Intracrine Androgens and AKR1C3 Activation Confer Resistance to Enzalutamide in Prostate Cancer

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Abstract

The introduction of enzalutamide and abiraterone has led to improvement in the treatment of metastatic castration-resistant prostate cancer. However, acquired resistance to enzalutamide and abiraterone therapies frequently develops within a short period in many patients. In the present study, we developed enzalutamide-resistant prostate cancer cell lines in an effort to understand the mechanism of resistance. Global gene-expression analysis showed that the steroid biosynthesis pathway is activated in enzalutamide-resistant prostate cancer cells. One of the crucial steroidogenic enzymes, AKR1C3, was significantly elevated in enzalutamide-resistant cells. In addition, AKR1C3 is highly expressed in metastatic and recurrent prostate cancer and in enzalutamide-resistant prostate xenograft tumors. LC/MS analysis of the steroid metabolites revealed that androgen precursors such as cholesterol, DHEA and progesterone, as well as androgens are highly upregulated in enzalutamide-resistant prostate cancer cells compared to the parental cells. Knockdown of AKR1C3 expression by shRNA or inhibition of AKR1C3 enzymatic activity by indomethacin resensitized enzalutamide-resistant prostate cancer cells to enzalutamide treatment both in vitro and in vivo. In contrast, overexpression of AKR1C3 confers resistance to enzalutamide. Furthermore, the combination of indomethacin and enzalutamide resulted in significant inhibition of enzalutamide-resistant tumor growth. These results suggest that AKR1C3 activation is a critical resistance mechanism associated with enzalutamide resistance; targeting intracrine androgens and AKR1C3 will overcome enzalutamide resistance and improve survival of advanced prostate cancer patients.

Intratumoral androgen biosynthesis has been well characterized as a mechanism of CRPC (9–12), but its role in enzalutamide resistance is yet to be understood. Clinical reports have shown that patients treated with enzalutamide have elevated testosterone levels in the bone marrow (13, 14). A cascade of enzymes is involved in the biosynthesis of intratumoral androgens, including CYP17A1, HSD3B and AKR1C3. A gain-of-function mutation in HSD3B1 (N367T) has been identified in CRPC patients recently, and was postulated to confer resistance to enzalutamide (15, 16). Aldo-keto reductase family 1 member C3 (AKR1C3) is a multifunctional enzyme and is one of the most important genes involved in androgen synthesis and metabolism. AKR1C3 facilitates the conversion of weak androgens androstenedione (A’-dione) and 5 α-androstenedione (5α-dione) to the more active androgens testosterone and DHT, respectively (17, 18). It catalyzes conversion of steroids and modulates transactivation of steroid receptors. Elevated expression of AKR1C3 has been associated with prostate cancer progression and aggressiveness (19, 20). The role of AKR1C3 in enzalutamide-resistant prostate cancer is unknown.

In the present study, we developed prostate cancer cell lines resistant to enzalutamide and found that intracrine androgen synthesis is activated in enzalutamide-resistant prostate cancer cells. Activation of one of the important steroidogenic enzymes, AKR1C3, was identified as a critical mechanism that confers resistance to enzalutamide. Inhibition of AKR1C3 activity using either shRNA or indomethacin resensitized enzalutamide-resistant prostate cancer cells to enzalutamide. Furthermore, the combination of indomethacin and enzalutamide resulted in...
significant inhibition of enzalutamide-resistant prostate cancer xenograft tumor growth.

Materials and Methods

Reagents and cell culture

LNCaP, CWR22Rv1, VCaP, and HEK293T cells were obtained from the 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). LN-95 cells were kindly provided and authenticated by Dr. Joel Nelson, University of Pittsburgh, Pittsburgh, PA. The cells were maintained in RPMI-1640 supplemented with 10% FBS, 100 U/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 RPMI-1640 medium containing 300 µg/ml G418. AKR1C3 shRNA (TRCN0000026561 and TRCN0000025694) were purchased from Sigma. Cells resistant to enzalutamide were referred to as C4-2B MDVR (C4-2B enzalutamide resistant) as described previously (2). All cells were maintained at 37°C in a humidified incubator with 5% carbon dioxide.

