}$, or 10 $\mu\text{mol/L}$ abiraterone acetate. The colonies were counted and results are presented as mean \pm SD of two experiments performed in duplicate. **C**, C4-2B parental cells and C4-2B MDVR cells were cultured in RPMI 1640 media containing 10% FBS for 3 days, total RNA was extracted, and AKR1C3, CYP17A1, HSD3B2, and AR mRNA levels were analyzed by qRT-PCR. Whole-cell lysates were immunoblotted with the indicated antibodies. **D**, C4-2B AbiR cells were transiently transfected with AKR1C3 shRNA (#561 and #694). Following treatment with 5 $\mu\text{mol/L}$ abiraterone acetate for 3 days, total cell numbers were counted and cell survival rate (%) was calculated. Knockdown effects were examined by Western blot. Abi: Abiraterone. *, $P < 0.05$.

combined with abiraterone further inhibited colony numbers and reduced colony size in CWR22Rv1 cells compared with either treatment on its own (Fig. 5B).

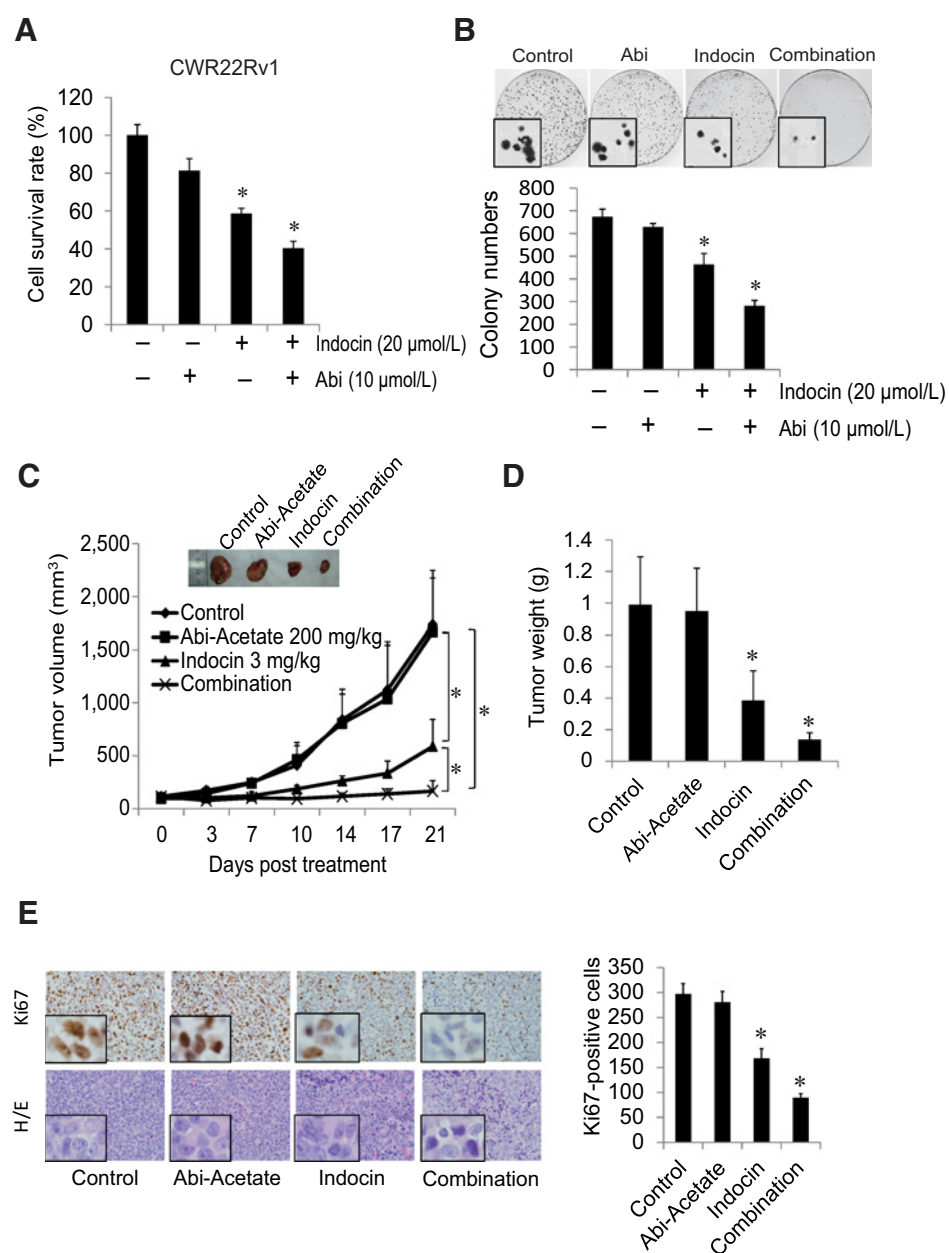
To test if inhibition of AKR1C3 activity enhances abiraterone treatment *in vivo*, a CWR22Rv1 xenograft model was developed. As shown in Fig. 5C and D, CWR22Rv1 tumors were resistant to abiraterone. Treatment with Indocin alone significantly inhibited tumor growth and combination of abiraterone and Indocin further inhibited tumor growth compared with either treatment alone. Indocin inhibited Ki67 expression while combination treatment further lowered Ki67 expression (Fig. 5E). These data demonstrate that inhibition of AKR1C3 activity by Indocin overcomes abiraterone resistance, providing preclinical data to support further clinical development of combination therapy of abiraterone and Indocin to treat advanced prostate cancer.

