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Small Molecule Therapeutics

Molecular Cancer Therapeutics

Niclosamide and Bicalutamide Combination Treatment Overcomes Enzalutamide- and Bicalutamide-Resistant Prostate Cancer

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Abstract

Activation of the androgen receptor (AR) and its splice variants is linked to advanced prostate cancer and drives resistance to antiandrogens. The roles of AR and AR variants in the development of resistance to androgen deprivation therapy (ADT) and bicalutamide treatment, however, are still incompletely understood. To determine whether AR variants play a role in bicalutamide resistance, we developed bicalutamide-resistant LNCaP cells (LNCaP-BicR) and found that these resistant cells express significantly increased levels of AR variants, particularly AR-V7, both at the mRNA and protein levels. Exogenous expression of AR-V7 in bicalutamide-sensitive LNCaP cells confers resistance to bicalutamide treatment. Knockdown of AR-V7 in bicalutamide- and enzalutamide-resistant CWR22Rv1, enzalutamide-resistant C4-2B (C4-2B MDVR), and LNCaP-BicR cells reversed bicalutamide resistance. Niclosamide, a potent inhibitor of AR variants, significantly enhanced bicalutamide treatment. Niclosamide and bicalutamide combination treatment not only suppressed AR and AR variants expression and inhibited their recruitment to the PSA promoter, but also significantly induced apoptosis in bicalutamide- and enzalutamide-resistant CWR22Rv1 and C4-2B MDVR cells. In addition, combination of niclosamide with bicalutamide inhibited the growth of enzalutamide-resistant tumors. In summary, our results demonstrate that AR variants, particularly AR-V7, drive bicalutamide resistance and that targeting AR-V7 with niclosamide can resensitize bicalutamide-resistant cells to bicalutamide treatment. Furthermore, combination of niclosamide with bicalutamide inhibits enzalutamide resistant tumor growth, suggesting that the combination of niclosamide and bicalutamide could be a potential cost-effective strategy to treat advanced prostate cancer in patients, including those who fail to respond to enzalutamide therapy. *Mol Cancer Ther; 16(8); 1521–30. ©2017 AACR.*

Introduction

Androgen signaling through the androgen receptor (AR) plays an important role not only in maintaining the function of the prostate, but also in promoting the development of castration-resistant prostate cancer (CRPC). A common treatment modality for prostate cancer is androgen deprivation, which is achieved either by surgical or medical castration (1). The nonsteroidal antiandrogen bicalutamide is often used in combination with androgen-deprivation therapy (2–4). Although bicalutamide treatment initially exhibits favorable responses, prostate cancers eventually become refractory and develop resistance to bicalutamide and progress to CRPC (5, 6). Enzalutamide, abiraterone, docetaxel, and cabazitaxel are standard treatments for CRPC (7, 8). Although both abiraterone and enzalutamide provide significant benefit to CRPC patients, resistance occurs frequently. In addition, both abiraterone and enzalutamide therapies are quite expensive. According to recent cost-effectiveness analysis, abiraterone treatment cost about $123,400/quality-adjusted life year compared with placebo, and the cost of enzalutamide is $437,600/quality-adjusted life year compared with abiraterone (9). The cost of these two drugs makes treating patients with metastatic CRPC (mCRPC) an extremely expensive process, and cost-effective therapeutic strategies are in urgent need.

Bicalutamide is a nonsteroidal antiandrogen drug used in the treatment of prostate cancer and is effective at blocking AR activity and tumor growth in androgen-responsive prostate cancer (10, 11). Although the TERRAIN and STRIVE clinical trials revealed enzalutamide had better outcomes compared with bicalutamide in CRPC patients (12), bicalutamide at a dose of 150 mg/day is regularly prescribed in men with locally advanced nonmetastatic prostate cancer either as a monotherapy or an adjuvant to surgical or medical castration (13). The mechanisms associated with bicalutamide resistance are still incompletely understood. To date, it is known that AR mutations and coactivators such as TIF2 can confer resistance to (14, 15). Reduced AR binding and hypersensitivity to low levels of endogenous androgens are also associated with bicalutamide resistance (16). Alternative mRNA splicing or genome rearrangements generate truncated and constitutively active AR variants which have been

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associated with enzalutamide and abiraterone resistance in late-stage prostate cancer patients (17–22). However, the role of AR variants in the development of resistance to bicalutamide has not been well studied.

In the current study, we found that bicalutamide-resistant prostate cancer cells express higher levels of AR variants than bicalutamide-responsive cells. Overexpression of AR-V7 confers resistance to bicalutamide and targeting AR-V7 with niclosamide, a potent inhibitor of AR-V7 (23, 24), can resensitize bicalutamide-resistant cells to bicalutamide treatment. Furthermore, combination of niclosamide with bicalutamide inhibits enzalutamide-resistant tumor growth, suggesting that the combination of niclosamide with bicalutamide could provide a potential cost-effective strategy to treat advanced prostate cancer that fails to respond to enzalutamide therapy.

Materials and Methods

Reagents and cell culture

LNCaP and CWR22Rv1 cells were obtained from the ATCC. All experiments with cell lines were performed within 6 months of receipt from ATCC or resuscitation after cryopreservation. ATCC uses short tandem repeat (STR) 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). LNCaP, C4-2B, and CWR22Rv1 were maintained in RPMI1640 media supplemented with 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. LNCaP AR-V7 cells were stably transfected with AR-V7 plasmid and maintained in 300 μg/mL G418 RPMI1640 medium. LNCaP cells were incubated with increasing concentrations of bicalutamide (1–40 μmol/L) over 12 months to generate bicalutamide-resistant LNCaP cells (LNCaP-BicR) and stored for further analysis. Parental LNCaP cells were passaged alongside the bicalutamide-treated cells as an appropriate control. C4-2B MDVR cells were maintained in 20 μmol/L enzalutamide containing medium as described previously (25). All cells were maintained at 37°C in a humidified incubator with 5% carbon dioxide. Niclosamide was purchased from Sigma.

Plasmids and cell transfection

For siRNA transfection, cells were seeded at a density of 1×10^5 cells per well in 12-well plates or 3×10^5 cells per well in 6-well plates and transfected with siRNA (Dharmacon) targeting the AR-V7 sequence (GUAGUUGUGAGUAUCAUGA) or a control sequence (CTTACGCTGAGTACTTCGA) using Lipofectamine 2000 (Invitrogen). Cells were transiently transfected with AR-V7 plasmids using Attractene (Qiagen).