Sample preparation and analysis of steroids

The steroid extraction and analysis has been described previously (21). Briefly, 50 million C4-2B parental and C4-2B MDVR cells were cultured in serum- and phenol-red-free RPMI-1640 medium for 5 days, then 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 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 before sample analysis. Also, 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, blank was injected to wash the injector and remove carry over effect.

UPLC/MS-MS analysis of steroid metabolites

All experiments were performed on a Waters Xevo-TQ triple quadruple mass spectrometer (Milford) and MS and MS-MS spectra were recorded using Electro Spray Ionization (ESI) in positive ion (PI) and negative ion (NI) mode, capillary voltage of 3.0 kV, extractor cone voltage of 3 V, and detector voltage of 650 V. Cone gas flow was set at 50 L/h and desolvation gas flow was maintained at 600 L/h. Source temperature and desolvation temperatures were set at 150°C and 350°C, respectively. The collision energy was varied to optimize daughter ions. The acquisition range was 20 to 500 Da. Analytic separations were conducted on the UPLC system using an Acquity UPLC HSS T3 1.8 µm 1 x 150-mm analytic column kept at 50°C and at a flow rate of 0.15 mL/min. The gradient started with 100% A (0.1% formic acid in H2O) and 0% B (0.1% formic acid in CH3CN), after 2 minutes, changed to 80% A over 2 minutes, then 45% A over 5 minutes, followed by 20% A in 2 minutes. Finally, it was changed over 1 minute to original 100% A, resulting in a total separation time of 15 minutes. The elutions from the UPLC column were introduced to the mass spectrometer and resulting data were analyzed and processed using MassLynx 4.1 software.

cDNA microarray analysis

The microarray analysis has been described previously (22). Briefly, 24 hours after plating of $5 \times 10^5$ C4-2B parental and C4-2B MDVR cells, total RNA was isolated using TRIzol Reagent (Invitrogen) and purified with Eppendorf phase-lock-gel tube. RNA quality of all samples was tested by RNA electrophoresis to ensure RNA integrity. Samples were analyzed by the Genomics Shared Resource (UC Davis Medical Center, Sacramento, CA) using the Affymetrix Human Gene 1.0 ST array. The data were analyzed by Subie platform and Gene Set Enrichment Analysis (23). Microarray data have been deposited in GEO with the accession number GSE64143.

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 [AKR1C3 (A6229, Sigma); CYP17A1 (SC-66849, Santa Cruz Biotechnology); HSD3B (SC-28206, Santa Cruz Biotechnology); AR (SC-815, 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 MDVR, CWR22Rv1 cells were seeded on 12-well plates at a density of $0.5 \times 10^5$ cells per well in RPMI-1640 media containing 10% FBS and transiently transfected with AKR1C3 shRNA or control shRNA following treatment with 20 µmol/L enzalutamide. Total cell numbers were counted after 3 or 5 days. LNCaP-neo, LNCaP-AKR1C3, or LN-95 cells were treated with different concentrations of enzalutamide for 48 hours. Total cell numbers were counted or the cell survival rate (%) was calculated. Cell survival rate (%) = (treatment group cell number/control group cell number) x 100%.