Discussion

Intratumor androgen biosynthesis is well characterized as a mechanism of CRPC (30–33). Many enzymes are involved in androgen synthesis, including CYP17A1, AKR1C3, and HSD3B. CYP17A1 can be inhibited by abiraterone in clinical treatments (4, 34). However, serum levels of androgens are still high in some

patients treated with abiraterone, suggesting that inhibition of androgen synthesis by abiraterone is incomplete, and sustained steroidogenesis contributes to resistance (10, 35, 36). In this study, we demonstrated that AKR1C3 is overexpressed in abiraterone-resistant prostate cancer cells. Overexpression of AKR1C3 increases intracrine androgens and AR expression and induces abiraterone resistance, while downregulation of AKR1C3 sensitizes resistant cells to abiraterone treatment. Furthermore, we demonstrated that Indocin (an AKR1C3 inhibitor) inhibits the levels of intracrine androgens, suppresses AR and AR variants expression, and overcomes abiraterone resistance and enhances abiraterone treatment both *in vitro* and *in vivo*.

Abiraterone inhibits CYP17A1, resulting in the reduction of testosterone levels in advanced prostate cancer patients. However, the serum level of DHEA-S is still in the high range, and may serve as an ample pool for intracrine androgen synthesis (29). Many enzymes involved in androgen synthesis, including AKR1C3 and HSD3B, are elevated in abiraterone-resistant C4-2B AbiR cells. AKR1C3 is one of the most important enzymes in androgen synthesis; it catalyzes the reduction of 5 α -dihydrotestosterone to 5 α -androstane-3 α ,17 β -dior by its 3 α -HSD activity and conversion of androstenedione to testosterone by its 17 β -HSD activity (16). We showed that the levels of intracrine androgens are

**Figure 5.**

Indocin enhances abiraterone treatment *in vitro* and *in vivo*. **A**, CWR22Rv1 cells were treated with 20 $\mu\text{mol/L}$ Indocin with or without 10 $\mu\text{mol/L}$ abiraterone for 2 days, and total cell numbers were counted and cell survival rate was calculated. **B**, CWR22Rv1 cells were treated with 20 $\mu\text{mol/L}$ Indocin with or without 10 $\mu\text{mol/L}$ abiraterone, and clonogenic assays were performed; colonies were counted and results are presented as mean \pm SD of 2 experiments performed in duplicate, the representative pictures were taken under a microscope. **C**, Mice bearing CWR22Rv1 xenografts were treated with vehicle control, abiraterone acetate (200 mg/Kg p.o), Indocin (3 mg/Kg i.p), or their combination for 3 weeks. Tumor volumes were measured twice weekly. **D**, Each group of tumors was weighed. **E**, IHC staining of Ki67 in each group was performed and quantified. *, $P < 0.05$. Abi, Abiraterone; Indocin, Indomethacin.

elevated in AKR1C3-overexpressing LNCaP-AKR1C3 cells compared with their parental cells, suggesting that AKR1C3 activation results in an increase in androgen synthesis in these cells.

In addition to its function as a critical enzyme in intracrine androgen synthesis, AKR1C3 also catalyzes the formation of prostaglandin (PG) $F_{2\alpha}$ and $11\beta\text{-PGF}_{2\alpha}$ from PGH_2 and PGD_2 , respectively. The $\text{PGF}_{2\alpha}$ and $11\beta\text{-PGF}_{2\alpha}$ can inactivate proliferator peroxisome activator receptor gamma (PPAR γ) and displays antiproliferative effects (37). Recently, the AKR1C3 has been found to directly interact with E3 ubiquitin ligase siah2 and control its stabilization, and thus regulate the siah2-dependent AR signaling (38). Furthermore, AKR1C3 has been found to specifically interact with the AR and is recruited to the ARE on the promoter of androgen responsive genes. AKR1C3 coactivating the AR in prostate cancer tissue might magnify androgen effects

(18). This makes the fact that AKR1C3 is pharmacologically targetable in prostate cancer highly important.

