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Authors

Liu, Chengfei
Lou, Wei
Zhu, Yezi
et al.

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Niclosamide inhibits androgen receptor variants expression and overcomes enzalutamide resistance in castration resistant prostate cancer

Chengfei Liu[#1], **Wei Lou**[#1], **Yezi Zhu**[1,2], **Nagalakshmi Nadiminty**[1], **Chad T. Schwartz**[1], **Christopher P. Evans**[1,3], and **Allen C. Gao**[1,2,3,4]

[1]Department of Urology, University of California Davis, CA, USA

[2]Graduate Program in Pharmacology and Toxicology, University of California Davis, CA, USA

[3] UC Davis Comprehensive Cancer Center, University of California Davis, CA, USA

[#] These authors contributed equally to this work.

Abstract

Purpose—Enzalutamide, a second-generation antiandrogen, was recently approved for the treatment of castration-resistant prostate cancer (CRPC) in patients who no longer respond to docetaxel. Despite these advances that provide temporary respite, resistance to enzalutamide occurs frequently. AR splice variants such as AR-V7 have recently been shown to drive castration resistant growth and resistance to enzalutamide. This study was designed to identify inhibitors of AR variants and test its ability to overcome resistance to enzalutamide.

Experimental Design—The drug screening was conducted using luciferase activity assay to determine the activity of AR-V7 after treatment with the compounds in the Prestwick Chemical Library, which contains about 1120 FDA-approved drugs. The effects of the identified inhibitors on AR-V7 activity and enzalutamide sensitivity were characterized in CRPC and enzalutamide-resistant prostate cancer cells *in vitro* and *in vivo*.

Results—Niclosamide, an FDA-approved anti-helminthic drug, was identified as a potent AR-V7 inhibitor in prostate cancer cells. Niclosamide significantly downregulated AR-V7 protein expression by protein degradation through a proteasome dependent pathway. Niclosamide also inhibited AR-V7 transcription activity and reduced the recruitment of AR-V7 to the PSA promoter. Niclosamide inhibited prostate cancer cell growth *in vitro* and tumor growth *in vivo*. Furthermore, the combination of niclosamide and enzalutamide resulted in significantly inhibition of enzalutamide-resistant tumor growth, suggesting that Niclosamide enhances enzalutamide therapy and overcomes enzalutamide resistance in castration resistant prostate cancer cells.

Conclusions—Niclosamide was identified as a novel inhibitor of AR variants. Our findings offer preclinical validation of niclosamide as a promising inhibitor of androgen receptor variants

[4]To whom correspondence should be addressed:Allen C. Gao Department of Urology University of California Davis Medical Center 4645 2nd Ave, Research III, Suite 1300 Sacramento, CA 95817 Phone: 916-734-8718 acgao@ucdavis.edu.

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to treat, either alone or in combination with current antiandrogen therapies, advanced prostate cancer patients, especially those resistant to enzalutamide.

Keywords

prostate cancer; enzalutamide; niclosamide; variants; drug resistance

Introduction

Next generation anti-androgens such as enzalutamide and inhibitors of androgen synthesis such as abiraterone have improved the standard of care for late-stage prostate cancer patients (1, 2). Despite their successes and continuing wide-spread use, development of resistance is inevitable (3, 4). Potential resistance mechanisms are emerging that perpetuate disease progression during effective AR blockade. Alternative mRNA splicing generates truncated and constitutively active AR variants which support the CRPC phenotype (5-8). The truncated AR variants which lack the LBD naturally occur in both prostate cancer clinical samples and cell lines (5, 6, 9, 10). Several groups showed that AR variants are up-regulated in CRPC patient samples compared with androgen sensitive patient samples and are associated with prostate cancer progression and resistance to AR-targeted therapy (3, 11-13). AR variants have been shown to induce ligand independent activation of ARE-driven reporters in the absence of androgen which indicates that those variants may have a distinct transcription program compared to the full length AR (9, 14), and the activity of AR variants is postulated to depend on the full length AR (15).

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 (7, 12, 16). AR-V7 functions as a constitutively active ligand independent transcription factor that can induce castration resistant cell growth *in vitro* and *in vivo* (7, 17). Recent studies have linked AR alternative splicing, particularly AR-V7, to the development of enzalutamide resistance (18-21). Thus, targeting AR-V7 would be a valuable strategy to treat CRPC patients.

In the present study, we screened the Prestwick Chemical Library and identified niclosamide, a FDA-approved drug effective against human tapeworms, as a potent AR-V7 inhibitor in prostate cancer cells. We found that niclosamide reduces AR-V7 recruitment to the PSA promoter and significantly inhibits AR-V7 protein expression by protein degradation via the proteasome dependent pathway. Niclosamide inhibits prostate cancer cell growth *in vitro* and tumor growth *in vivo*. Furthermore, niclosamide overcomes enzalutamide resistance and significantly enhances enzalutamide therapy in prostate cancer cells, suggesting that niclosamide can be used to treat, either alone or in combination with current antiandrogen therapies, advanced prostate cancer patients, especially those resistant to enzalutamide.

Materials and Methods

Reagents and Cell Culture

LNCaP, VCaP, CWR22Rv1, PC3 and HEK293 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). All experiments with cell line 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. C4-2 and C4-2B cells were kindly provided and authenticated by Dr. Leland Chung, Cedars-Sinai Medical Center, Los Angeles, CA. The cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 0.1 mg/ml streptomycin. VCaP cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 0.1 mg/ml streptomycin. C4-2-neo and C4-2 AR-V7 cells were generated by stable transfection of C4-2 cells with either empty vector pcDNA3.1 or pcDNA3.1 encoding AR-V7 and were maintained in RPMI1640 medium containing 300 μg/mL G418. HEK293-AR-V7-PSA-E/P-LUC cells were generated by stable transfection of HEK293 cells with plasmids encoding AR-V7 and PSA-E/P-LUC reporter and were maintained in RPMI1640 medium containing 300 μg/mL G418. C4-2B cells were chronically exposed to increasing concentrations of enzalutamide (5 ~ 40 μM) by passage in media containing enzalutamide for >12 months in complete FBS and stored for further analysis. Cells resistant to enzalutamide were referred to as C4-2B MR (C4-2B enzalutamide resistance)(18). Parental C4-2B cells were passaged alongside the enzalutamide treated cells as an appropriate control. C4-2B MR cells were maintained in 20 μM enzalutamide containing medium. All cells were maintained at 37°C in a humidified incubator with 5% carbon dioxide.