Clonogenic assay

C4-2 parental or C4-2B MDVR cells were treated with DMSO, 10 µmol/L or 20 µmol/L enzalutamide in media containing 10% FBS. CWR22Rv1 cells or C4-2B MDVR cells were treated with 10 or 20 µmol/L indomethacin with or without 20 µmol/L enzalutamide, cells were plated at equal density (1500 cells/dish) in 100 mm dishes for 14 days, the medium was changed every 3 days; LNCaP-neo or LNCaP-AKR1C3 cells were treated with DMSO or 10 µmol/L enzalutamide in media containing 10% complete FBS, cells were plated at equal density (10,000 cells/dish) in 100-mm
disks for 28 days, the 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 RT-PCR using Sso Fast Eva Green Supermix (Bio-Rad) according to the manufacturer’s instructions and as described previously (24). Each reaction was normalized by coamplification of actin. Triplicates of samples were run on default settings of Bio-Rad CFX-96 real-time cycler. Primers used for Real-time PCR are: AKR1C3, 5′-gagaagtaagcgctggacagca-3′ (forward) and 5′-caacgctctcataaagtaaatc-3′ (reverse); AKR1C1/2, 5′-gtcactcgctctgctct-3′ (forward) and 5′-actcggctcgggaatgt-3′ (reverse); HSD3B1, 5′-agatatacgacactcttgcaagcttt-3′ (forward) and 5′-cttgtaactcaggtggatgaa-3′ (reverse); HSD3B2, 5′-cgggcaccactctcacaag-3′ (forward) and 5′-tttcccaggtctctgg-3′ (reverse); CYP17A1, 5′-agggcgctcaataagcagggc-3′ (forward) and 5′-ccagggctcataaccttc-3′ (reverse); HSD17B3, 5′-tggggacagtgggcagagtga-3′ (forward) and 5′-cagcgaaggcgaaggcga-3′ (reverse); SRD5A1, 5′-actcggcacttcatgcctgtcct-3′ (forward) and 5′-ccagggctcataaccttc-3′ (reverse); and Actin, 5′-actctggtcgatgggaattg-3′ (forward) and 5′-taccctta-3′ (reverse).

**Measurement of PSA**

PSA levels were measured in sera from scC4-2B parental or C4-2B MDV tumor-bearing mice using the PSA ELISA Kit (KA0208, Abnovo, Inc.) according to the manufacturer’s instructions.

**In vivo tumorigenesis assay**

C4-2B parental or C4-2B MDV tumors (4 million) were mixed with Matrigel (1:1) and injected into the prostates of 6- to 7-week-old male SCID mice. When the serum PSA level reached 5 ng/ml, mice were randomized into two groups (4 mice in each group) and treated as follows: (i) vehicle control (0.5% weight/volume (w/v) Methocel A4M orally), (ii) enzalutamide (25 mg/kg, orally). Tumors were monitored by PSA level. All tumor tissues were harvested after 3 weeks of treatment.

CWR22Rv1 cells (4 million) were mixed with Matrigel (1:1) and injected s.c. into the flanks of 6- to 7-week-old male SCID mice. Tumor-bearing mice (tumor volume around 50–100 mm³) were randomized into four groups (5 mice in each group) and treated as follows: (i) vehicle control (5% Tween 80 and 5% ethanol in PBS, i.p.), (ii) enzalutamide (25 mg/kg, orally), (iii) indomethacin (3 mg/kg, i.p.), and (iv) enzalutamide (25 mg/kg, orally) + indomethacin (3 mg/kg, i.p.). Tumors were measured using calipers twice a week and tumor volumes were calculated using length × width²/2. Tumor tissues were harvested after 3 weeks of treatment.

**Immunohistochemistry**

Tumors were fixed by formalin and paraffin-embedded tissue blocks were dewaxed, rehydrated, and blocked for endogenous peroxidase activity. Antigen retrieving 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), anti-AKR1C3 (at 1:100; Sigma) 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 cells was scored and counted in five different vision areas. Images were taken with an Olympus BX51 microscope equipped with DP27 camera.

**Statistical analysis**

All data are presented as means ± SD of the mean. Statistical analyses were performed with Microsoft Excel analysis tools. Differences between individual groups were analyzed by one-way ANOVA followed by the Scheffe procedure for comparison of means. A P value of <0.05 was considered statistically significant.