Chromatin immunoprecipitation assay

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 antibodies against AR (AR-C19; Santa Cruz Biotechnology) or AR-V7 (AG10008, precision antibody) 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 $NaHCO_3$), crosslinking was reversed, and DNA was extracted. The resulting chromatin preparations were analyzed by PCR using primers spanning either the proximal or the distal enhancer AREs of the PSA promoter (24, 26). Isotype-matched IgG was used as control.

Western blot analysis

Whole-cell 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, mouse mAb, 1:1,000 dilution, Santa Cruz Biotechnology); AR-V7 (AG10008, mouse monoclonal antibody, 1:1,000 dilution, precision antibody); Tubulin (T5168, monoclonal anti-α-tubulin antibody, 1:5,000 dilution, Sigma-Aldrich)]. Tubulin was used as loading control. Following secondary antibody incubation, immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (Millipore).

Luciferase assay

LNCaP cells were transfected with pGL3-PSA6.0-Luc reporters along with AR-V7 as indicated in the figures in charcoal-stripped FBS (CS-FBS) conditions. Cell lysates were subjected to luciferase assays with the Luciferase Assay System (Promega) as described previously (23).

Cell growth assay

Cells were treated with the indicated compounds and total cell numbers were counted and the cell survival rate [(treatment group cell number/control group cell number) × 100%] was calculated after 3 or 5 days.

Clonogenic assay

Cells were plated at equal density (2,000 cells/dish) and treated with the indicated compounds in 100-mm dishes for 14 days. The colonies were rinsed with PBS before staining with 0.5% crystal violet/4% formaldehyde for 30 minutes and the number of colonies was counted.

Cell death ELISA

CWR22Rv1, C4-2B MDVR, and LNCaP-BicR cells were seeded on 12-well plates (1×10^5 cells/well) in RPMI1640 media containing 10% FBS and treated with DMSO or 0.5 μmol/L niclosamide for 3 days. Mono- and oligonucleosomes in the cytoplasmic fraction were measured by the Cell Death Detection ELISA Kit (Roche, catalog. no. 11544675001) as described previously (27). Briefly, cells were collected and homogenized in 400 μL of incubation buffer. The wells were coated with anti-histone antibodies and incubated with the lysates, horseradish peroxidase–conjugated anti-DNA antibodies, and the substrate. Absorbance was measured at 405 nm.

Real-time quantitative RT-PCR

Total RNA was 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 (28). 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 RT-PCR were: AR-full length: forward (F)-AAG CCA GAG CTG

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TGC AGA TGA, reverse (R)-TGT CCT GCA GCC ACT GGT TC; AR-V1: F-AAC AGA AGT ACC TGT GCG CC, R-TGA GAC TCC AAA CAC CCT CA; AR-V3: F-TGG ATG GAT AGC TAC TCC GG, R-GTT CAT TCT GAA AAA TCC TTC AGC; AR-V7: F-AAC AGA AGT ACC TGT GCG CC, R-TCA GGG TCT GGT CAT TTT GA; AR-V9: F-TGC GCC AGC AGA AAT GAT TG, R-GCA GCT GCT CAG GTA AGT TG; PSA: F-GCC CTG CCC GAA AGG, R-GAT CCA CTT CCG GTA ATG CA; FKBP5: F-AGA ACC AAA CGG AAA GGA GA, R-GCC ACA TCT CTG CAG TCA AA; UBE2C: F-TGG TCT GCC CTG TAT GAT GT, R-AAA AGC TGT GGG GTT TTT CC; Myc: F-TGA GGA GAC ACC GCC CAC, R-CAA CAT CGA TTT CTT CCT CAT C; Actin: F-AGA ACT GGC CCT TCT TGG AGG, R-GTT TTT ATG TTC CTC TAT GGG.

In vivo tumorigenesis assay

CWR22Rv1 cells (4×10^6) were mixed with Matrigel (1:1) and injected subcutaneously into the flanks of 6–7 weeks old male SCID mice. Tumor-bearing mice (tumor volume around 50–100 mm^3) were randomized into four groups ($n = 5$ per group) and treated as follows: (i) vehicle control (5% Tween 80 and 5% ethanol in PBS, intraperitoneally), (ii) bicalutamide (25 mg/kg, orally), (iii) niclosamide (25 mg/kg, intraperitoneally), (iv) bicalutamide (25 mg/kg, orally) + niclosamide (25 mg/kg, intraperitoneally). Tumors were measured using calipers twice a week and tumor volumes were calculated using length $\times$ width^2/2. Tumor tissues and vital organs were harvested after 3 weeks of treatment. All the animal experiments were performed in accordance with the approved guidelines. The experimental animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC), University of California, Davis, and satisfied all University and NIH rules for the humane use of laboratory animals.

IHC

Tumors and vital organs were fixed by formalin- and 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 incubation in 10% FBS in PBS for 30 minutes at room temperature. Slides were then incubated with anti–Ki-67 (at 1:500; NeoMarker), or anti-AR-V7 (at 1:250; AG10008, precision antibody) at room temperature for 30 minutes. 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 were scored and counted in 5 different vision areas. Images were taken with an Olympus BX51 microscope equipped with DP72 camera.

Statistical analysis

All data are presented as means $\pm$ 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 Scheffé procedure for comparison of means. $P < 0.05$ was considered statistically significant.

Results

Bicalutamide-resistant prostate cancer cells express AR variants

Numerous reports show that enzalutamide and abiraterone treatment induce expression of AR variants, particularly AR-V7, in prostate cancer cells (20, 21, 23, 29). But, to date, the effect of bicalutamide treatment on AR variants expression in prostate cancer cells or patients is unknown. To address this issue, we generated bicalutamide-resistant cells (LNCaP-BicR) by chronically treating LNCaP cells with bicalutamide for more than 6 months. As shown in Fig. 1A, after 12 months of continuous culture in media containing bicalutamide, LNCaP-BicR, cells exhibited increased resistance to bicalutamide compared with LNCaP parental cells. These results were confirmed by clonogenic assay (Fig. 1B and C). Next, we examined the expression of AR variants in LNCaP parental and LNCaP-BicR cells. LNCaP-BicR cells express higher levels of AR variant mRNAs than LNCaP parental cells, including AR-V1, AR-V3, AR-V7, and AR-V9 (Fig. 1D). The levels of AR-V7 protein expression were confirmed by Western blot analysis (Fig. 1E). LNCaP-BicR cells express significantly higher levels of AR-V7 protein compared with LNCaP parental cells, particularly in CS-FBS conditions. We also examined the location of AR-V7 expression in LNCaP-BicR cells. As shown in Fig. 1F, AR-V7 was highly expressed in the nucleus in LNCaP-BicR cells. Full-length AR was also upregulated in LNCaP-BicR cells compared with the parental LNCaP cells. Collectively, these results demonstrated that bicalutamide-resistant LNCaP cells express higher levels of AR and AR variants than parental cells.