Cell transfection and luciferase assay

For small interfering RNA (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 exon7 sequence (UCAAGGAACUCGAUCGUAU) (22) or AR-V7 sequence (GUAGUUGUAAGUAUCAUGA) (22) or a control siRNA targeting the luciferase (Luc) gene, sicontrol (CTTACGCTGAGTACTTCGA), using lipofectamine-RNAiMAX (invitrogen). Cells were transiently transfected with plasmids expressing wild-type (WT)-AR, AR-V7 or pcDNA3.1 using Attractene transfection reagent (QIAGEN). For luciferase assay, LNCaP or PC3 cells (1×10^5 cells per well of 12-well plate) were transfected with 0.5 μg of pGL3-PSA6.0-Luc reporter plasmid or the control plasmid along with WT-AR or AR-V7. The luciferase activity was determined 24–48 hr after transfection using a dual-luciferase reporter assay system as described previously (Promega) (23), the signal was normalized to *Renilla* luciferase control as relative luciferase units (RLU).

Chromatin immunoprecipitation assay

C4-2 neo and C4-2 AR-V7 cells were cultured in CS-FBS condition for 3 days. DNA-AR protein complexes were cross-linked inside the cells by the addition of 1% formaldehyde. Whole-cell extracts were prepared by sonication, and an aliquot of the cross-linked DNA-protein complexes was immunoprecipitated by incubation with the AR-specific antibody

(AR-441; Santa Cruz Biotechnology) overnight at 4°C with rotation. Chromatin-antibody complexes were isolated from solution by incubation with protein A/G agarose beads for 1 hour at 4°C with rotation. The bound DNA-protein complexes were washed and eluted from beads with elution buffer (1% SDS and 0.1 mol/L $NaHCO_3$), crosslinking was reversed, and DNA was extracted. The resulting chromatin preparations were analyzed by PCR using primers spanning AREs of the PSA promoter as described previously(24). Isotype-matched IgG was used as control.

Preparation of nuclear and cytosolic extracts

C4-2B parental and C4-2B MR cells were cultured in media containing charcoal-stripped FBS (CS-FBS) for 4 days. Cells were harvested, washed with PBS twice, and resuspended in a low salt buffer [10 mmol/L HEPES-KOH (pH 7.9), 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, and 0.1% NP40] and incubated on ice for 30 minutes. Nuclei were precipitated by centrifugation at 3,000 × g at 4°C for 10 minutes. The supernatants were collected as the cytosolic fraction. After washing once with the low salt buffer, the nuclei were lysed in a high salt lysis buffer [50 mmol/L Tris-HCl (pH 8), 150 mmol/L NaCl, 1% Triton X-100] with vigorous shaking at 4 ° for 30 minutes. The nuclear lysates were precleared by centrifugation at 10,000 rpm at 4° for 15 minutes. Protein concentration was determined using the Coomassie Plus protein assay kit (Pierce, Rockford, IL).

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 [AR441, (SC-7305, Santa Cruz Biotechnology, Santa Cruz, CA); AR-V7 (AG10008, Precision antibody); PSA (SC-7316, Santa Cruz Biotechnology, Santa Cruz, CA); Tubulin (T5168, Sigma-Aldrich, St. Louis, MO)]. Tubulin was used as loading control. Following secondary antibody incubation, immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (Millipore, Billerica, MA).

Cell growth assay

C4-2 neo, C4-2 AR-V7, CWR22Rv1 or PZ-HPV-7 cells were seeded on 12-well plates at a density of 1×10^5 cells/well in RPMI 1640 media containing 10% FBS and treated with 0.5 μM niclosamide for 48 hours. Total cell numbers were counted and the cell survival rate (%) was calculated. Cell survival rate (%) = (Treatment group cell number / Control group cell number) ×100%. CWR22Rv1 cells were transient transfected with AR exon7 siRNA or AR-V7 siRNA in CS-FBS condition, cell numbers were counted on different days. C4-2B or C4-2B MR cells were seeded on 12-well plates at a density of 0.5×10^5 cells/well in RPMI 1640 media containing 10% FBS and treated with 20 μM enzalutamide. Total cell numbers were counted after 3 and 5 days. CWR22Rv1 cells or C4-2B MR cells were seeded on 12-well plates at a density of 0.5×10^5 cells/well in RPMI 1640 media containing 10% FBS and co-treated with 0.25 μM niclosamide and 20 μM enzalutamide in media containing FBS. Total cell numbers were counted after 3 and 5 days.

Clonogenic Assay

C4-2 neo, C4-2 AR-V7, CWR22Rv1 or C4-2B MR cells were treated with DMSO, 0.5 μM or 1.0 μM niclosamide in media containing 10% complete FBS. CWR22Rv1 cells or C4-2B MR cells were treated with 0.25 μM niclosamide with or without 20 μM enzalutamide. Cells were plated at equal density (1500 cells/dish) in 100 mm dishes for 14 days; the medium was changed every 7 days. The colonies were rinsed with PBS before staining with 0.5% crystal violet/4% formaldehyde for 30 min and the numbers of colonies were counted.

Cell death ELISA

C4-2 neo, C4-2 AR-V7, CWR22Rv1 or pzHPV7 cells were seeded on 12-well plates (1×10^5 cells/well) in RPMI 1640 media containing 10% FBS and treated with DMSO or 0.5 μM niclosamide for 48 hours. Mono- and oligonucleosomes in the cytoplasmic fraction were measured by the Cell Death Detection ELISA kit (Roche, Cat#11544675001) according to the manufacturer's instructions. Briefly, floating and attached cells were collected and homogenized in 400 μL of incubation buffer. The wells were coated with antihistone antibodies and incubated with the lysates, horseradish peroxidase–conjugated anti-DNA antibodies, and the substrate, in that sequence. Absorbance was measured at 405 nm.