**Results**

**Identification of AKR1C3 activation in enzalutamide-resistant prostate cancer cells**

In our previous study, we generated enzalutamide-resistant prostate cancer cells, named C4-2B-MDVR, by chronic culture of C4-2B cells in media containing enzalutamide (2). As shown in Fig. 1A and B, enzalutamide significantly inhibited proliferation and clonogenic ability of C4-2B parental cells. To verify the gene-expression data, CYP17A1, HSD3B1, HSD3B2, HSD17B3, AKR1C3, AKR1C1, AKR1C2, HSD3B1, CYP17A1, and SRD5A1 in C4-2B MDVR cells compared with C4-2B parental cells. These results suggested that C4-2B-MDVR cells are resistant to enzalutamide both in vitro and in vivo. We also characterized several other prostate cancer cell lines in response to enzalutamide treatment. As shown in Fig. 1D, LNCaP cells are sensitive to enzalutamide, whereas CWR22Rv1 and LN-95 cells are resistant to enzalutamide treatment, consistent with previously published studies (25–27). Intratumoral androgen biosynthesis has been well characterized as a mechanism of CRPC (9–12, 28), but its role in enzalutamide resistance remains unknown. To further understand potential mechanisms that underlie enzalutamide resistance, we performed microarray analysis of the enzalutamide-resistant C4-2B-MDVR versus C4-2B parental cells. Expression of transcripts encoding for steroid hormone biosynthesis was analyzed by gene set enrichment. Among 45 genes involved in hormone biosynthesis, 31 genes were upregulated whereas 14 genes were downregulated in C4-2B MDVR cells. As shown in Fig. 2A, we found increased expression of AKR1C3, AKR1C1, AKR1C2, HSD3B1, CYP17A1, and SRD5A1, and decreased expression of UGT2B15, UGT2B17, CYP39A1, HSD17B6, and SRD5A1 in C4-2B MDVR cells compared with C4-2B parental cells. To verify the gene-expression data, CYP17A1, HSD3B1, HSD3B2, HSD17B3, SRD5A1, AKR1C1/2, and AKR1C3 mRNA levels were measured using specific primers by qRT-PCR. As shown in Fig. 2B left, the levels of mRNA expression were consistent with the microarray data. We also confirmed the results by Western blot analysis, as shown in Fig. 2B right, C4-2B MDVR cells express significantly higher levels of AKR1C3, HSD3B, and CYP17A1 proteins compared with C4-2B parental cells. These results suggested that
acquired androgen synthesis signaling was upregulated in enzalutamide-resistant prostate cancer cells.

AKR1C3 is highly expressed in metastatic and recurrent prostate cancer and enzalutamide-resistant prostate xenograft tumors.

We found that AKR1C3 was upregulated by more than 16-fold in enzalutamide-resistant C4-2B MDVR cells compared with the C4-2B parental cells. We examined AKR1C3 expression in different prostate cancer cell lines, including VCaP, CWR22Rv1, LNCaP, LN-95, C4-2B, and C4-2B-MDVR cells. C4-2B-MDVR, VCaP, CWR22Rv1, and LN-95 cells express higher levels of AKR1C3; C4-2B MDVR, CWR22Rv1, and LN-95 cells express significantly higher levels of AKR1C3. C4-2B parental and C4-2B-MDVR cells are resistant to enzalutamide, whereas C4-2B and LNCaP cells are sensitive to enzalutamide. As shown in Fig. 3A, C4-2B MDVR, VCaP, CWR22Rv1, and LN-95 cells express higher levels of CYP17A1. We also examined AKR1C3 expression in tumor xenografts by IHC, as shown in Fig. 3B, C4-2B MDVR and CWR22Rv1 tumors express higher levels of AKR1C3 compared with C4-2B parental tumors.

The intracrine androgen synthesis pathway activated in enzalutamide-resistant prostate cancer cells. A, expression of transcripts encoding genes involved in steroid hormone biosynthesis was analyzed by gene set enrichment. Genes that were regulated 1.3-fold between C4-2B parental cells and C4-2B MDVR cells were enriched and heatmap was generated by Subio platform. B, C4-2B parental cells and C4-2B MDVR cells were cultured in RPMI-1640 media containing 10% FBS for 3 days, total RNAs were extracted, and CYP17A1, HSD3B1, HSD3B2, HSD17B3, SRD5A1, AKR1C1/2 or AKR1C3 mRNA levels were analyzed by qRT-PCR. AKR1C3, HSD3B, and CYP17A1 protein levels were examined by Western blot analysis (right).
We performed data-mining using the Oncomine and GEO databases to compare the expression of AKR1C3 in normal prostate and prostate cancer. Primary prostate cancer and normal prostate express similar AKR1C3 levels in two independent prostate datasets, whereas AKR1C3 was significantly elevated in metastatic prostate cancer in GEO datasets (Fig. 3C), which is consistent with the previous reports (29, 30). We further examined the correlation between AKR1C3 and prostate cancer disease progression. As shown in Fig. 3D, AKR1C3 was significantly correlated with Gleason score and recurrence status in prostate cancer patients in two independent prostate datasets in Oncomine. Collectively, these results demonstrate that AKR1C3 is highly expressed in late-stage prostate cancer.