AR deregulation has been found in 80% of CRPC patients (39). AR overexpression can confer AR hypersensitivity to low levels of androgen and may contribute to drug resistance (40). In the present study, we found that AR is overexpressed in abiraterone-resistant C4-2B-AbiR and AKR1C3-overexpressing LNCaP-AKR1C3 cells. Knockdown of AKR1C3 expression down regulates full-length AR as well as AR variants expression in these abiraterone-resistant cell lines, suggesting that AKR1C3 regulates AR and AR variants expression. The effects of altered AKR1C3 expression on androgen signaling can be due to a number of potential mechanisms including changes in AR and AR variants expression (41–43), changes in cellular concentration of androgens (12), and/or AKR1C3 functions as a coregulator for AR (18).

Taken together, AKR1C3 is not only an enzyme that catalyzes testosterone synthesis but also affects AR expression and/or functions as an AR coactivator to regulate AR activity.

Both abiraterone and enzalutamide are FDA approved. However, when a patient becomes resistant to one drug, the subsequent response rate to the other drug is 20% or less (43–45). This suggests common resistance mechanisms exist for the observed cross resistance. In previous studies, we demonstrated that AKR1C3 activation increases intracrine androgens and confers resistance to enzalutamide (12). Here, we showed that AKR1C3 activation also induces resistance to abiraterone. Thus, AKR1C3 activation could represent a common resistance mechanism for cross resistance between enzalutamide and abiraterone. We showed that enzalutamide-resistant C4-2B MDVR cells are also resistant to abiraterone treatment, knocking down AKR1C3 expression blocked the recruitment of AR to the AREs and reduced the levels of intracrine androgens, thus resensitizing the cells to abiraterone treatment. These results further the understanding of cross-resistance between abiraterone and enzalutamide in prostate cancer, and provide the groundwork for the development of meaningful treatment strategies by targeting AKR1C3 to enhance next generation antiandrogen therapies.

Indocin, a NSAID used for reducing fever, pain and inflammation, has been shown to inhibit AKR1C3 activity (12, 46). Indocin blocks AKR1C3 function through binding with the AKR1C3 active site (20). Indocin exhibits a strong selectivity for AKR1C3 (8.2 $\mu\text{mol/L}$) over AKR1C1 and AKR1C2 (over 100 $\mu\text{mol/L}$; ref. 46). Recently, several derivatives of Indocin, as well as other compounds, were developed that exhibit higher potency to inhibit AKR1C3 activity (47, 48). These, in addition to baccharin (49) and other more specific AKR1C3 inhibitors such as (3-(4-(Trifluoromethyl)phenylamino)benzoic acid), have been identified and characterized (48). We showed that Indocin is able to inhibit the levels of intracrine androgens in C4-2B MDVR cells and CWR22Rv1 cells and suppress prostate cancer tumor growth and enhance enzalutamide and abiraterone treatment, suggesting that Indocin is more potent than SN33638, an inhibitor of AKR1C3, which has limited activity in the inhibition of testosterone production and cell proliferation (50). Indocin has been on the market and safely taken by patients, including prostate cancer

patients, for decades. Together, this study and the previous report of Indocin synergizing with enzalutamide therapy pave the way for the development of Indocin as a potential treatment strategy to block AKR1C3 activation to overcome treatment resistance and enhance the therapeutic effect of enzalutamide and abiraterone.

In summary, we have identified AKR1C3 activation as a critical mechanism of resistance to abiraterone through increasing intracrine androgen synthesis and enhancing androgen signaling. Furthermore, this study provides a preclinical proof-of-principle for clinical trials investigating combination treatment of Indocin with abiraterone for advanced prostate cancer.

Disclosure of Potential Conflicts of Interest

C. Liu has ownership interest (including patents) in Patent application covering the use of indomethacin. C.P. Evans reports receiving commercial research grant from, has received honoraria from the speakers bureau of, and is a consultant/advisory board member for, Medivation/Astellas. A.C. Gao has ownership interest (including patents) in a patent application. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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Development of methodology: C. Liu, A.C. Gao

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Liu, C.M. Armstrong, A. Lombard, A.C. Gao

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Liu, A. Lombard, C.P. Evans, A.C. Gao

Writing, review, and/or revision of the manuscript: C. Liu, C.M. Armstrong, C.P. Evans, A.C. Gao

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Liu, W. Lou, A.C. Gao

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