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14. ABSTRACT

Enzalutamide (Enza) and abiraterone (Abi) were approved for the treatment of metastatic castration resistant prostate cancer (mCRPC) patients. Resistance to Enza and Abi occurs frequently and renders mCRPC patients incurable. Therefore, there is great unmet medical need to identify resistant mechanisms to improve the treatment outcome of mCRPC. We have shown that overexpression of AKR1C3 is responsible for the elevated intracrine androgen biosynthesis in prostate cancer cells. Up-regulation of AKR1C3 is correlated with anti-androgen resistance. We therefore sought to knock down AKR1C3 with specific siRNA/shRNA and small molecule drug to confirm its role in androgen synthesis and drug resistance. In this report, we used siRNA, shRNA specific to AKR1C3 and a small molecule inhibitor Indomethacin to target AKR1C3. At cellular level, we demonstrated that knockdown AKR1C3 may 1. Restore sensitivity to anti-androgen drugs such as enzalutamide and abiraterone. 2. Reduce AR-V7 level. 3. Inhibit AR transactivation activity. 4. Abate intratumoral androgen synthesis. Using Indocin to target AKR1C3 in vivo is efficient in reducing tumor sizes and further successful in blocking tumor growth when combined with either enzalutamide or abiraterone. Our results confirmed that blocking AKR1C3 restores drug sensitivity of CRPC or drug resistant cells to anti-androgen treatments. Targeting AKR1C3 with Indomethacin in combination with enzalutamide shows great potential in advanced prostate cancer treatment.

15. SUBJECT TERMS
AKR1C3, intracrine androgens, enzalutamide, resistance

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TABLE OF CONTENTS

1. INTRODUCTION:

Aldo-keto reductase family 1 member C3 (AKR1C3), also named 17BHSD5, 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- androstanedione (5α-dione) to the more active androgens, testosterone and DHT respectively, which cannot be inhibited by abiraterone. It catalyzes steroids conversion and modulates steroid receptors trans-activation. AKR1C3 is the major AKR1C isozyme expressed in the human prostate; and elevated expression of this enzyme has been associated with PCa progression and aggressiveness. We have demonstrated that AKR1C3 was up-regulation in anti-androgen resistant prostate cancer cells. This overexpression conferred resistance to enzalutamide and was reversible by either AKR1C3 inhibitor or RNA interference. Our hypothesis is that targeting AKR1C3 decreases intracrine androgens and AR variants and improves enzalutamide therapy against metastatic CRPC. The work of this section of the project has been completed, and this semi-annual report is for administrative purposes.

2. KEYWORDS:

Androgen synthesis pathways; tet-inducible AKR1C3 expression; steroid measurement by LC-MS analysis, Abiraterone resistance, orthotopic animal model

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1 To determine the mechanisms of AKR1C3-mediated resistance to enzalutamide	**Timeline**	**Site 1**	**Site 2**
Major Task 3: AKR1C3 increases ARv7 expression through upregulation of hnRNPA			
Subtask 1: Test overexpression of AKR1C3 increases ARv7 and hnRNPA expression in LNCaP and C4-2B cells	25-27	Drs. Gao, Evans	
Subtask 2: Test if downregulation of hnRNPA in AKR1C3 overexpressing LNCaP or C4-2B cells decreases ARv7 expression	27-30	Drs. Gao, Evans	
Subtask 3: Test if knocking down ARv7 expression in AKR1C3 overexpressing LNCaP or C4-2B cells alters the sensitivity to enzalutamide treatment	30-36	Drs. Gao, Evans	
Milestone(s) Achieved: AKR1C3 increases ARv7 expression by enhancing hnRNPA expression	36	Drs. Gao, Evans	

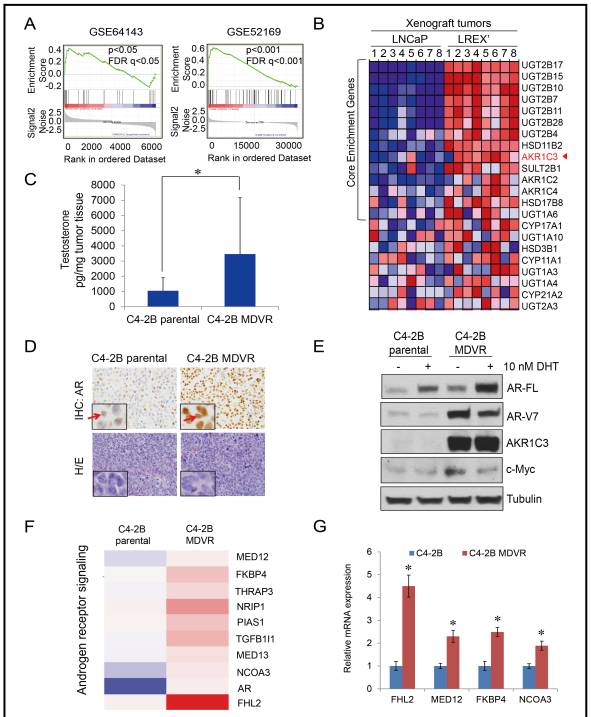

Figure 1 Comparisons between C4-2B parental vs. MDVR cells or tumors (A) steroid hormone biosynthetic pathway (B) steroidogenesis in LNCaP vs. LREX' xenograft tumors (C) Testosterone levels (D) AR IHC staining (E) WB of DHT stimulation after CS medium (F) androgen receptor signaling genes.