Real-Time quantitative RT-PCR

Total RNAs were extracted using TriZOL reagent (Invitrogen). cDNAs were prepared after digestion with RNase-free RQ1 DNase (Promega). The cDNAs were subjected to real-time reverse transcription-PCR (RT-PCR) using Sso Fast Eva Green Supermix (Bio-Rad) according to the manufacturer's instructions and as described previously (25). Each reaction was normalized by co-amplification of actin. Triplicates of samples were run on default settings of Bio-Rad CFX-96 real-time cycler. Primers used for Real-time PCR were: AR-full length: 5’-AAG CCA GAG CTG TGC AGA TGA, 3’-TGT CCT GCA GCC ACT GGT TC; AR-V1: 5’-AAC AGA AGT ACC TGT GCG CC, 3’-TGA GAC TCC AAA CAC CCT CA; AR-V7: 5’-AAC AGA AGT ACC TGT GCG CC, 3’-TCA GGG TCT GGT CAT TTT GA; AR V1/2/2b: 5’-TGG ATG GAT AGC TAC TCC GG, 3’-GTT CAT TCT GAA AAA TCC TTC AGC; AR1/2/3/2b: 5’-AAC AGA AGT ACC TGT GCG CC, 3’-TTC TGT CAG TCC CAT TGG TG; Actin: 5’-AGA ACT GGC CCT TCT TGG AGG, 3’-GTT TTT ATG TTC CTC TAT GGG.

Measurement of PSA

PSA levels were measured in the culture supernatants using PSA ELISA Kit (United Biotech, Inc., Mountain View, CA) according to the manufacturer's instructions.

In vivo tumorigenesis assay

CWR22Rv1 cells (3 million) were mixed with matrigel (1:1) and injected subcutaneously into the flanks of 6-7 week male SCID mice. Tumor-bearing mice (tumor volume around 50-100 mm^3) were randomized into four groups (with 10 tumors in each group) and treated as follows: (1) vehicle control (5% Tween 80 and 5% ethanol in PBS, i.p.), (2) enzalutamide (25 mg/kg, p.o.), (3) niclosamide (25 mg/kg, i.p.), (4) enzalutamide (25 mg/kg, p.o.) + niclosamide (25 mg/kg, i.p.). Tumors were measured using calipers twice a week and tumor

volumes were calculated using length × width^2/2. 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 min and then at 100 W for 20 min. Nonspecific antibody binding was blocked by incubating with 10% fetal bovine serum in PBS for 30 min at room temperature. Slides were then incubated with anti-Ki-67 (at 1:500; NeoMarker) at room temperature for 30 min. Slides were then washed and incubated with biotin-conjugated secondary antibodies for 30 min, followed by incubation with avidin DH-biotinylated horseradish peroxidase complex for 30 min (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 5 different vision areas. Images were taken with an Olympus BX51 microscope equipped with DP72 camera.

Statistical Analysis

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

Results

AR-V7 is constitutively activated in prostate cancer cells

The deletion of LBD results in constitutive activation of the AR (26, 27). AR-V7, a C-terminally truncated AR splice variant, has been linked to castration resistant prostate cancer and enzalutamide resistance (5, 7, 18). We detected AR-V7 mRNA in different prostate cancer cell lines, as shown in Fig.1A left. CWR22Rv1 and VCaP cells expressed significantly higher AR-V7 than LNCaP and C4-2 cells. The results were also confirmed by Western blot, as shown in Fig.1A right, in which CWR22Rv1 and VCaP cells expressed higher protein expression levels of AR variants, including AR-V7, than LNCaP and C4-2 cells. To examine the transcriptional activity of AR-V7 in PC-3 and LNCaP cells, we transiently transfected WT-AR, AR-V7 or pcDNA3.1 along with PGL3-PSA 6.0 luciferase reporter plasmids. As shown in Fig.1B, expression of AR-V7 was able to activate PSA promoter in both PC-3 and LNCaP cells in the absence of DHT, whereas, expression of WT-AR could not activate PSA promoter in PC-3 cells in the absence of DHT, consistent with reports that AR-V7 is constitutively activated in the absence of ligand (6). To further understand the function of AR-V7 in prostate cancer cells, we stably transfected AR-V7 into C4-2 cells, as shown in Fig.1C left panel, C4-2 AR-V7 stable clone expressed significantly higher AR-V7 mRNA and protein level compared with C4-2 neo cells. Furthermore, AR-V7 functionally increased PSA mRNA and protein expression in C4-2 cells cultured in media containing androgen deprived charcoal-stripped FBS conditions (Fig.1C right panel). To

examine the effect of AR-V7 on cell growth in prostate cancer cells, CWR22Rv1 cells were transient transfected with AR exon7 siRNA (which targets full-length AR, but not AR variants) or AR-V7 siRNA in CS-FBS condition, cell numbers were determined on different days. As shown in Fig.1D left panel, knocked down full length AR had moderate growth inhibition on CWR22Rv1 cells, while knocked down AR-V7 significantly inhibited cell growth, consistent with previous reports that AR-V7 but not full length AR plays dominant role in growth of CWR22Rv1 cells (19, 22). The knocked down efficiency was confirmed by western blot (Fig.1D right panel). Collectively, these data suggested that AR-V7 is constitutively activated in prostate cancer cells and targeting AR-V7 could inhibit cell growth.