Intracrine androgens are elevated in enzalutamide-resistant prostate cancer cells

AKR1C3 (also named 17βHSD5) is one of the most important genes involved in androgen synthesis and metabolism. AKR1C3 facilitates the conversion of weak androgens Aβ-dione and 5α-dione to the more active androgens, testosterone, and DHT, respectively. To further confirm that intracellular androgen synthesis was acquired by C4-2B MDVR cells, steroid metabolism in C4-2B parental and C4-2B MDVR cells was analyzed by LC/MS. C4-2B parental and C4-2B MDVR cells were cultured in serum-free medium for 5 days, and steroid metabolites were extracted from 50 × 10⁶ cells and subjected to LC/MS analysis. As shown in Fig. 4A–C, C4-2B MDVR cells synthesize extremely high levels of testosterone (131.025 vs. 0.15 pg/50 million cells), dihydrotestosterone (17.55 vs. 0 pg/50 million cells), and DHEA (72.075 vs. 0 pg/50 million cells), compared with C4-2B parental cells. Intriguingly, the active estrogen metabolite estradiol was significantly reduced (82.725 vs. 207.3 pg/50 million cells) in C4-2B MDVR cells, suggesting that the biosynthesis of androgens was activated whereas transformation of estrogen from androgens was suppressed in C4-2B MDVR cells. Of note, the precursors involved in intracrine androgen synthesis such as cholesterol, DHEA, and progesterone are also elevated in C4-2B MDVR cells compared with C4-2B parental cells (Fig. 4C).
The steroidogenic enzymes involved in androgen synthesis and metabolism are illustrated in Fig. 4D; bold arrows and bold font indicate upregulation in enzalutamide-resistant prostate cancer cells compared with C4-2B parental cells (Fig. 4D). Collectively, these results suggest that intracrine-acquired androgen synthesis was elevated in prostate cancer cells resistant to enzalutamide.

**AKR1C3 confers resistance to enzalutamide in prostate cancer cells**

Having demonstrated that AKR1C3 is upregulated in enzalutamide-resistant prostate cancer cells and in late-stage prostate cancer patients, we next examined whether AKR1C3 could confer resistance to enzalutamide. We found that AKR1C3 was sufficient to confer resistance to enzalutamide in prostate cancer cells. CWR22Rv1 or C4-2B MDVR cells were transiently transfected with control shRNA or AKR1C3 shRNA following treatment with enzalutamide for 3 days. As shown in Fig. 5A and B, CWR22Rv1 and C4-2B MDVR cells are resistant to enzalutamide, whereas knockdown of AKR1C3 expression by two independent shRNAs (#561 and #694) restored their sensitivity to enzalutamide. The downregulation of AKR1C3 by shRNA was confirmed by Western blot analysis (Fig. 5C). We also generated LNCaP cells stably expressing AKR1C3 (LNCaP-AKR1C3) to test whether exogenous expression of AKR1C3 induces enzalutamide resistance. LNCaP-AKR1C3 and LNCaP-neo vector control cells were treated with different concentrations of enzalutamide for 48 hours and cell numbers were counted. As shown in Fig. 5D, LNCaP-AKR1C3 cells exhibited greater resistance to enzalutamide than LNCaP-neo cells. These results were also confirmed by clonogenic ability assay. LNCaP-AKR1C3 cells showed significantly more clonogenic ability than the control LNCaP-neo cells in response to enzalutamide treatment (Fig. 5E and F). Collectively, these results demonstrate that overexpression of AKR1C3 confers resistance to enzalutamide, whereas downregulation of AKR1C3 resensitizes enzalutamide-resistant prostate cancer cells to enzalutamide treatment.