We examined C4-2B MDVR (enzalutamide resistant) cells closer and found that in addition to AKR1C3, a whole array of steroid biosynthetic genes were also upregulated. C4-2B MDVR xenograft tumors expressed significantly higher testosterone levels compared to C4-2B parental xenograft tumors (Figure 1C). IHC staining of AR in C4-2B MDVR xenografts was predominately in the nucleus compared to C4-2B parental tumors (Figure 1D). Addition of DHT into C4-2B cells maintained in androgen-deprived medium stimulated AR/AR-V7 protein levels and its signaling pathways with upregulation of co-activators (Figures 1E-G)

AKR1C3 binds with AR-V7 and induces AR/AR-V7 protein overexpression (led by Dr. Gao and assisted by Dr. Evans' labs)

Overexpression of AKR1C3 in LNCaP and C4-2B cells enhanced AR-V7 protein expression; full length AR (AR-FL) was also significantly in C4-2B-ARK1C3 cells (Figures 2A, 2B). However, no significant difference in the mRNA levels of AR-FL and AR-V7 in these cells. This induction might be due to the binding of AKR1C3 to AR-V7 demonstrated by both co-immunoprecipitation and dual immunofluorescence staining (Figures 2C, 2D). AKR1C3 overexpression also enabled LNCaP cells to grow in CS-FBS condition (Figure 3A). These cells once grown in to tumors, responded to castration with slight regression and delay in tumor volume increase for two weeks. Once relapse, tumor progression was not affected by daily treatment of enzalutamide (20 mg/kg), almost identical to that in the control group. Western blot analysis showed an induction of AR-V7 post castration, suggesting enzalutamide resistance in castrated LNCaP-AKR1C3 tumors (Figures 3B-D). In parallel, knocking down AR-V7 in LNCaP-AKR1C3 and C4-2B-AKR1C3 cells slightly decreased cell proliferation and combination with enzalutamide significantly suppressed cell growth (Figure 3E, 3F).

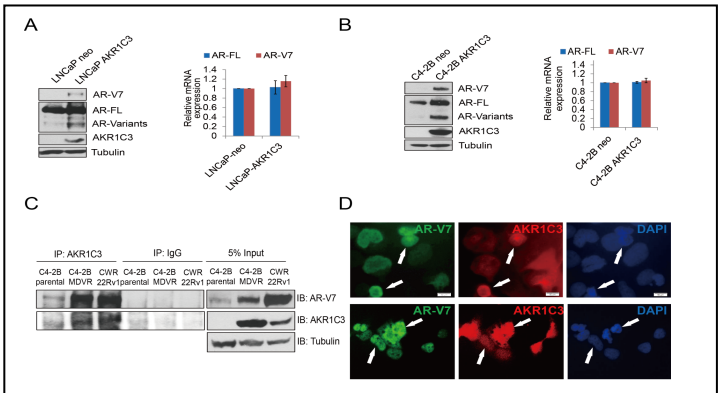

Figure 2 Effect of AKR1C3 overexpression (A) LNCaP (B) C4-2B cells (C) MDVR and Rv1 cells with AKR1C3 co-IP (D) dual IF stainingin MDVR and Rv1 cels.

AKR1C3 controls AR and AR-V7 protein stabilization in resistant prostate cancer cells

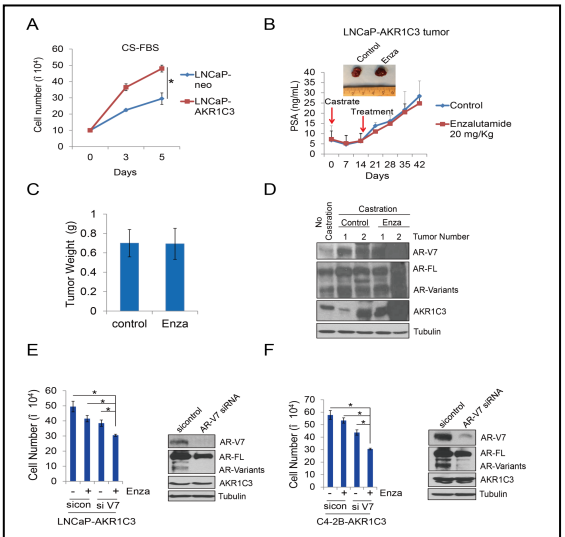

Figure 3 (A) LNCaP neo and AKR1C3 cells growth curves in CS medium (B) LNCaP AKR1C3 tumor growth after castration and treatment with or without enzalutamide (C) tumor weights (D) WB analysis of tumors. AR-V7 knockdown effect on cell growth and protein (E) LNCaP-AKR1C3 (F) C4-2B-AKR1C3