Identification of niclosamide as a novel inhibitor of AR-V7

To identify potential inhibitors of AR-V7, we generated an AR-V7 expression cell system and used it to screen the Prestwick Chemical Library®, which contains about 1120 small molecules and approved drugs (FDA, EMEA and other agencies). The drug screening was conducted using luciferase activity assay to determine the activity of AR-V7 after treatment with the compounds in the library. To avoid interference from expression of the full length AR, we used the HEK293 cell line which lacks the AR. The HEK293 cells were stably co-transfected with AR-V7 plasmid and PGL3-PSA6.0 luciferase reporter plasmid, and stable clones were selected using G418. To perform compound screening, HEK293 AR-V7-PSA-luc stable clones were seeded in 96-well plates following treatment with each compound in the library for 24 hrs and luciferase activities were measured. Niclosamide, an FDA-approved anti-helminthic drug, was identified as being able to inhibit AR-V7-mediated luciferase activity. Niclosamide has been used to treat human tapeworm infections for around 50 years.

Niclosamide inhibited AR-V7 transcriptional activity and reduced recruitment of AR-V7 to PSA promoter

To further examine whether niclosamide inhibits AR-V7 transcriptional activity, we first validated in HEK293 AR-V7-PSA-E/P-LUC cell systems. As shown in Fig.2A, niclosamide significantly inhibited AR-V7 transcriptional activity while enzalutamide had no effect. To examine whether the effects could be reproduced in a prostate cancer cell system, LNCaP cells were transiently transfected with AR-V7, followed by treatment with niclosamide or enzalutamide with or without DHT overnight. As shown in Fig.2B, both niclosamide and enzalutamide dramatically inhibited DHT induced AR transcriptional activity, but only niclosamide inhibited AR-V7 transcriptional activity. To further determine whether the inhibition of luciferase activity could be translated to inhibition of protein expression, PSA ELISA was performed, as shown in Fig.2C. C4-2 AR-V7 cells were cultured in CS-FBS condition and shown to express higher PSA levels than C4-2 neo cells. Niclosamide significantly inhibited PSA levels in C4-2 AR-V7 cells. To further dissect the mechanism of AR-V7 inhibition by niclosamide, a ChIP assay was performed. C4-2 neo and C4-2 AR-V7 cells were cultured in CS-FBS condition for 3 days and whole cell lysates were subjected to ChIP assay, as shown in Fig.2D left, AR-V7 was recruited to the PSA promoter. Next, we cultured C4-2 AR-V7 cells in CS-FBS condition followed by treatment with niclosamide (0.5 μM and 1 μM) or 20μM enzalutamide overnight and whole cell lysates was subjected to

ChIP assay. As shown in Fig.2D right, niclosamide significantly reduced recruitment of AR-V7 to PSA promoter while enzalutamide had no effect. Collectively, these results demonstrate that niclosamide but not enzalutamide was able to inhibit AR-V7 transactivation.

Niclosamide inhibits AR-V7 protein expression through enhancing protein degradation

To determine whether niclosamide inhibits AR-V7 expression, CWR22Rv1 cells, which express endogenous AR-V7, were treated with different concentrations of niclosamide. As shown in Fig. 3A left, niclosamide inhibited endogenous AR-V7 protein in a dose dependent manner. 0.5 μM niclosamide significantly inhibited AR-V7 expression but had little effect on full length AR (AR FL) expression, which suggested that niclosamide is more potent in inhibition of the truncated AR. Niclosamide inhibited AR-V7 protein expression starting at 4 h treatment (Fig. 3A right). To further clarify how niclosamide decreases AR-V7 protein expression, we first determined the effects of niclosamide on AR-V7 expression at the transcriptional level. As shown in Fig.3B, niclosamide did not affect AR-V7 or full length AR mRNA level, suggesting that niclosamide did not affect AR-V7 expression at the transcriptional level. Next, we examined the effect of niclosamide on AR-V7 protein degradation after new protein synthesis was blocked by cycloheximide. The protein synthesis inhibitor cycloheximide (50 μg/mL) was added with or without 2 μM Niclosamide at time 0 hour. At specified time points, cells were harvested, and the levels of AR-V7 protein were measured by Western blot using antibodies specific against AR-V7. As shown in Fig. 3C, niclosamide increased AR-V7 protein degradation compared to the untreated control cells. To examine whether niclosamide induced AR-V7 protein degradation via the ubiquitin-proteasome system, the 26S proteasome inhibitor MG132 (5 μM) was added to the cells treated with niclosamide. MG132 was able to reduce the ability of niclosamide-mediated inhibition of AR-V7 protein expression (Fig. 3D), suggesting that niclosamide induced AR-V7 degradation via a proteasome-dependent pathway.

Niclosamide inhibited prostate cancer cell growth and induced cell apoptosis

To examine whether niclosamide affects prostate cancer cell growth, C4-2 neo, C4-2 AR-V7, CWR22Rv1 and PZ-HPV-7 cells were treated with DMSO or 0.5 μM niclosamide for 48 hours and cell numbers were determined. As shown in Fig.4A. 0.5 μM niclosamide significantly inhibited cell growth in prostate cancer cells, with little effect on PZ-HPV-7 normal prostate epithelial cells. To further examine the anti-cancer effects of niclosamide, cell death ELISA was performed. As shown in Fig.4B, 0.5 μM niclosamide significantly induced cell apoptosis in prostate cancer cells, but had little effect on PZ-HPV-7 cells. We also examined the effect of niclosamide on clonogenic ability. As shown in Fig.4C and 4D, niclosamide significantly inhibited clonogenic ability of prostate cancer cells in a dose dependent manner. Collectively, these results revealed that niclosamide inhibited prostate cancer cell growth and induced cell apoptosis with minimal effects on normal prostate epithelial cells.

C4-2B cells chronically treated with enzalutamide express AR variants and are sensitive to niclosamide

We generated an enzalutamide resistant prostate cancer cell line by continuous culture of C4-2B cells in media containing enzalutamide. As shown in Fig. 5A, after 12 months of being cultured in media containing enzalutamide, C4-2B MR (C4-2B enzalutamide resistant) cells exhibited more resistance to enzalutamide treatment than C4-2B parental cells. Next, we examined the expression levels of AR variants in C4-2B parental and C4-2B MR cells. As shown in Fig.5B left panel, C4-2B MR cells express higher levels of AR variant mRNAs and protein than C4-2B parental cells, including AR-V1, AR-V7, AR1/2/2b and AR1/2/3/2b. Furthermore, AR-V7 was constitutively expressed in the nucleus but not in cytoplasm (Fig.5B right panel). Since niclosamide can inhibit AR-V7 expression, we examined whether Niclosamide inhibits C4-2B MR cell growth. As shown in Fig.5C, C4-2B MR cells were resistant to enzalutamide but significantly inhibited by niclosamide in a dose dependent manner. The results were also confirmed by clonogenic assay. As shown in Fig. 5D, niclosamide but not enzalutamide significantly inhibited colony formation in C4-2B MR cells.