Overexpression of AR-V7 confers resistance to bicalutamide treatment

Previous studies revealed that AR-V7 confers resistance to enzalutamide and abiraterone (21, 29, 30). To determine whether overexpression of AR-V7 also confers resistance to bicalutamide in bicalutamide-resistant LNCaP-BicR cells, we knocked down AR-V7 by transfecting siRNA specific to AR-V7, and then treated the cells with 20 μmol/L bicalutamide for 3 days and cell numbers were counted. As shown in Fig. 2A, downregulation of AR-V7 expression resensitized LNCaP-BicR cells to bicalutamide treatment. The effects of AR-V7 knockdown by AR-V7 siRNA were validated by Western blot analysis (Fig. 2B). To further examine whether AR-V7 drives bicalutamide resistance, we generated AR-V7–overexpressing LNCaP AR-V7 cells by stably expressing AR-V7 in LNCaP cells (Fig. 2C). LNCaP-neo and LNCaP AR-V7 cells were treated with different concentrations of bicalutamide for 3 days and cell numbers were determined. Two independent LNCaP AR-V7–stable cell lines exhibited resistance to bicalutamide treatment in a dose-dependent manner compared with LNCaP-neo cells (Fig. 2D). Collectively, these results suggest that overexpression of AR-V7 confers resistance to bicalutamide.

Both CWR22Rv1 and C4-2B MDVR cells are resistant to enzalutamide and bicalutamide and express higher levels of AR-V7 than cells that respond to enzalutamide or bicalutamide treatment (21, 23). We have previously shown that overexpression of AR-V7 drives resistance to enzalutamide in CWR22Rv1 and C4-2B MDVR cells (23), and downregulation of AR-V7 resensitizes these cells to enzalutamide treatment. To test whether downregulation of AR-V7 in CWR22Rv1 and C4-2B MDVR cells can resensitize these enzalutamide-resistant cells to bicalutamide treatment as well, we knocked down AR-V7 by transfecting AR-V7 siRNA to

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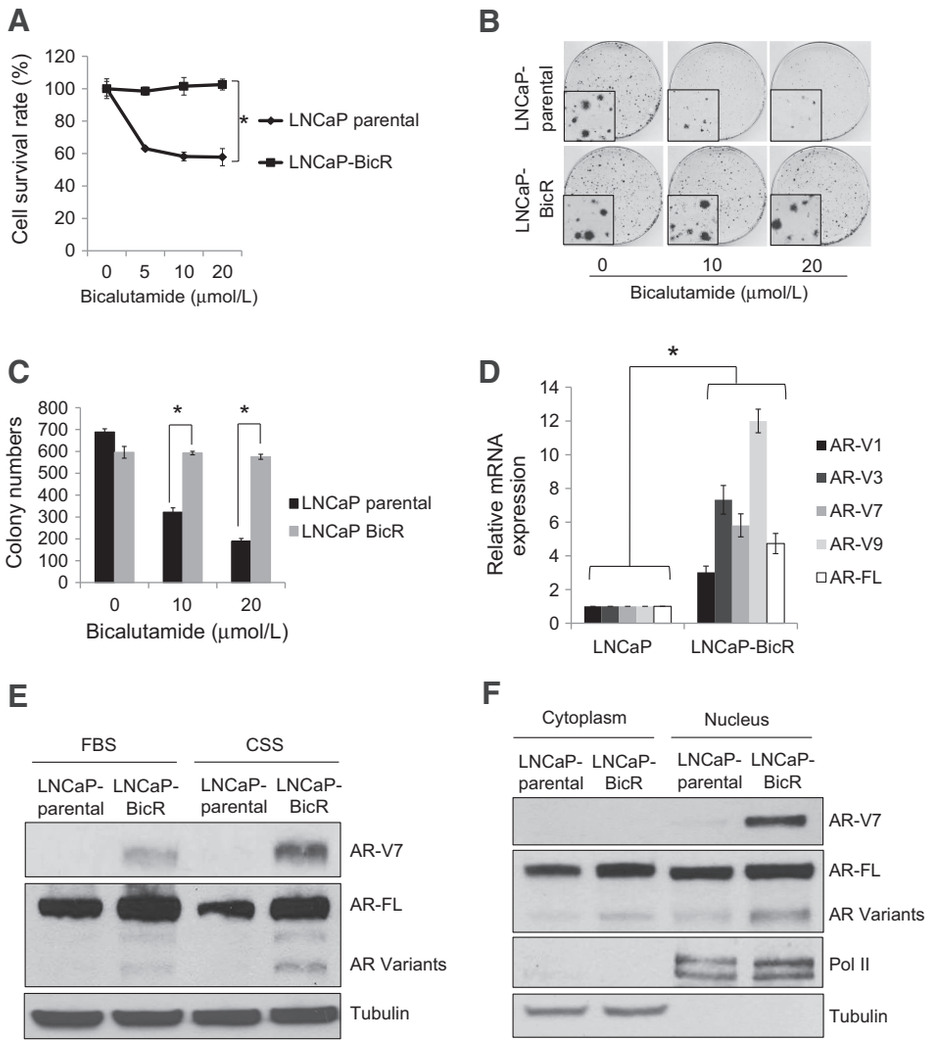

Figure 1.
Chronic bicalutamide treatment in LNCaP cells increases expression of AR and AR variants. **A,** LNCaP parental or LNCaP-BicR cells were treated with different concentrations of bicalutamide in RPMI 1640 media containing 10% FBS, and total cell numbers were counted after 3 days. **B–C,** The clonogenic ability of LNCaP parental and LNCaP-BicR cells treated with 10 μmol/L and 20 μmol/L bicalutamide was analyzed. Colonies were counted, and results are presented as means ± SD of 2 experiments performed in duplicate. **D,** LNCaP parental cells and LNCaP-BicR cells were cultured in RPMI 1640 media containing 10% FBS for 3 days, total RNA was extracted and AR-V1, AR-V3, AR-V7, AR-V9, and full-length AR mRNA levels were analyzed by qRT-PCR. **E,** LNCaP parental cells and LNCaP-BicR cells were cultured in media containing 10% FBS or 10% CS-FBS for 3 days, the total cell lysates were harvested and analyzed by Western blotting using antibodies against AR-V7. **F,** LNCaP parental cells and LNCaP-BicR cells were cultured in media containing 10% CS-FBS for 3 days, the cells were harvested for preparation of cytosolic and nuclear fractions and analyzed by Western blotting using antibodies against AR-V7, AR, RNA polymerase II, or Tubulin. The expression of RNA polymerase II and tubulin was used as markers for the integrity of the nuclear and cytosolic fractions, respectively. *, $P < 0.05$.