We reasoned that the underlying mechanism of AKR1C3 mediated AR-V7 upregulation may be at the protein level since there was no significant change in the AR-FL or AR-V7 mRNA levels. AR/AR-V7 protein stability was determined in C4-2B-neo and C4-2B-AKR1C3 cells. In C4-2B neo cells, AR-FL protein levels were apparently decreased when cells were maintained in the presence of cycloheximide (CHX), the inhibitor for protein synthesis. When translation elongation was inhibited by CHX, no new protein was synthesized while the old one continued to be turned over. AR level dropped to 50% within 4 hours after cycloheximide addition (Figure 4A). AR-V7 was scant in the neo cells and diminished within 2 hours from the beginning of incubation. In contrast, AR protein level was steadily maintained throughout 8 hours of test period due to the high level of AKR1C3 in C4-2B-AKR1C3 cells. Both the levels of AR-V7 and variants were near constant as well. Knockdown of AKR1C3 in cells with high level of AKR1C3 such as CWR22Rv1 (Figure 4B) and C4-2B MDVR (Figure 4C) cells reverted AR/AR-7 protein stability to the state as that in neo cells. Inhibition of AKR1C3 with the potent inhibitor indomethacin suppressed AR and AR variants expression in C4-2B MDVR cells (Fig.4D). Proteasome inhibitor MG132 maintained AR/AR-V7 protein expression in C4-2B MDVR cells treated with indomethacin (Fig.4E). Immunoprecipitation of AR-V7 with its antibody in C4-2B MDVR cells showed that the variant was heavily ubiquitinated upon indomethacin treatment, suggesting AKR1C3 protects AR-V7 from ubiquitination degradation in the enza resistant cells (Figure 4F).

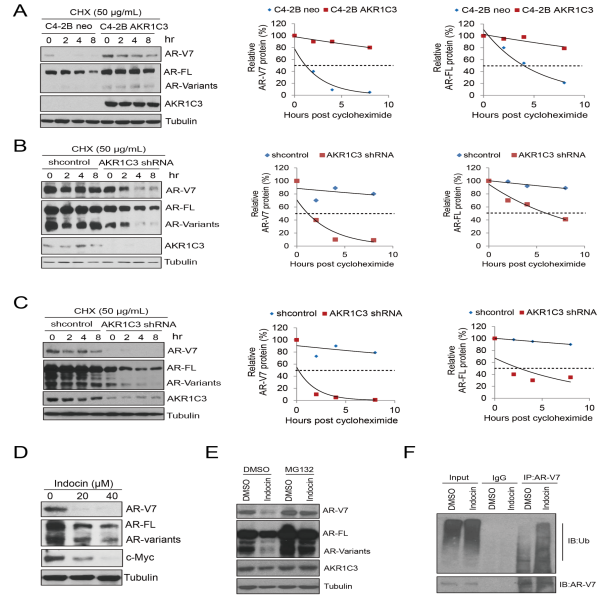

Figure 4 AR-FL/AR-V7 protein stability (A) C4-2B neo vs. AKR1C3 cells (B) knockdown of AKR1C3 in CWR22Rv1 cells (C) in C4-2B MDVR cells (D) treatment with indomethacin in MDVR cells (E) with MG132 inhibition on the effect of Indocin treatment (F) ubiquitination of AR-V7 by co-IP.

Inhibition of AKR1C3 with indomethacin disrupts gene programs and suppresses AR/AR-V7 signaling in resistant cells

We use RNA-seq analysis to scrutinize the effect of indomethacin. By GSEA, the top pathways upregulated in indomethacin treated C4-2B MDVR cells are unfolded protein response (UPR), p53 signaling, apoptosis and hypoxia pathways; and downregulated, E2F targets, cell cycle and Myc

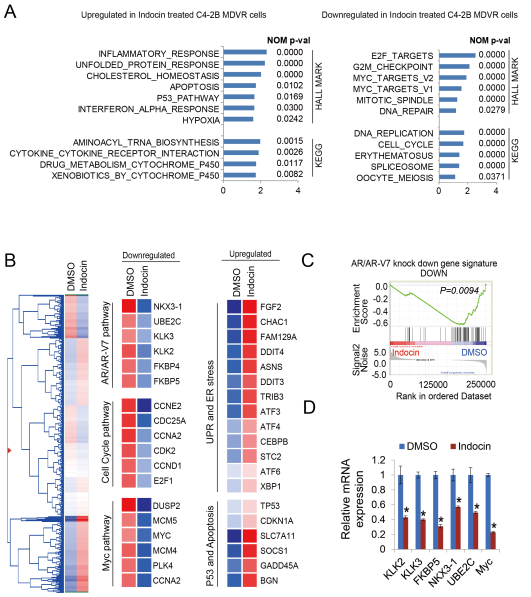

Figure 5 RNA-seq analysis of C4-2B MDVR cells treated with indomethacin (A) GSEA showed up and down-regulated pathways (B) heatmap plots showed individual genes (C) significant downregulation of AR/AR-v7 pathway by indomethacin (D) qRT-PCR validation of representative genes .