Niclosamide enhances enzalutamide treatment

To examine whether niclosamide could enhance enzalutamide therapy in prostate cancer cells, CWR22Rv1 cells were treated with niclosamide in combination with or without enzalutamide for 48 hours. As shown in Fig.6A left panel, single agent treatments with low dose of niclosamide or enzalutamide had moderate effects on CWR22Rv1 cells while combination treatments significantly inhibited cell growth in a time dependent manner. We also tested the effects in enzalutamide resistant cell line (C4-2B MR). Combination therapy of niclosamide with enzalutamide significantly inhibited C4-2B MR cell growth compared to single agent treatments (Fig. 6A right panel). The synergistic effects were also confirmed by clonogenic assay (Fig. 6B).

To test whether niclosamide overcomes enzalutamide resistance of prostate cancer *in vivo*, xenografts generated from CWR22Rv1 cells were treated with vehicle, enzalutamide, niclosamide or their combination for 3 weeks as described in methods. As shown in Fig.6C, CWR22Rv1 cells were resistant to enzalutamide treatment with tumor volumes comparable to those in the vehicle treated control group. Niclosamide alone decreased the tumor volume while combination of niclosamide and enzalutamide synergistically decreased CWR22Rv1 tumors, indicating that niclosamide could overcome enzalutamide resistance and restore sensitivity of CWR22Rv1 xenografts to enzalutamide in *vivo*. Representative tumor samples were analyzed by IHC for Ki67. As shown in Fig.6D, niclosamide inhibited Ki67 expression while combination treatment further decreased Ki67 expression. In summary, these results suggested that niclosamide can improve enzalutamide treatment and overcome enzalutamide resistance.

Discussion

Development of resistance to enzalutamide is eventually inevitable with the development of several potential pathways of resistance (4, 28). Recent studies have linked AR alternative

splicing, particularly AR-V7, to the development of enzalutamide resistance (18, 20, 21, 29). Targeting of AR signaling, especially AR variants, would improve current anti-androgen therapies for advanced prostate cancer. In the present study, we identified niclosamide as a potent AR-V7 inhibitor in prostate cancer cells. We found that niclosamide significantly inhibits AR-V7 protein expression and AR-V7 transcription activity and reduces AR-V7 recruitment to the PSA promoter. Niclosamide inhibits prostate cancer cell growth *in vitro* and tumor growth *in vivo*. Furthermore, niclosamide significantly enhanced enzalutamide therapy in prostate cancer cells, suggesting that niclosamide can be used to treat, either alone or in combination with current antiandrogen therapies, advanced prostate cancer patients, especially those resistant to enzalutamide.

The potential molecular mechanisms underlying the development of resistance to enzalutamide are under intensive investigation. Recent report showed that increased expression of glucocorticoid receptor (GR) can bypass AR signaling and induces resistance to enzalutamide (30). A novel F876L mutation in AR was also identified as a potent driver of resistance to enzalutamide, and AR variants such as AR-V7 induced by NF-κB/p52 can drive prostate cancer cells to develop enzalutamide resistance (18-21). We confirmed increased expression of AR-V7 in both CWR22Rv1 and VCaP cells but not in LNCaP and C4-2 cells. We demonstrated that overexpressing AR-V7 in C4-2 cells significantly enhanced the expression level of PSA. In addition, C4-2B cells chronically treated with enzalutamide exhibited significantly higher levels of full length AR and AR variants, suggesting that persistent activation of signaling by AR variants is important during the development of enzalutamide resistance. Therefore, targeting both full length AR and AR variants may prove to be an effective strategy to treat advanced prostate cancer. Several compounds have been recently found to be able to affect AR variants. The analogs of EPI-001 were developed to treat the castration resistant prostate cancer, including those driven by AR variants expression (31, 32). Methylselenol prodrug methylseleninic acid (MSA) can down regulate both full length AR and AR variants expression (33). A protein kinase C (PKC) inhibitor Ro31-8220 also showed to be able to down regulate both full length AR and AR variants (34). In the present study, we identified niclosamide as a novel inhibitor of AR-V7 and found that niclosamide reversed enzalutamide resistance in prostate cancer cells through inhibition of AR variants. Niclosamide significantly down regulates AR-V7 protein expression in a dose and time dependent manner by protein degradation through a proteasome dependent pathway. Niclosamide also inhibits AR-V7 transcriptional activity and reduces the recruitment of AR-V7 to the PSA promoter. Enzalutamide effectively blocks the recruitment of AR but not of AR-V7 to the PSA promoter, indicating that enzalutamide cannot inhibit AR-V7-mediated transcriptional activity. A combination of niclosamide to target AR-V7 and enzalutamide to target AR could provide an ideal strategy to treat advanced prostate cancer and prevent the development of resistance to enzalutamide driven by AR variants. This combination strategy was validated in this study in an animal model to show that niclosamide inhibits CWR22Rv1 tumor growth and enhances enzalutamide therapy. These results may warrant a combination treatment strategy using niclosamide to improve the efficacy of enzalutamide therapy.