**Indomethacin, an inhibitor of AKR1C3 activity, overcomes enzalutamide resistance**

Indomethacin, an NSAID used for reducing fever, pain, and inflammation, has been shown to be able to inhibit AKR1C3 activity (9, 31, 32). To further examine the role of AKR1C3 in
enzalutamide resistance, we used indomethacin to hinder AKR1C3 activation and examined the effects on the response of prostate cancer cells to enzalutamide treatment in vitro and in vivo. As shown in Fig. 6A left, indomethacin did not have an effect on CWR22Rv1 cell growth at 10 μmol/L, but inhibited cell growth marginally at 20 μmol/L. However, combination of indomethacin with enzalutamide significantly inhibited the growth of enzalutamide-resistant CWR22Rv1 cells. The results were also confirmed by clonogenic assay. As shown in Fig. 6A right, combination of indomethacin with enzalutamide significantly inhibited colony numbers and reduced colony size in CWR22Rv1 cells. Similar results were also obtained in C4-2B MDVR cells (Fig. 6B). To test whether inhibition of AKR1C3 by indomethacin overcomes resistance to enzalutamide treatment in vivo, CWR22Rv1 xenograft model was used. As shown in Fig. 6C, although CWR22Rv1 tumors were resistant to enzalutamide treatment, indomethacin significantly inhibited tumor growth. Combination of indomethacin with enzalutamide further inhibited tumor growth of CWR22Rv1 xenografts. Immunohistochemical staining of Ki-67 showed that cell proliferation was significantly inhibited by indomethacin, and further inhibited by the combination treatment (Fig. 6D). Collectively, these results suggest that inhibition of AKR1C3 by indomethacin reduced enzalutamide-resistant tumor growth, and that combination of enzalutamide with indomethacin further reduced the tumor growth of enzalutamide-resistant prostate cancer. These results indicate that inhibition of AKR1C3 by indomethacin potentiates the cell killing effect of enzalutamide.

Discussion
The second-generation androgen antagonist enzalutamide represents an improvement in therapy options for late stage metastatic CRPC (33, 34). However, the initial responders develop resistance inevitably. The potential mechanisms associated with enzalutamide resistance have been the focus of intense investigation. We previously identified several novel mechanisms involved in enzalutamide resistance, including activation of NF-κB2/p52 (27, 35), AR-V7 (2, 27), Stat3 (4), and induction of autophagy (36). In this study, we have identified AKR1C3 activation and elevated intracrine androgens as potential mechanisms contributing to enzalutamide resistance. We demonstrate that AKR1C3 is overexpressed in enzalutamide-resistant prostate cancer cells. Overexpression of AKR1C3 confers resistance to
Figure 6.
Indomethacin, an inhibitor of AKR1C3 activity, overcomes enzalutamide resistance. A, CWR22Rv1 cells were treated with 10 or 20 μmol/L indomethacin with or without 20 μmol/L enzalutamide for 2 days, total cell numbers were counted (left), and clonogenic assay was performed; the colony size pictures were taken under a microscope (middle). Colonies were counted and results are presented as means ± SD of two experiments performed in duplicate (right).

B, C4-2B MDVR cells were treated with 10 or 20 μmol/L indomethacin with or without 20 μmol/L enzalutamide for 2 days, total cell numbers were counted (left), and clonogenic assay was performed; the colony size pictures were taken under a microscope (middle). Colonies were counted and results are presented as means ± SD of two experiments performed in duplicate (right).

C, mice bearing CWR22Rv1 xenografts were treated with vehicle control, enzalutamide (25 mg/kg orally), indomethacin (3 mg/kg i.p.), or their combination for 3 weeks, tumor volumes were measured twice weekly, and the tumors were collected and weighed.

D, IHC staining of Ki-67 and hematoxylin and eosin (H&E) staining in each group was performed and quantified as described in Materials and Methods. *, P < 0.05. Enza, enzalutamide; indocin, indomethacin.
enzalutamide, whereas downregulation of AKR1C3 sensitizes prostate cancer cells to enzalutamide treatment. In addition, overexpression of AKR1C3 has been demonstrated in clinical metastatic prostate cancer and correlated with disease progression. We also demonstrated that intracrine steroids, including androgens, are elevated in enzalutamide-resistant cells, possibly through increased expression of steroidogenic enzymes such as AKR1C3. We further demonstrated that indomethacin, a potent inhibitor of AKR1C3, could be used to overcome enzalutamide resistance. The discovery of elevated intracrine androgen synthesis and enhanced AKR1C3 activation in enzalutamide-resistant cells reveal a novel mechanism for the development and progression of resistant CRPC. Cotargeting AKR1C3 will provide proof-of-concept experiments to overcome resistance and achieve durable responses in men with second-generation antiandrogen treatment.