targets (Figure 5A). Heatmap plots (Figure 5B) reveal the individual genes, especially those downregulated in AR/AR-V7 (such as KLK2, KLK3, FKBP5, Nkx3-1 and Ube2C), cell cycle and Myc pathways. On the other hand, indomethacin upregulated an array of genes in UPR and ER stress pathway, as well as those responsible for P53 and apoptosis pathway. The inhibitory effect of indomethacin on AR/AR-V7 pathway is significant (Figure 5C). This was validated by qRT-PCR, expression of AR/AR-V7 downstream genes and Myc were significantly decreased by indomethacin (Figure 5D).

Orally administered of indomethacin enhances enzalutamide treatment through AKR1C3/AR-V7 inhibition (led by Dr. Evans' and assisted by Dr. Gao's Labs)

Previous data suggested indomethacin enhanced enzalutamide treatment when administered through intraperitoneal (i.p.) injection. To further identify the potential activity of indomethacin *in vivo*, we determined its tumor inhibition effects though oral administration. As shown in Fig.6A-6C, CWR22Rv1 tumors were completely resistant to enzalutamide treatment, orally administered indomethacin significantly reduced tumor growth, and indomethacin combined with enzalutamide treatment further suppressed tumor growth. However, all treatment did not affect the mice body weight (Fig.6D). We also determined the intratumoral testosterone level of each group, as shown in Fig.6E, enzalutamide slightly decreased the testosterone level, however, indomethacin and indomethacin plus enzalutamide treatment group significantly decreased the tumor testosterone level. We also extracted tumor proteins and found that indomethacin alone and the combination treatment groups significantly decreased AR/AR-V7, c-Myc and Bcl-2 expression in these tumors. These results suggest that targeting AKR1C3 with small molecule indomethacin enhances enzalutamide treatment *in vivo*

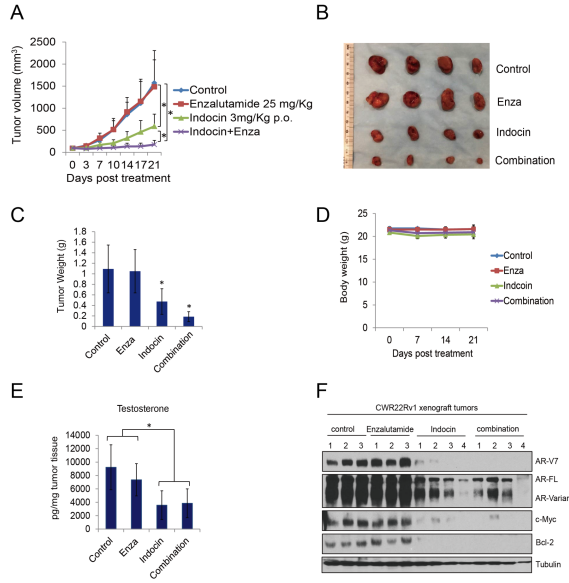

Figure 6 CWR22Rv1 xenograft model treated with control, enza, Indocin or combination. (A) Tumor growth curve (B) Representative tumor pictures (C) Tumor weights (D) Mouse body weight in each group (E) Intratumoral testosterone (F) Western blots of AR-FL and AR-V7 from all treatment groups.

through suppressing both intratumoral testosterone and AR-V7 expression.

Correlation of hnRNPA1 with AKR1C3 in AR-V7 upregulation (led by Dr. Gao and assisted by Dr. Evans' labs)