Niclosamide is a FDA approved anthelminthic drug to treat tapeworm infection in humans for about 50 years, and has rich repository of pharmacokinetic data accumulated *in vivo*. It has low toxicity in mammals, with the oral median lethal dose calculated to be above 5000 mg/Kg (35). A single oral dose of 5 mg/Kg niclosamide in rats generates a maximum plasma concentration of 1.08 μmol/ml (36). Niclosamide has been recently demonstrated to exhibit anti-tumor activity in several cancers such as colorectal cancer (37-39), ovarian cancer (40), acute myeloid leukemia (AML) (41), breast cancer and prostate cancer (20). Niclosamide has been shown to promote Fzd1 endocytosis, down regulate Dvl2 protein, and inhibit Wnt3A-stimulated β-catenin stabilization and downstream β-catenin signaling (38). Niclosamide can also block TNFα induced IκBα phosphorylation, translocation of p65, and the expression of NF-κB regulated genes (41). Niclosamide is a selective inhibitor of Stat3 and can overcome acquired resistance to erlotinib through suppression of Stat3 in non-small cell lung cancer and head-neck cancer (42, 43). Niclosamide can also induce autophagy and inhibit mTROC1 (44-46). Niclosamide treatment in colon cancer cells inhibited S100A4-induced metastasis in *vivo* (37). In this study we showed that niclosamide exhibits anti-androgenic activity by inhibition of AR transcriptional activity and AR-V7 expression.

In summary, our study identified niclosamide, as a novel AR-V7 inhibitor which inhibits prostate cancer cell growth and induces apoptosis. Niclosamide can inhibit enzalutamide resistant prostate cancer cell growth and tumor growth. Furthermore, niclosamide exhibits synergistic effect with enzalutamide and resensitizes treatment resistant prostate cancer cells to Enzalutamide therapy. Our studies suggest that niclosamide has great potential as an effective and orally bioavailable drug candidate either as monotherapy or in combination with current antiandrogen therapies for treatment of advanced metastatic prostate cancer.

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Translational Relevance

Development of resistance to enzalutamide is eventually inevitable. Recent studies have linked AR alternative splicing, particularly AR-V7, to the development of enzalutamide resistance. Targeting of AR signaling, especially AR variants, would improve current anti-androgen therapies for advanced prostate cancer. In the present study, we identified niclosamide as a potent AR-V7 inhibitor in prostate cancer cells. We found that niclosamide significantly inhibits AR-V7 protein expression and AR-V7 transcription activity and reduces AR-V7 recruitment to the PSA promoter. Niclosamide inhibits prostate cancer cell growth *in vitro* and tumor growth *in vivo*. Furthermore, the combination of niclosamide and enzalutamide resulted in a significantly inhibition of enzalutamide-resistant tumor growth, suggesting that niclosamide enhances enzalutamide therapy and overcomes enzalutamide resistance in castration resistant prostate cancer cells. These findings offer preclinical validation of niclosamide as a promising inhibitor of androgen receptor variants to treat, either alone or in combination with current antiandrogen therapies, advanced prostate cancer patients, especially those resistant to enzalutamide.

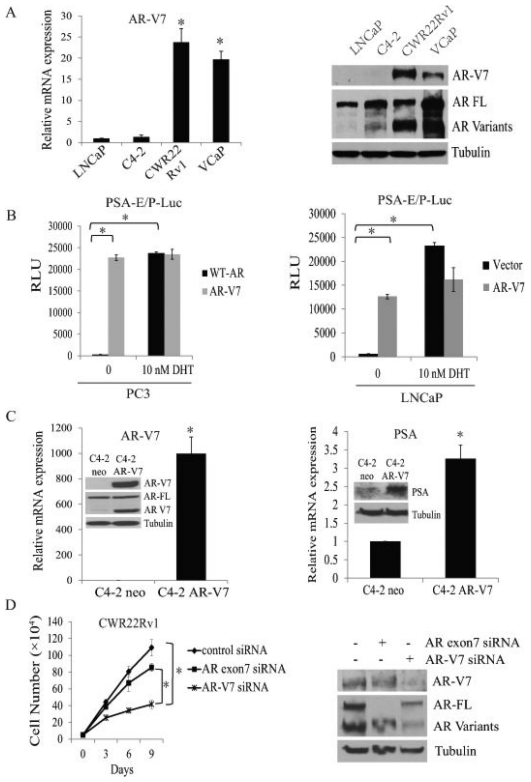

Figure 1.

AR-V7 is constitutively active in prostate cancer cells. **A.** LNCaP, C4-2, CWR22Rv1 and VCaP cells were cultured in CS-FBS condition for 3 days. AR-V7 mRNA level were analyzed by qRT-PCR, whole cell protein was extracted and immunoblotted with indicated antibodies. Results are presented as means ± SD of 3 experiments performed in duplicate. **B.** PC-3 cells and LNCaP cells were cultured in CS-FBS condition and transiently transfected with WT-AR or AR-V7 plasmid for 3 days followed by treatment with 10nM DHT for another 24 hours and whole cell lysates were subjected to luciferase assay. **C.** C4-2 neo and C4-2 AR-V7 stable clone were cultured in CS-FBS condition, AR-V7 and PSA mRNA level was determined by qRT-PCR, the AR-V7 and PSA protein expression was detected by western blot (insert). **D.** CWR22Rv1 cells were transient transfected with control siRNA, AR exon7 siRNA or AR V7 siRNA in CS-FBS condition, cell numbers were counted on different days and the knock down efficiency was examined by western blot. Results are presented as means ± SD of 3 experiments performed in duplicate. * $P<0.05$

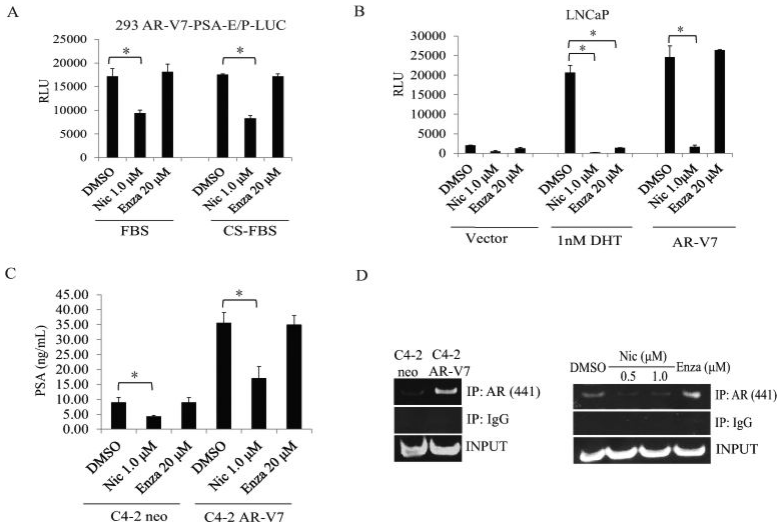

Figure 2.