both CWR22Rv1 and C4-2B MDVR cells, and then treated the cells with 20 μmol/L bicalutamide for 3 days and cell numbers were counted. As shown in Fig. 2E, knockdown of AR-V7 resensitized enzalutamide-resistant CWR22Rv1 and C4-2B MDVR cells to bicalutamide. The effects of AR-V7 knockdown were confirmed by the Western blot analysis (Fig. 2F). These results suggest that bicalutamide can be combined with AR-V7–targeting agents to treat advanced prostate cancer cell resistance to either enzalutamide or bicalutamide.

Combination of niclosamide with bicalutamide overcomes treatment resistance to enzalutamide *in vitro*

In previous studies, we identified niclosamide as a potent inhibitor of AR-V7, and demonstrated that niclosamide can reverse enzalutamide resistance in advanced prostate cancer cells (23). Having demonstrated that downregulation of AR-V7 can resensitize enzalutamide-resistant CWR22Rv1 and C4-2B MDVR cells to bicalutamide treatment, we next determined if niclosamide, which targets AR-V7, could resensitize enzalutamide-resistant cells to bicalutamide treatment. CWR22Rv1 and C4-2B MDVR cells were treated with 0.5 μmol/L niclosamide with or without 20 μmol/L bicalutamide for 3 or 5 days and total cell numbers were counted. As shown in Fig. 3A, 20 μmol/L bicalutamide had no effect on cell growth and 0.5 μmol/L niclosamide had some degree of growth inhibition in both CWR22Rv1 and C4-2B MDVR cells. However, combination treatment of niclosamide with bicalutamide further inhibited cell growth. The results were confirmed by clonogenic assay. As shown in Fig. 3B, combination of bicalutamide with niclosamide significantly inhibited colony formation in CWR22Rv1 and C4-2B MDVR cells. These results suggest that bicalutamide can be combined with niclosamide to effectively treat enzalutamide-resistant prostate cancer cells.

To further examine niclosamide and bicalutamide combination effects in enzalutamide-resistant prostate cancer cells, cell apoptosis was examined by cell death ELISA. CWR22Rv1 and C4-2B MDVR cells were treated with 0.5 μmol/L niclosamide with or without 20 μmol/L bicalutamide for 3 days and then the cell lysates were collected and subjected to cell death ELISA. Bicalutamide did not induce apoptosis in these two cell lines and niclosamide slightly induced apoptosis. The combination treatment significantly induced cell death (Fig. 3C). Cell survival genes

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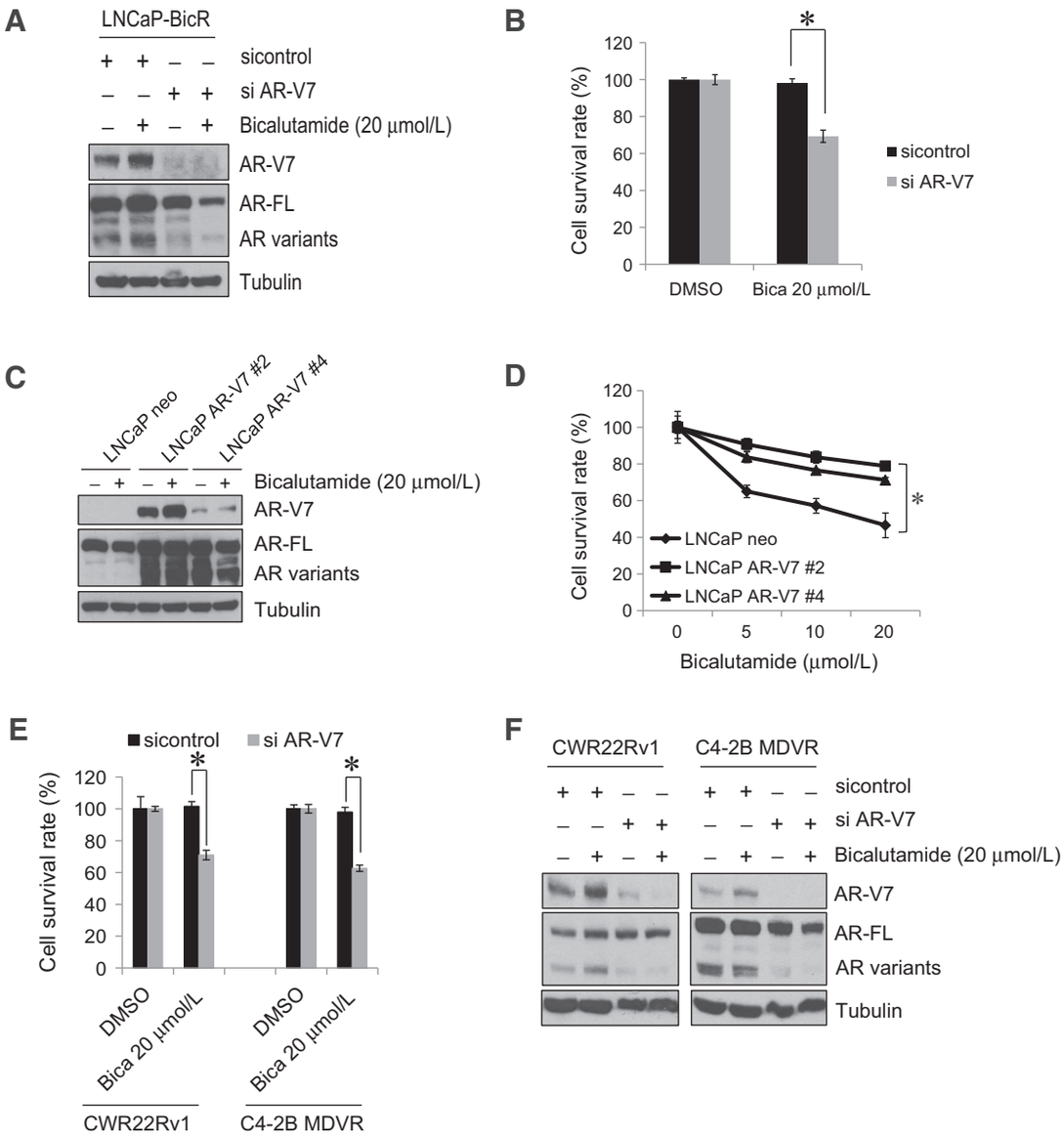

Figure 2.