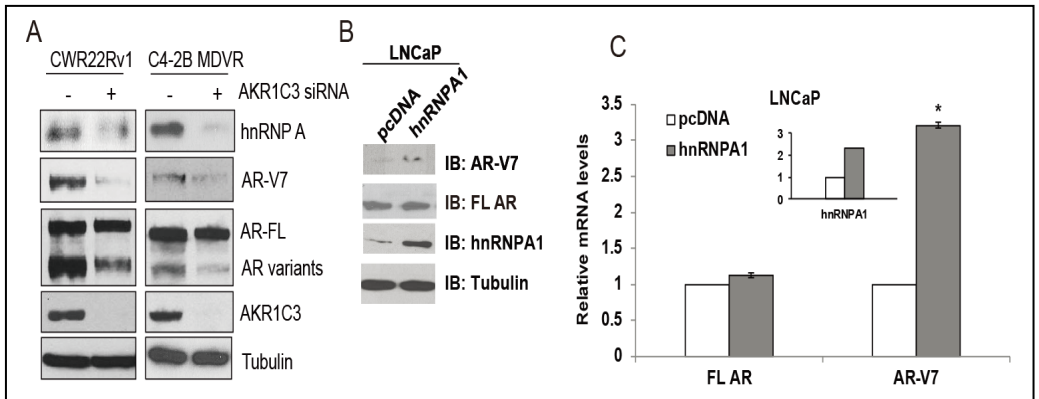

Figure 7 (A) Knockdown of AKR1C3 in CWR22Rv1 and C4-2B MDVR cells decreased hnRNPA1 expression. Overexpression of hnRNPA1 in LNCaP cells upregulated AR-V7 in (B) WB and (C) RT-αPCR

One possible mechanism for the increase in AR variants expression could be changes in expression of factors such as RNA binding proteins that regulate AR slicing patterns. Alternative splicing leads to the production of multiple mRNAs from a single gene. Our preliminary data suggest that AKR1C3 regulates ARv7 expression. Knock down of AKR1C3 expression reduced ARv7 expression in both CWR22rv1 and C4-2BMDVR cells (Figure 7A), which correlated with down regulation of hnRNPA expression (Figure 7A). In addition, overexpression of HnRNPA1 in LNCaP cells increased ARv7 protein (Figure 7B**)** and mRNA expression (Figure 7C). These data suggest that AKR1C3 increases ARv7 expression through upregulation of HnRNPA.

Treatment of Qucertin decreases and AR-V7 expression through regulating hnRNP

Qucertin in one of the components of fruit and seed extracts used in food supplements. Treatment of C4-2B MDVR cells with Qucertin over various time downregulated expression of hnRNPA1, AR-V7 and AR-FL both at the mRNA and protein levels (Figure 8).

In summary, we examined closer on the underlying mechanisms how AKR1C3 regulates the upregulation of AR/AR-V7 expression in enzalutamide resistant prostate cancer cell models. Changes in gene profiling supported AR/AR-V7 downstream gene signaling. And AKR1C3 functions through binding to AR/AR-V7 and protecting them from ubiquitination degradation. AKR1C3 inhibitor indomethacin inhibited tumor growth of a CRPC line, CWR22Rv1; combination use of indomethacin and enzalutamide completed blocked

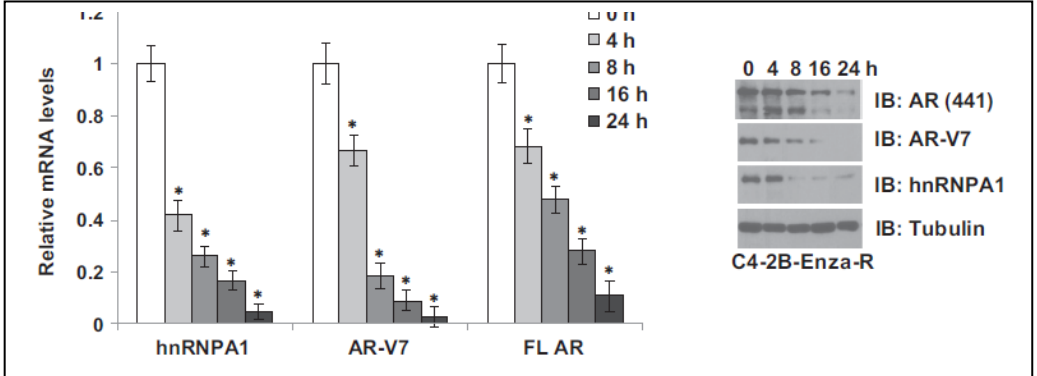

Figure 8 Treatment of Qucertin in C4-2B MDVR cells decreased hnRNPA1, AR-V7 and AF-FL at mRNA and protein levels.

tumor progression. Targeting hnRNPA1, an RNA splicing enzyme, with food supplement Qucertin also downregulated AR/AR-V7 in enzalutamide resistant cells. Overall, we show therapeutic potential of using AKR1C3 inhibitor to treat anti-androgen resistant prostate cancer patients.