Niclosamide inhibited AR-V7 transcription activity. **A.** 293-AR-V7-PSA luciferase promoter stable clone was treated with 1.0 μM niclosamide or 20μM enzalutamide overnight in media containing 10% FBS or 10% CS-FBS and whole cell lysates were subjected to luciferase assay. **B.** LNCaP cells were co-transfected with PSA luciferase promoter and AR V7 in CS-FBS condition for 24 hours, followed by treatment with 1.0 μM niclosamide or 20μM enzalutamide overnight and whole cell lysates were subjected to luciferase assay. **C.** C4-2 neo and C4-2 AR-V7 cells were cultured in CS-FBS condition for 3 days, followed by treatment with 1.0 μM niclosamide or 20μM enzalutamide overnight and the supernatants were subjected to PSA ELISA.. **D.** C4-2 neo and C4-2 AR-V7 cells were cultured in CS-FBS condition for 3 days, whole cell lysis was subjected to ChIP assay (left). C4-2 AR-V7 cells were treated with 0.5 μM, 1.0 μM niclosamide or 20μM enzalutamide overnight and whole cell lysates were subjected to ChIP assay (right). Results are presented as means ± SD of 3 experiments performed in duplicate. * $P<0.05$. Enza: Enzalutamide, Nic: Niclosamide, RLU: relative luciferase unit.

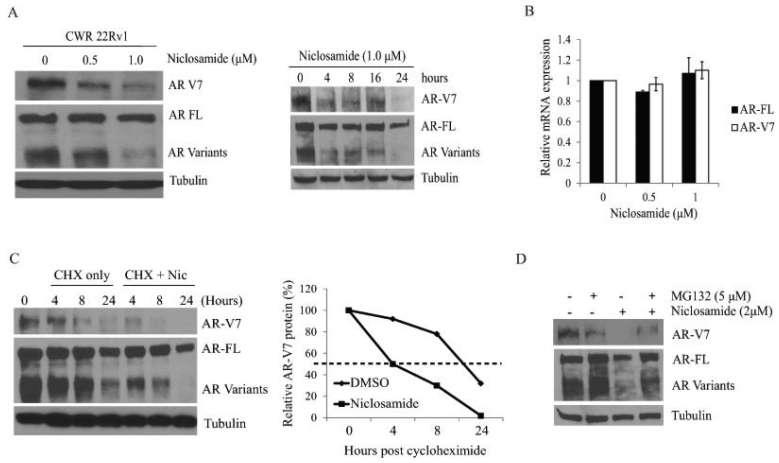

Figure 3.

Niclosamide inhibited AR-V7 protein expression through enhancing protein degradation. **A.** CWR22Rv1 cells were treated with 0 μM, 0.5 μM or 1.0 μM niclosamide in RPMI 1640 media containing 10% FBS overnight and the whole cell lysates were immunoblotted with the indicated antibodies (left). CWR22Rv1 cells were treated with 1.0 μM niclosamide in RPMI 1640 media containing 10% FBS, whole cell lysates were extracted at different time points and immunoblotted with the indicated antibodies (right). **B.** CWR22Rv1 cells were treated with 0 μM, 0.5 μM or 1.0 μM niclosamide in RPMI 1640 media containing 10% FBS overnight, total RNAs were extracted and AR or AR-V7 mRNA levels were analyzed by qRT-PCR. **C.** 50 μg/mL cycloheximide (CHX) was added with or without 2 μM Niclosamide (Nic) at time 0 hour. At specified time points, cells were harvested, and the levels of AR-V7 protein were measured by Western blot using antibodies specific against AR-V7. Plotted on semilog scale relative to respective time 0 AR-V7 value as 100%, dashed line indicates 50% half-life. **D.** Effect of MG132 on niclosamide–induced AR protein degradation. MG132 (5 μmol/L) was added to CWR22Rv1 cells together with cycloheximide (50 μg/mL) in the presence or absence of 2 μM niclosamide. The cell lysates were prepared at 8h. AR-V7 protein levels were determined by Western blot analysis using antibodies specifically against AR-V7 and tubulin as a control. Nic: Niclosamide, CHX: Cycloheximide.

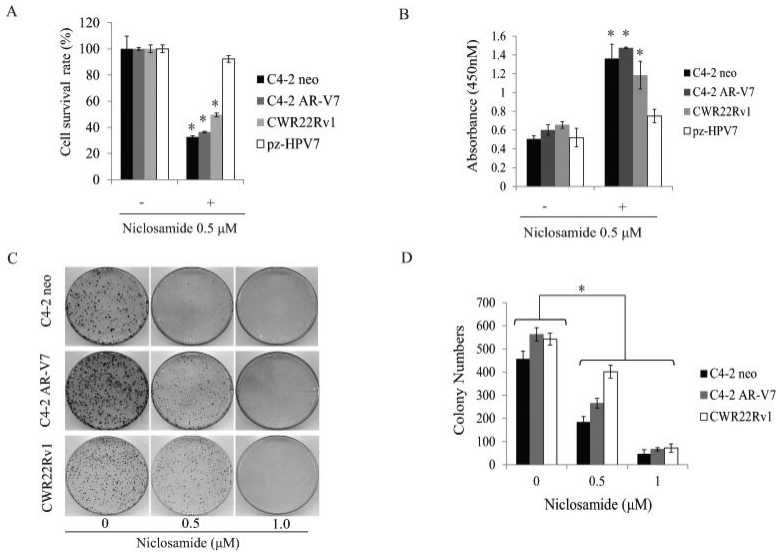

Figure 4.