AR-V7 is involved in bicalutamide resistance. **A–B,** LNCaP-BicR cells were transiently transfected with AR-V7 siRNA, and then treated with 20 μmol/L bicalutamide for 3 days. AR-V7 and AR expression were examined by Western blot. Total cell numbers were counted, and cell survival rate (%) was calculated. **C,** LNCaP-neo, LNCaP AR-V7 #2, and #4 clones were treated with 20 μmol/L bicalutamide for 3 days, the total cell lysates were harvested and analyzed by Western blotting using antibodies against AR and AR-V7. **D,** LNCaP-neo, LNCaP AR-V7 #2, and #4 clones were treated with 20 μmol/L bicalutamide in media containing FBS, and cell numbers were counted after 3 and 5 days. Results are presented as means ± SD of 3 experiments performed in duplicate. **E–F,** CWR22Rv1 and C4-2B MDVR cells were transiently transfected with AR-V7 siRNA, and then treated with 20 μmol/L bicalutamide for 3 days. Total cell numbers were counted, and cell survival rate (%) was calculated. AR-V7 and AR expression were examined by Western blot. *, $P < 0.05$. Bica, Bicalutamide.

downstream of AR and AR variants such as c-Myc, CyclinD1 and survivin were also examined. Combination of niclosamide and bicalutamide significantly inhibited cell survival gene expression. The levels of c-Myc, Cyclin D1, survivin, Bcl-2, and Bcl-xl expression were reduced and cleaved caspase activation and cleaved PARP expression were increased (Fig. 3D). In summary, combination of niclosamide and bicalutamide treatment significantly induced apoptosis in enzalutamide-resistant cell lines. Taken together, the above results show that combination of niclosamide with bicalutamide can overcome resistance to enzalutamide treatment *in vitro*.

To explore the combinatory effects of niclosamide and bicalutamide on androgen signaling, CWR22Rv1, C4-2B MDVR, and LNCaP-BicR cells were treated with DMSO, 0.5 μmol/L niclosamide, 20 μmol/L bicalutamide, or their combination for 3 days and total cell lysates were collected and subjected to Western blot. As shown in Fig. 4A, bicalutamide treatment had no effect on AR-V7 expression, niclosamide reduced the AR-V7 expression and the

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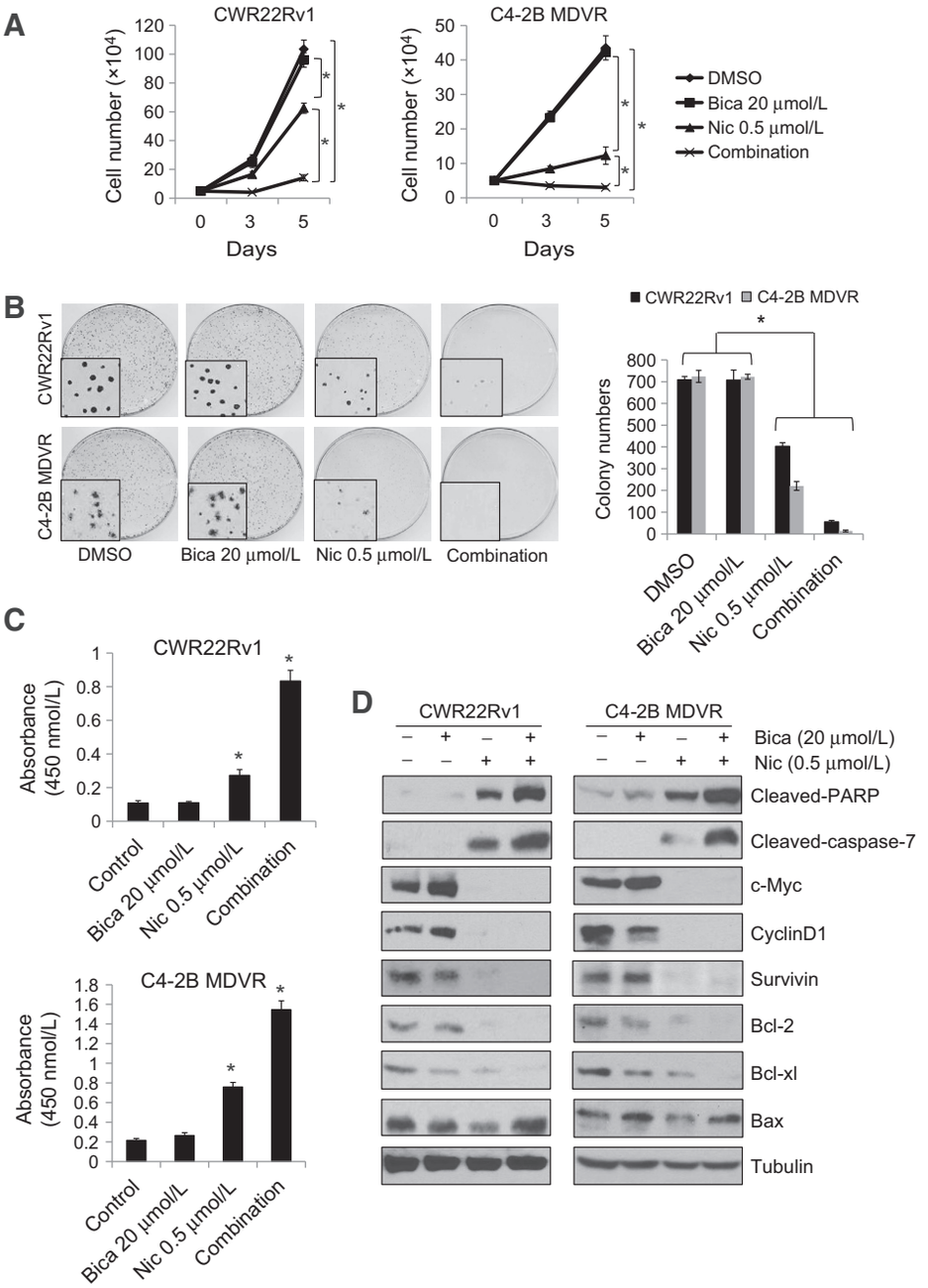

Figure 3.