AKR1C3/AR-V7 axis confers cross-resistance to apalutamide and darolutamide

C4-2B MDVR cells with elevated levels of AR-V7 and AKR1C3 display cross resistance to two new anti-androgen drugs, Apalutamide and Darolutamide. To test if the AKR1C3/AR-V7 axis confers cross-resistance to apalutamide and darolutamide, we determined if knockdown of AKR1C3 re-sensitizes the resistant cells to apalutamide and darolutamide. As shown in **Fig.9A**, knockdown of AKR1C3 by lenti-AKR1C3 shRNA inhibited cell growth. AKR1C3 knockdown combined with apalutamide/darolutamide further reduced cell number compared to the control group. We furthed confirmed that knockdown of AKR1C3 significantly down-regulated AR, AR-V7, and c-Myc expression in C4-2B MDVR cells **(Fig.9B)**. To further confirm AKR1C3 is involved in apalutamide and darolutamide resistance, we used C4-2B-AKR1C3 cells to test whether exogenous expression of AKR1C3 induces apalutamide or darolutamide resistance. As shown in **Fig.9C**, C4-2B-AKR1C3 cells exhibited greater resistance to apalutamide and darolutamide than C4-2B-neo cells. Finally, AKR1C3 inhibitor indomethacin significantly enhanced both apalutamide and darolutamide treatment in C4-2B MDVR cells in a time dependent manner **(Fig.9D)**. At 5 days treatment, the coefficient of drug interaction (CDI) of indomethacin with apalutamide and darolutamide combination treatment were 0.56 and 0.64 respectively, suggesting synergistic interactions (CDI<1). In light of these results, our data indicated that the AKR1C3/AR-V7 axis is one of the major mechanisms conferring apalutamide/darolutamide cross-resistance to enzalutamide in advanced prostate cancer.

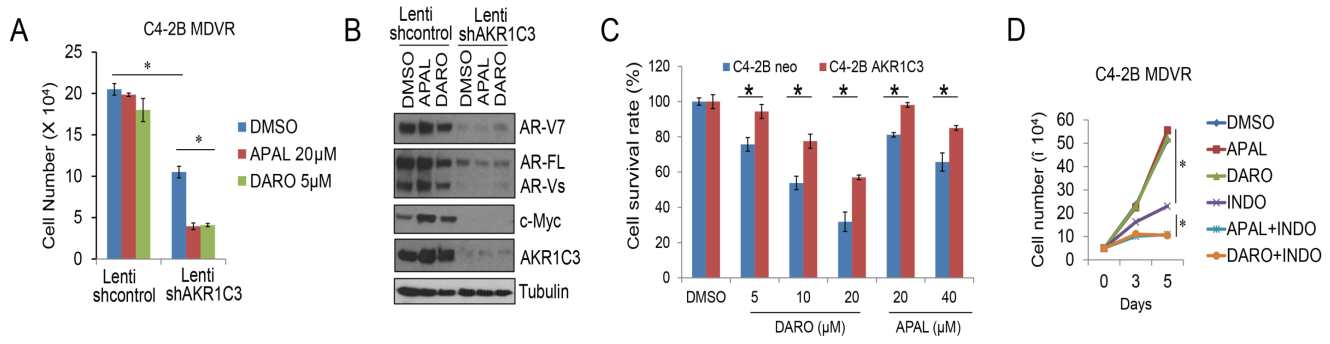

Figure 9. AKR1C3 confers apalutamide and darolutamide resistance through AR-V7 regulation. A-B. C4-2B MDVR cells were infected with lenti-control shRNA or lenti-AKR1C3 shRNA and then treated with 20 μM apalutamide or 5 μM darolutamide and cell numbers were determined after 5 days. Whole cell lysates were collected and subjected to western blot. C. C4-2B neo or C4-2B AKR1C3 cells were treated with different concentrations of apalutamide or darolutamide for 3 days, total cell numbers were counted and cell survival rate (%) was calculated. D. C4-2B MDVR cells were treated with 20 μM apalutamide or 5 μM darolutamide or 20 μM indomethacin, alone or in combination, total cell numbers were determined on 3 and 5 days. APAL: apalutamide, DARO: darolutamide. INDO: indomethacin * $p<0.05$.

Chronic apalutamide treatment in C4-2B cells upregulates the steroid biosynthesis pathway