Niclosamide inhibited prostate cancer cell growth and induced cell apoptosis. **A.** C4-2 neo, C4-2 AR-V7, CWR22Rv1 and PZ-HPV-7 cells were treated with 0.5 μM niclosamide in media containing FBS, cell numbers were counted and cell survival rate was calculated after 48 hours. **B.** C4-2 neo, C4-2 AR-V7, CWR22Rv1 and PZ-HPV7 cells were treated with 0.5 μM niclosamide in media containing FBS, and apoptosis was analyzed by Cell death ELISA after 48 hours. **C.** C4-2 neo, C4-2 AR-V7 or CWR22Rv1 cells were treated with 0, 0.5 μM or 1.0 μM niclosamide and clonogenic assays were performed. **D.** Colonies were counted and results are presented as means ± SD of 2 experiments performed in duplicate. Niclosamide inhibited colony formation in a dose dependent manner. * $P<0.05$

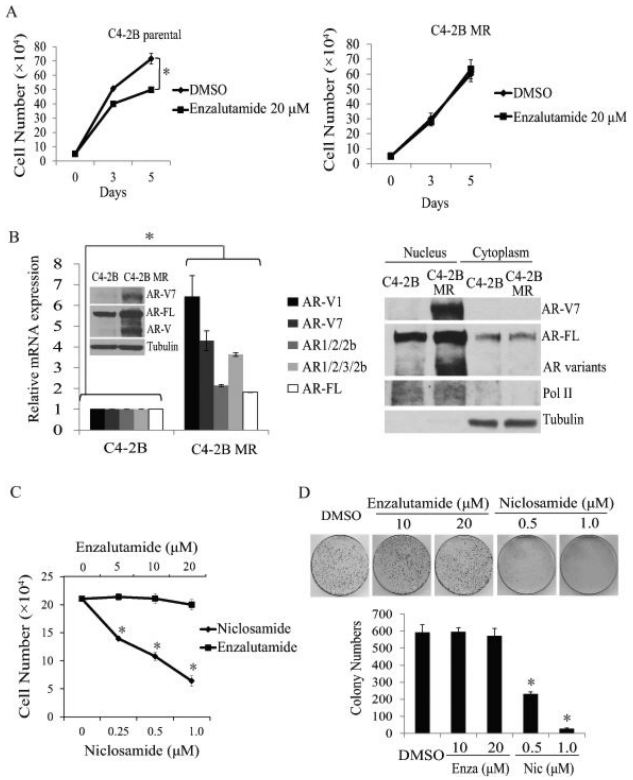

Figure 5.

C4-2B cells chronically treated with enzalutamide express AR variants and are sensitive to Niclosamide. **A.** C4-2B parental or C4-2B MR cell was treated with 20μM enzalutamide in RPMI 1640 media containing 10% FBS and total cell numbers were counted at different time points as indicated. **B.** C4-2B parental cells and C4-2B MR cells were cultured in RPMI 1640 media containing 10% FBS for 3 days, total RNAs were extracted and AR-V1, AR-V7, AR1/2/2b, AR1/2/3/2b or AR full length mRNA levels were analyzed by qRT-PCR. AR-V7 protein level was examined by western blot (inside panel).C4-2B parental cells and C4-2B MR 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 (right panel). The expression of RNA polymerase II and tubulin were used as markers for the integrity of the nuclear and cytosolic fractions, respectively. **C.** C4-2B MR cells were cultured in media containing 10% FBS and treated with different concentrations of enzalutamide or niclosamide as indicated and total cell numbers were counted after 48 h. **D.** C4-2B MR cells were treated with DMSO, 10 μM or 20 μM enzalutamide, 0.5 μM or 1.0 μM niclosamide and clonogenic assays were performed. Colonies were counted and results are presented as means ± SD of 2 experiments performed in duplicate. * P<0.05. Enza: Enzalutamide, Nic: Niclosamide.

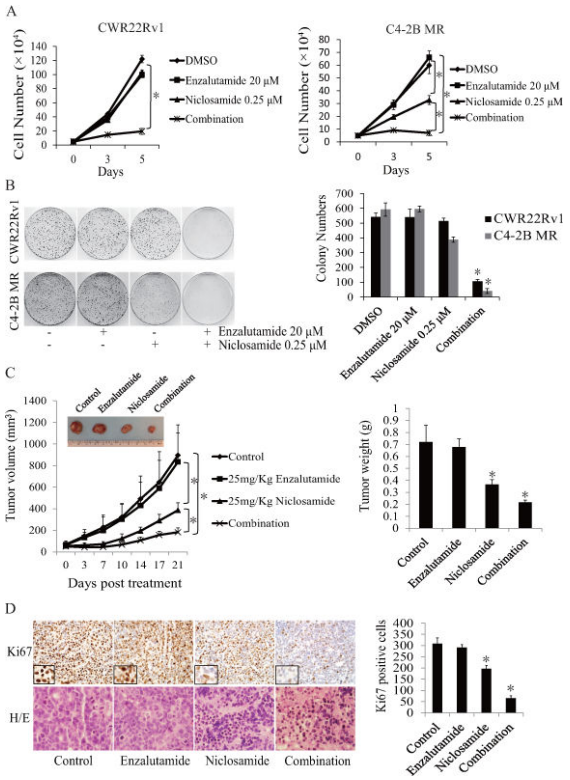

Figure 6.

Niclosamide enhances enzalutamide effects both in *vitro* and *in vivo.* **A.** CWR22Rv1 cells or C4-2B MR cells were treated with 0.25 μM niclosamide with or without 20 μM enzalutamide 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.** CWR22Rv1 cells or C4-2B MR cells were treated with 0.25 μM niclosamide with or without 20 μM enzalutamide in media containing FBS and clonogenic assays were performed. Colonies numbers were counted and results are presented as means ± SD of 2 experiments performed in duplicate. **C.** Mice bearing CWR22Rv1 xenografts were treated with vehicle control, enzalutamide, niclosamide or their combination for 3 weeks, tumor volumes were measured twice every week and the tumors were collected and weighed. **D.** Ki67 was analyzed in tumor tissues by IHC staining and quantified as described in methods * P<0.05.