Niclosamide and bicalutamide treatment suppresses enzalutamide-resistant cell growth and induces apoptosis *in vitro*. **A,** CWR22Rv1 cells and C4-2B MDVR cells were treated with 0.5 μmol/L niclosamide with or without 20 μmol/L bicalutamide in media containing FBS, and cell numbers were counted after 3 and 5 days. Results are presented as means ± SD of 3 experiments performed in duplicate. **B,** Clonogenic assays were performed in CWR22Rv1 and C4-2B MDVR cells, colony pictures were taken under microscope and the number of colonies was counted. Results are presented as means ± SD of 2 experiments performed in duplicate. **C,** CWR22Rv1 cells and C4-2B MDVR cells were treated with 0.5 μmol/L niclosamide with or without 20 μmol/L bicalutamide in media containing FBS for 3 days, cell lysates were subjected to cell death ELISA. Results are presented as means ± SD of 3 experiments performed in duplicate. **D,** CWR22Rv1 cells and C4-2B MDVR cells were treated with 0.5 μmol/L niclosamide with or without 20 μmol/L bicalutamide in media containing FBS for 3 days, total cell lysates were collected and subjected to Western blot. *, $P < 0.05$. Bic, Bicalutamide; Nic, Niclosamide.

combination further inhibited both AR and AR-V7 protein expression. Knockdown of AR-V7 expression using AR-V7 siRNA suppressed PSA luciferase activity in C4-2B MDVR cells, and combination of AR-V7 siRNA and bicalutamide further reduced PSA luciferase activity (Supplementary Fig. S1). To examine if niclosamide combined with bicalutamide could affect AR and AR-V7 transcriptional activity, LNCaP cells were transiently transfected with PSA luciferase plasmid with or without AR-V7 or 1 nmol/L DHT and then treated with 0.5 μmol/L and 1 μmol/L niclosamide with or without 20 μmol/L bicalutamide overnight. As shown in Fig. 4B, both niclosamide and bicalutamide significantly inhibited DHT induced PSA activity. While niclosamide inhibited the AR-V7 transcriptional activity, combined niclosamide and bicalutamide further inhibited both DHT and AR-V7 induced AR transcriptional activity. We also examined the full-length AR and AR-V7 target genes by niclosamide and bicalutamide combination treatment in C4-2B MDVR cells. As shown in supplementary Fig. S2, bicalutamide treatment suppressed full-length AR target genes, such as PSA, NKX3.1 and FKBP5 but not AR-V7 target genes, such as UBE2C and Myc. However, niclosamide treatment suppressed the expression of both full-length AR and AR-V7 target genes, and the niclosamide and bicalutamide combination further suppressed the expression of these target genes. These results were confirmed in a C4-2 AR-V7 stable clone by ChIP assay. As

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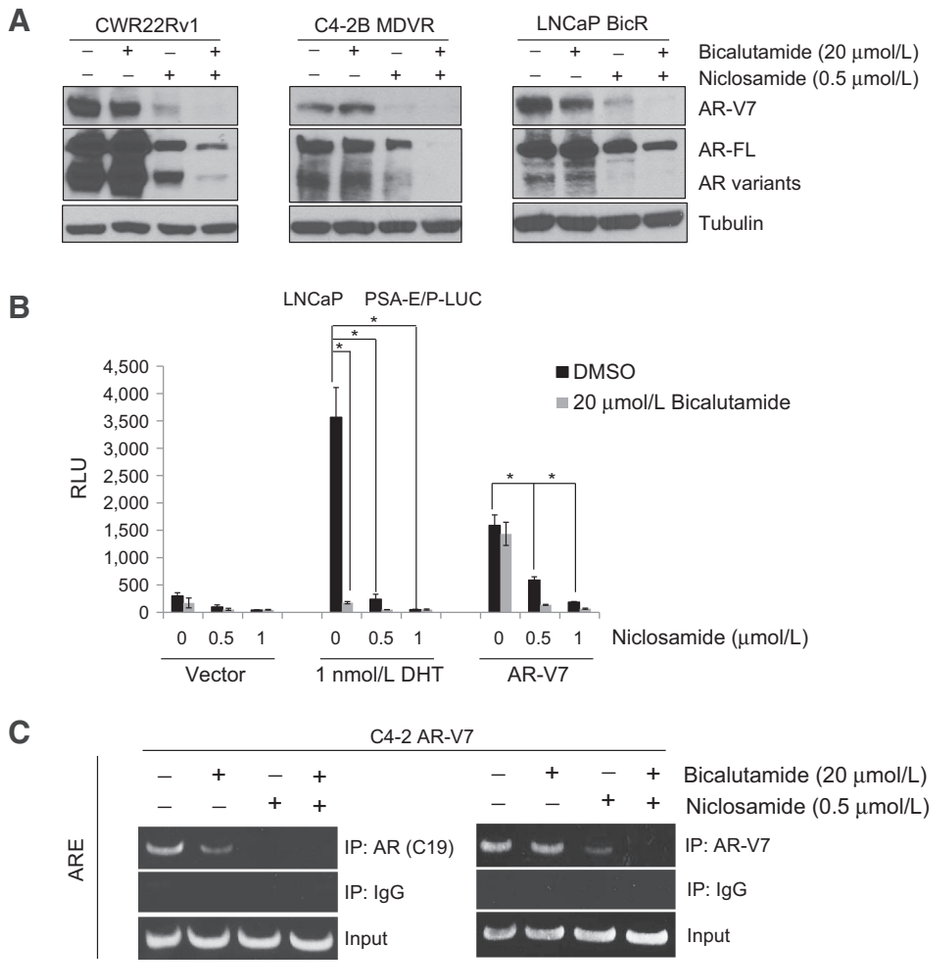

Figure 4.
Niclosamide and bicalutamide combination treatment suppresses AR and AR variants signaling in enzalutamide-resistant prostate cancer. **A,** CWR22Rv1, C4-2B MDVR, and LNCaP-BicR cells were treated with 0.5 μmol/L niclosamide with or without 20 μmol/L bicalutamide in media containing FBS for 3 days, total cell lysates were collected and subjected to Western blot. **B,** LNCaP cells were transiently transfected with PSA-E/P luciferase with 0.5 μg AR-V7 plasmid or control plasmid for 24 hours, cells were then treated with DMSO, 0.5 μmol/L niclosamide, 20 μmol/L bicalutamide, or their combination with or without 1 nmol/L DHT overnight and luciferase activity was measured. Results are presented as means ± SD of 3 experiments performed in duplicate. **C,** C4-2 AR-V7 cells were treated with DMSO, 0.5 μmol/L niclosamide, 20 μmol/L bicalutamide, or their combination for 2 days. Recruitment of AR to AREs in the PSA promoter was analyzed by ChIP assay. *, $P < 0.05$.

shown in Fig. 4C, both bicalutamide and niclosamide reduced the recruitment of full-length AR to the PSA promoter, however, only niclosamide significantly reduced the recruitment of AR-V7 to the PSA promoter and combination treatment further reduced the recruitment. Taken together, these results show that combination of niclosamide with bicalutamide suppresses AR-V7 expression and transcriptional activity and induces cell growth inhibition and apoptosis.