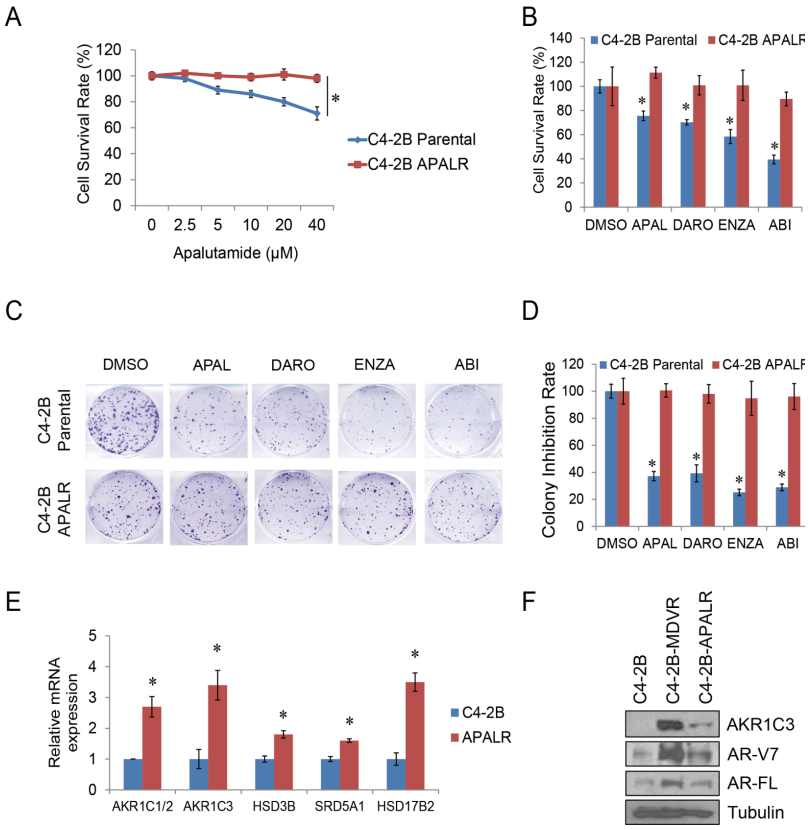

Figure 10. Chronic apalutamide treatment in C4-2B cells activates the steroid biosynthesis pathway through AKR1C3 upregulation. A. C4-2B parental and C4-2B APALR cells were treated with different concentration of apalutamide for 3 days, total cell numbers were determined and cell survival rate was calculated. B. C4-2B parental and C4-2B APALR cells were treated with 20 μM apalutamide, 5 μM darolutamide, 20 μM enzalutamide or 10 μM abiraterone and cell numbers were determined after 3 days. C-D. The clonogenic ability of C4-2B parental and C4-2B APALR cells treated with 20 μM apalutamide, 5 μM darolutamide, 20 μM enzalutamide or 10 μM abiraterone was analyzed. E. The genes involved in steroid hormone biosynthesis pathway were determined by qRT-PCR. F. Whole cell lysates were collected from C4-2B parental, C4-2B MDVR and C4-2B ARNR cells and subjected to western blot. Enza: enzalutamide, APAL: apalutamide, Abi: abiraterone acetate, DARO: darolutamide. * $p<0.05$

Apalutamide is a new next-generation anti-androgen with a molecular structure very similar to enzalutamide (6). To investigate apalutamide resistance, we have successfully generated a C4-2B apalutamide resistant cell line (C4-2B APALR). As shown in **Fig.10A**, C4-2B parental cells were seneitive to apalutamide treatment in a dose dependent manner; however, C4-2B APALR cells were resistant. To test if cross-resistance exists between apalutamide and other androgen signal-targeting agents in this cell model, C4-2B APALR cells were treated with apalutamide, darolutamide, enzalutamide and abiraterone. Both cell growth **(Fig.10B)** and colony formation assays **(Fig.10C-D)** confirmed that C4-2B APALR cells exhibited robust resistance not only to apalutamide but also to darolutamide, enzalutamide and abiraterone. We then examine the transcriptional alterations of steroid hormone biosynthesis related genes in C4-2B APALR cells compared to the parental ones. The steroid hormone biosynthesis related genes were significantly upregulated in C4-2B APALR cells compared to C4-2B parental cells **(Fig.10E)**. Results from western blot indicated that the protein levels of both AKR1C3 and AR-V7 are overexpressed in C4-2B APALR cells . However, these levels were lower than those in C4-2B MDVR cells (**Fig.10F**). These results strongly imply that the AKR1C3/AR-V7 axis is critical in conferring cross-resistance between apalutamide and other anti-androgens.

Targeting AKR1C3 resensitize apalutamide resistant cells to apalutamide and enzalutamide

To confirm the contribution of the AKR1C3/AR-V7 axis in cross-resistance between enzalutamide and apalutamide, C4-2B APALR cells were treated with enzalutamide or apalutamide with or without the knockdown of AKR1C3 by lenti-AKR1C3 shRNA **(Fig.11A)**. Growth of C4-2B APALR cells was largely inhibited by AKR1C3 knockdown. AKR1C3 knockdown in combination with enzalutamide/apalutamide treatment further reduced cell growth. The knockdown of AKR1C3 was accompanied with reduced expression levels of AR-FL, AR-V7 and c-Myc **(Fig.11B)**, which is consistent with what is observed in C4-2B MDVR cells (18). Finally, C4-2B APALR cells were treated with the AKR1C3 inhibitor indomethacin or apalutamide, alone and in combination. Indomethacin effectively resensitized C4-2B APALR cells to apalutamide treatment **(Fig11C),** the coefficient of drug interaction (CDI) of 40 μM apalutamide with 20 μM indomethacin or 40 μM indomethacin combination treatment were 0.61 and 0.63 respectively, suggesting drug synergism (CDI<1). The results were confirmed by clonogenic assay. 40 μM apalutamide has no effects on colony formation in C4-2B APALR cells, single indomethacin treatment reduced the colony formation and combination treatment significantly suppressed colony size and numbers **(Fig11D-11E)**. Collectively, our data suggest that targeting AKR1C3 in apalutamide resistant cells could efficiently reverse cross- resistance between apalutamide and enzalutamide.