Intracrine androgen biosynthesis has been well characterized as a mechanism of CRPC (9–12, 28). Many enzymes are involved in androgen synthesis, including CYP17A1, AKR1C3, and HSD3B. CYP17A1 can be inhibited by abiraterone in clinical treatments (37, 38). AKR1C3 is a steroidogenic enzyme involved in steroid biosynthesis and mediates the last step of testosterone biosynthesis from A1-dione. It catalyzes conversion of steroids and modulates transactivation of steroid receptors. Elevated expression of AKR1C3 has been associated with prostate cancer progression and aggressiveness (19, 20). AKR1C3 has also been identified as an AR coactivator (39). In this study, we used gene enrichment analysis to compare enzalutamide-resistant cells with enzalutamide sensitive cells, and found that the steroid biosynthesis genes were highly enriched in C4-2B MDVR cells, and several important genes involved in androgen synthesis, such as AKR1C3, HSD3B, and CYP17A1, were upregulated in enzalutamide-resistant cells. In another de novo enzalutamide-resistant cell line CWR22Rv1, AKR1C3 was highly expressed compared with C4-2B or LNCaP cells, suggesting that AKR1C3 might play a pivotal role in enzalutamide resistance. To further confirm that intracrine androgen synthesis was acquired by C4-2B MDVR cells, steroid levels in C4-2B parental and C4-2B MDVR cells were determined by LC/MS. In addition to the higher levels of testosterone and DHT in enzalutamide-resistant cells, the levels of the precursors of testosterone such as cholesterol, DHEA, and progesterone were all elevated in C4-2B-MDVR cells compared with C4-2B parental cells. These results demonstrate that AKR1C3 was significantly elevated in enzalutamide-resistant prostate cancer cells, which potentially resulted in higher levels of testosterone and DHT in enzalutamide-resistant cells. The de novo intratumoral steroid synthesis has also been shown as a potential mechanism contributing to abiraterone resistance (9). Long-term treatment of abiraterone in patients resulted in an increase in steroids biosynthesis through deregulated steroid enzymes, such as AKR1C3 (40).

Several inhibitors have been developed to target AKR1C3 activation, including indomethacin (32). Indomethacin is an NSAID used for reducing fever, pain, and inflammation. Several studies revealed that indomethacin might have the potential to increase the sensitivity of cancer cells to anticancer agents, such as that of human melanoma cells to TRAIL-induced apoptosis (41), and of colon cancer cells to cisplatin (42). Indomethacin also has the ability to inhibit PSA and ERG protein expression and decreased testosterone and DHT levels in relapsed VCaP xenograft tumors (9). In the present study, we showed that inhibition of AKR1C3 enzyme activity by indomethacin restored enzalutamide sensitivity in enzalutamide-resistant prostate cancer cells both in vitro and in vivo. Furthermore, the combination of indomethacin and enzalutamide resulted in significantly greater inhibition of enzalutamide-resistant tumor growth. Our data suggest that inhibition of AKR1C3 holds promise as a sensitizing strategy to restore antitumor effects in patients resistant to enzalutamide.

Taken together, we found that AKR1C3 activation and the resultant intracrine androgen synthesis confers resistance to enzalutamide in prostate cancer cells. Inhibition of AKR1C3 by shRNA or indomethacin overcomes resistance to enzalutamide. Furthermore, the combination of indomethacin and enzalutamide resulted in significant inhibition of enzalutamide-resistant tumor growth. Targeting AKR1C3 may provide an effective treatment strategy for patients resistant to enzalutamide.

Disclosure of Potential Conflicts of Interest
C. Liu has ownership interest (including patents) in patent application covering the use of indomethacin. W. Lou and A.C. Gao have ownership interest (including patents) in patent application. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: C. Liu, W. Lou, A.C. Gao
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