Combination of niclosamide with bicalutamide suppresses tumor growth of enzalutamide-resistant prostate cancer

To test whether niclosamide plus bicalutamide can overcome resistance to enzalutamide *in vivo*, xenografts generated from enzalutamide-resistant CWR22Rv1 cells were treated with vehicle, bicalutamide, niclosamide, or their combination for 3 weeks as described in the methods. As shown in Fig. 5A–C, CWR22Rv1 cells were resistant to bicalutamide treatment with tumor volumes comparable with those in the vehicle treated control group. Niclosamide alone decreased the tumor volume while combination of niclosamide and bicalutamide synergistically decreased CWR22Rv1 tumors, indicating that niclosamide plus bicalutamide could overcome enzalutamide resistance and restore sensitivity of CWR22Rv1 xenografts to bicalutamide treatment *in vivo*. The treatment did not affect animal weight (Fig. 5D). To determine whether niclosamide alone or in combination with bicalutamide represses AR-V7 expression and tumor proliferation *in vivo*, representative tumor samples were analyzed by IHC for AR-V7 and Ki67. As shown in Fig. 5E, niclosamide inhibited AR-V7 and Ki67 expression while combination treatment further decreased the levels of their expression. These results suggest that niclosamide can improve bicalutamide treatment, and combination of niclosamide with bicalutamide can be used to treat enzalutamide-resistant prostate cancer.

To examine the treatment toxicity in each group, histopathologic examination of the vital organs was performed. Overall, no significant pathological changes were noted in any group. As shown in Fig. 5F, all the vital organs were normal after 3 weeks of treatment; the livers did not show any vacuolar changes, and there was no sign of inflammation at the renal pelvis in single treatment or combination treatment group. In conclusion, both the niclosamide and combination treatment were well tolerated.

Discussion

In this study, we demonstrated that AR-V7 expression confers resistance to bicalutamide and that downregulation of AR-V7

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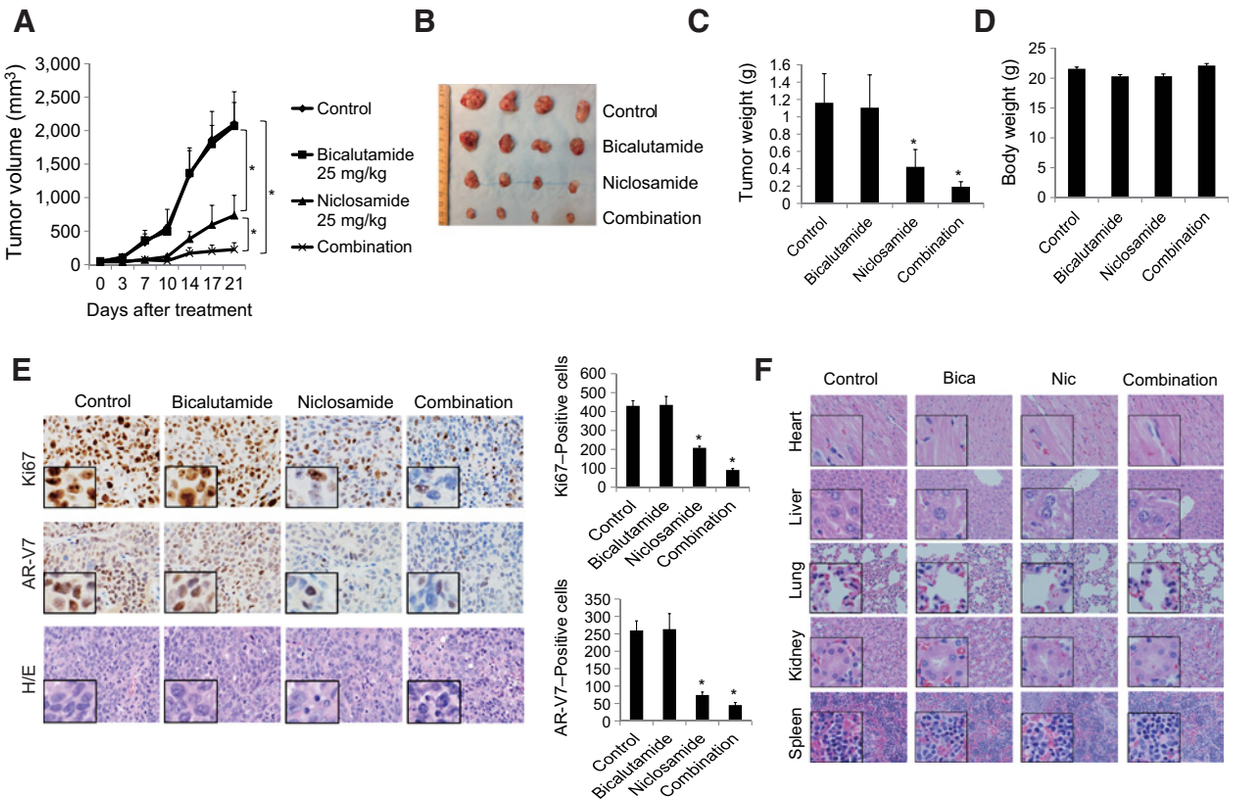

Figure 5.

Niclosamide and bicalutamide treatment suppressed enzalutamide-resistant tumor growth. **A,** Mice bearing CWR22Rv1 xenografts were treated with vehicle control, bicalutamide (25 mg/kg orally), niclosamide (25 mg/kg intraperitoneally), or their combination for 3 weeks. Tumor volumes were measured twice weekly. **B–C,** Tumors were photographed and weighed. **D,** Body weight for each group was calculated. **E,** IHC staining of Ki-67 and AR-V7 in each group was performed and quantified. **F,** Hematoxylin and eosin (H&E) staining of vital organs from each group was performed. *, $P < 0.05$. Bic, Bicalutamide; Nic, Niclosamide.

expression resensitizes bicalutamide and enzalutamide resistance cells to bicalutamide treatment. In addition, combination of bicalutamide with niclosamide, a potent inhibitor of AR-V7, suppresses tumor growth of enzalutamide-resistant prostate cancer *in vitro* and *in vivo*. These results indicate the potential therapeutic efficacy of niclosamide in combination with bicalutamide to treat advanced prostate cancer that is resistant to enzalutamide.