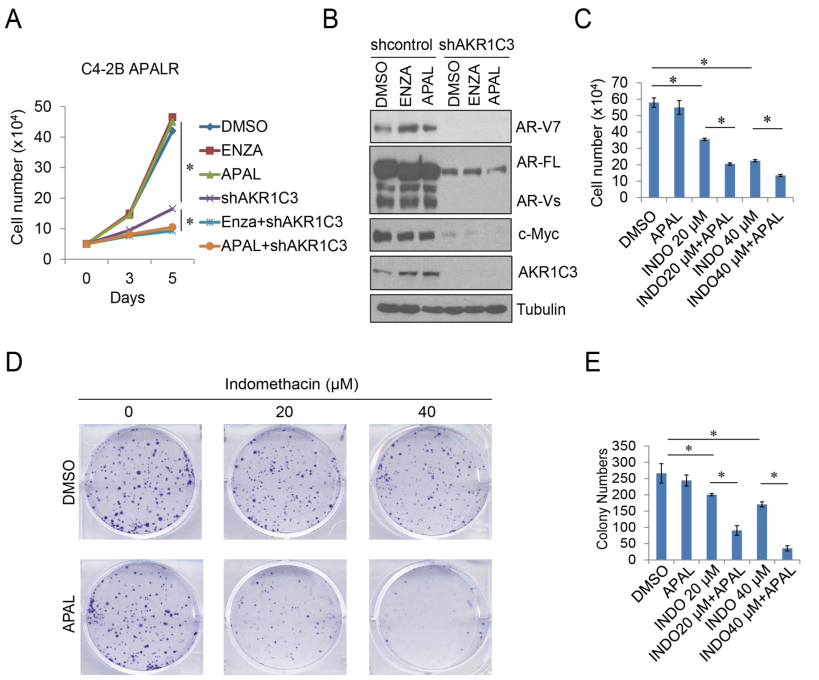

Figure 11. Targeting AKR1C3 resensitizes apalutamide resistant cells to apalutamide and enzalutamide. A. C4-2B APALR cells were treated with 20 μM enzalutamide, 20 μM apalutamide or infected with lenti-AKR1C3 shRNA, alone or in combination. Total cell numbers were determined on 3 and 5 days. B. C4-2B APALR cells were infected with lenti-control shRNA or lenti-AKR1C3 shRNA and then treated with 20 μM enzalutamide or 20 μM apalutamide. After 5 days, whole cell lysates were collected and subjected to western blot. C. C4-2B APALR cells treated with 0, 20, 40 μM indomethacin with or without 20 μM apalutamide and cell numbers were determined after 5 days. D-E. The clonogenic ability of C4-2B ARNR cells treated with 0, 20, 40 μM indomethacin with or without 20 μM apalutamide was analyzed. APAL: apalutamide, ENZA: enzalutamide. INDO: indomethacin * $p<0.05$.

4. PRODUCTS:

Publications, conference papers, and presentations

1. Zhao J, Ning S, Lou W, Yang JC, Armstrong CM, Lombard AP, D'Abronzo LS, Evans CP, Gao AC, Liu C. Cross-resistance Among Next Generation Anti-Androgen Drugs Through the AKR1C3/AR-V7 Axis in Advanced Prostate Cancer. Mol Cancer Ther. 2020 May 19; PMID: 32430485. Online ahead of print.

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5. IMPACT

Our study discovered a novel mechanism by which AKR1C3 induces AR-V7 protein stabilization via activation of the ubiquitin proteasome pathway system activation. We show that AKR1C3 reprograms AR/AR-V7 signaling in enzalutamide resistant cells. AKR1C3 induces AR-V7 overexpression and

stabilizes AR-V7 protein in resistant cells through alteration of the ubiquitin proteasome system. Targeting AKR1C3 by indomethacin activates UPR and p53 pathways but suppresses AR/AR-V7 signaling. Orally administrated indomethacin significantly enhances enzalutamide treatment through AKR1C3/AR-V7 signaling suppression. Additionaly, we also found out that AKR1C3 regulated RNA splicing factor hnRNPA1 expression. HnRNPA1 regulated AR-V7 expression in resistant cells. Additionally, we found apalutamide and darolutamide share similar resistant mechanisms with enzalutamide and abiraterone. The AKR1C3/AR-V7 complex confers cross-resistance to second generation AR-targeted therapies in advanced prostate cancer. Our results highlight the role of AKR1C3/AR-V7 complex in enzalutamide and abiraterone resistance.

6. CHANGES/PROBLEMS

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

(1) Allen Gao, MD PhD (PI) No change

(2) Christopher P. Evans MD (Co-PI) No Change

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Contribution to Project:	*No change*
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Researcher Identifier (e.g. ORCID ID):	975862723
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Contribution to Project:	*No change*
Funding Support:	*N/A*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

N/A

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report

9. APPENDICES:

N/A