Bicalutamide has been widely used as the first line treatment for prostate cancer that has failed androgen deprivation therapy. Although initially effective, resistance frequently occurs, most likely due to structural changes within the AR or modifications in androgen-dependent signaling cascades. AR mutation has been linked to anti-androgen resistance. Mutations in the ligand-binding domain that alter ligand specificity and sensitivity frequently increase after androgen ablation. The AR F877L mutation has been found in enzalutamide-resistant patients and might allow for enzalutamide acting as an AR antagonist, especially when this mutation co-occurs with the T878A mutation in prostate cancer cells (31–33). Prolonged enzalutamide or apalutamide treatment might reposition the drug to eliminate steric clashes that promoted helix 12 terminus (H12) dislocation in AR (34). The T878A and H875Y AR mutants have been reported to be activated by flutamide leading to anti-androgen withdrawal syndrome (35–37). Similarly, bicalutamide is an agonist to the W741L mutant AR in prostate cancer (14). In addition to AR mutations, other mechanisms involved in bicalutamide resistance have been discovered. The CAMK2N1 gene, an inhibitor of CaMKII (calcium/calmodulin-dependent protein kinase II), has been shown to induce bicalutamide resistance and is inversely correlated with AR in prostate cancer (38). TIF2/glucocorticoid receptor-interacting protein (TIF2/GRIP1/SRC2) is a member of the steroid receptor coactivator (SRC/p160) family and has also been found to contribute to bicalutamide resistance (15). In the present study, we provide evidence that AR variants, particularly AR-V7, confer resistance to bicalutamide treatment. Overexpression of AR-V7 in bicalutamide-sensitive LNCaP cells confers resistance to bicalutamide, while knockdown AR-V7 in bicalutamide-resistant cells resensitizes them to bicalutamide treatment. Chronic bicalutamide treatment of LNCaP cells significantly increased expression of several AR variants including AR-V1, AR-V7, and AR-V9. Among the identified AR variants, AR-V7, which is encoded by contiguous splicing of AR exons 1/2/3/CE3, has been well studied mainly due to its prevalence in prostate cancer samples (17–19). AR-V7 is a constitutively active, ligand independent transcription factor that can induce castration-resistant cell growth *in vitro* and *in vivo* (19, 39, 40). AR-V7 also may heterodimerize with full-length androgen receptor (AR-FL) in an androgen-independent manner (41). Recent studies have linked AR alternative splicing, particularly AR-V7, to the development of enzalutamide, abiraterone and docetaxel resistance (21, 29, 42). Clinical studies suggest that

AR-V7 in circulating tumor cells from patients with CRPC is associated with resistance to enzalutamide and abiraterone (22, 43). Several preclinical studies revealed that AR variants might play certain roles in taxane resistance which could be through blocking nuclear translocation via AR. AR-V7 lacks the hinge region that mediates the microtubule binding, which reduces the association of AR-V7 with microtubules (42, 44). Thus, targeting AR-V7 could be a valuable strategy to treat CRPC patients. The challenge to designing a drug that targets AR-V7 in patients is mainly drug toxicity and clinical efficacy. The ideal strategy to target AR-V7 is to decrease AR-V7 expression level or suppress its transcriptional activity by targeting the N-terminal domain. Our previous studies revealed that niclosamide targets AR variants and enhances enzalutamide and abiraterone treatment (23, 24). In the present study, we demonstrated that niclosamide could be used to resensitize bicalutamide-resistant cells via downregulation of AR-V7 expression. Combination of niclosamide with bicalutamide significantly suppressed AR and AR-V7 transcriptional activity and blocked their recruitment to the PSA promoter.

A key finding of our study is that combination of niclosamide with bicalutamide inhibits enzalutamide-resistant prostate cancer cell growth *in vitro* and tumors *in vivo*, suggesting that the combination of niclosamide with bicalutamide could be used to treat enzalutamide-resistant prostate cancer. This finding could have both clinical and economic significance toward the management of mCRPC. Although enzalutamide is effective in the treatment of mCRPC, resistance eventually occurs. The recent PLATO trial of continued treatment with enzalutamide plus abiraterone and prednisone in chemotherapy-naïve patients whose disease progressed on enzalutamide alone failed to show improvement in progression-free-survival (PFS), highlighting an urgent need to identify methods of successfully treating enzalutamide-resistant tumors. Our preclinical data showing the ability to inhibit the growth of enzalutamide-resistant tumors with niclosamide plus bicalutamide could pave the way for a novel strategy to address the unmet needs of men with mCRPC who fail to respond to enzalutamide treatment. Furthermore, bicalutamide is widely available in most developed and developing countries throughout the world and due to the fact that its patent protection has expired, the drug is available in low-cost generic formulations. This makes the niclosamide plus bicalutamide treatment not only widely accessible, but also would provide an economical cost benefit compared with current treatment strategies.

In summary, our results demonstrate that AR variants, particularly AR-V7, drive bicalutamide resistance. Targeting AR-V7 with niclosamide can resensitize bicalutamide-resistant cells to bicalutamide treatment. Furthermore, combination of niclosamide with bicalutamide inhibits enzalutamide-resistant tumor growth, suggesting that the combination of niclosamide with bicalutamide could provide a potential cost-effective strategy to treat advanced prostate cancer that fails to respond to enzalutamide therapy. A clinical trial of niclosamide plus bicalutamide to treat mCRPC patients is planned.

Disclosure of Potential Conflicts of Interest

C. Liu, W. Lou, and A.C. Gao have ownership interest (including patents) in Patent application covering the use of niclosamide. C.-x. Pan has ownership interest (including patents) in Pandomedx. C.P. Evans has received speakers bureau honoraria from, and is a consultant/advisory board member for Astellas. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Liu, C.M. Armstrong, A.P. Lombard, C.P. Evans, A.C. Gao
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Liu, C.-x. Pan, C.P. Evans, A.C. Gao
Writing, review, and/or revision of the manuscript: C. Liu, C.M. Armstrong, C.-x. Pan, C.P. Evans, A.C. Gao
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Liu, W. Lou, V. Cucchiara, X. Gu, J.C. Yang, N. Nadiminty
Study supervision: C. Liu, C.-x. Pan, A.C. Gao

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