Niclosamide Inhibits Androgen Receptor Variants Expression and Overcomes Enzalutamide Resistance in Castration-Resistant Prostate Cancer

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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. Androgen receptor (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 1,120 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 antihelminthic 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 significant inhibition of enzalutamide-resistant tumor growth, suggesting that niclosamide enhances enzalutamide therapy and overcomes enzalutamide resistance in CRPC cells.

Conclusions: Niclosamide was identified as a novel inhibitor of AR variants. Our findings offer preclinical validation of niclosamide as a promising inhibitor of AR variants to treat, either alone or in combination with current antiandrogen therapies, patients with advanced prostate cancer, especially those resistant to enzalutamide.

Introduction

Next generation anti-androgens such as enzalutamide and inhibitors of androgen synthesis such as abiraterone have improved the standard of care for patients with late-stage prostate cancer (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 that support the castration-resistant prostate cancer (CRPC) phenotype (5–8). The truncated androgen receptor (AR) variants that lack the ligand binding domain (LBD) naturally occur in both prostate cancer clinical samples and cell lines (5, 6, 9, 10). Several groups showed that AR variants are upregulated 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 androgen responsive element (ARE)-driven reporters in the absence of androgen, which indicates that those variants may have a distinct transcription program compared with the full-length AR (9, 14), and the activity of AR variants is postulated to depend on the full-length AR (15).
Translational Relevance

Development of resistance to enzalutamide is eventually inevitable. Recent studies have linked androgen receptor (AR) alternative splicing, particularly AR-V7, to the development of enzalutamide resistance. Targeting of AR signaling, especially AR variants, would improve current antiandrogen therapies for advanced prostate cancer. In this 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 significant 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 AR variants to treat, either alone or in combination with current antiandrogen therapies, patients with advanced prostate cancer, especially those resistant to enzalutamide.

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 because of 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 tumor growth in vivo. Furthermore, the combination of niclosamide and enzalutamide resulted in significant 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 AR variants to treat, either alone or in combination with current antiandrogen therapies, patients with advanced prostate cancer, 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). 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. C4-2 and C4-2B cells were kindly provided and authenticated by Dr. L. 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% 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 RPMI 1640 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 RPMI 1640 medium containing 300 μg/mL G418. C4-2B cells were chronically exposed to increasing concentrations of enzalutamide (5–40 μmol/L) 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 resistant; ref. 18). Parental C4-2B cells were passaged alongside the enzalutamide-treated cells as an appropriate control. C4-2B MR cells were maintained in 20 μmol/L 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 exon 7 sequence (UCAAGGAAACUCCGUUCGUA; ref. 22) or AR-V7 sequence (GUAGUUGUAAGUAUCAUGA; ref. 23) or a control siRNA targeting the luciferase (Luc) gene, sicontrol (CTTACGCGTACGTTCGCA), 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 and were maintained in RPMI 1640 medium containing 300 μg/mL G418. HEK293 cells expressing PSA-E/P-LUC and C4-2B cells were treated with 100 μg/mL G418. 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 RPMI 1640 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 RPMI 1640 medium containing 300 μg/mL G418. C4-2B cells were chronically exposed to increasing concentrations of enzalutamide (5–40 μmol/L) 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 resistant; ref. 18). Parental C4-2B cells were passaged alongside the enzalutamide-treated cells as an appropriate control. C4-2B MR cells were maintained in 20 μmol/L enzalutamide-containing medium. All cells were maintained at 37°C in a humidified incubator with 5% carbon dioxide.

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 mmol/L NaHCO3), 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 MgCl2, 10 mmol/L NaCl, 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, and 1% Triton X-100] with vigorous shaking at 4°C for 30 minutes. The nuclear lysates were pre cleared by centrifugation at 10,000 rpm at 4°C for 15 minutes. Protein concentration was determined using the Coomassie Plus Protein Assay Kit (Pierce).

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); AR-V7 (AG10008; Precision antibody); PSA (SC-7316, Santa Cruz Biotechnology); Tubulin (T5168, Sigma–Aldrich)]. Tubulin was also used as loading control. Following secondary antibody incubation, immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (Millipore).

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 × 105 cells/well in RPMI 1640 media containing 10% FBS and treated with 0.5 mmol/L niclosamide for 48 hours. Mono- and oligonucleosomes in the cytoplasmic fraction were measured by the Cell Death Detection ELISA Kit (Roche, Catalog No. 11546473001) 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 reverse transcription-PCR
Total RNAs were extracted using TRizol reagent (Invitrogen). DNAs were prepared after digestion with RNase-free RNQ1 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 TGG, 3'-TTC TGT CAG TCC -GTT CAT TCT GAA AAA TCC TTC AGC; AR1/2/3/2b: 5'-GTT CAT TCT GAA AAA TCC TTC AGC; AR-V7: 5'-AAC AGA AGT ACC TGT GGG CC, 3'-TGA GAC TCC AAA CAC CCT CA; AR-V7: 5'-AAC AGA AGT ACC TGT GGG CC, 3'-TCA GGG TTG GGT CAT TTT GA; AR-V1/2/2b: 5'-TGG ATG GAT AGC TAC TCC GG, 3'-GTT CAT TCT GAA AAA CAC CCT CA; AR-V7: 5'-AAC AGA AGT ACC TGT GGG CC, 3'-TCA GGG TTG GGT CAT TTT GA; Actin: 5'-AGA ACT GGC CCT TCT TGG AGG, 3'-GTT TTT ATG GAT AGC TAC TCC GG.
In vivo tumorigenesis assay

CWR22Rv1 cells (3 million) were mixed with matrigel (1:1) and injected subcutaneously into the flanks of 6- to 7-week male SCID mice. Tumor-bearing mice (tumor volume around 50–100 mm³) were randomized into 4 groups (with 10 tumors in each group) and treated as follows: (i) vehicle control (5% Tween 80 and 5% ethanol in PBS, i.p.; ref. 2), (ii) enzalutamide (25 mg/kg, p.o.; ref. 3), (iii) niclosamide (25 mg/kg, i.p.; ref. 4), and (iv) 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 × width²/2. Tumor tissues were harvested after 3 weeks of treatment.

Immunohistochemistry

Tumors were fixed by formalin and paraffin-embedded tissue blocks were dewaxed, rehydrated, and blocked for
endogenous peroxidase activity. Antigen retrieving was performed in sodium citrate buffer (0.01 mol/L, pH 6.0) in a microwave oven at 1,000 W for 3 minutes and then at 100 W for 20 minutes. Nonspecific antibody binding was blocked by incubating with 10% FBS in PBS for 30 minutes at room temperature. Slides were then incubated with anti-Ki67 (at 1:500; NeoMarker) 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 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 (SD) of the mean. 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 CRPC 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

![Figure 2. Niclosamide inhibited AR-V7 transcription activity. A, 293-AR-V7-PSA luciferase promoter stable clone was treated with 1.0 μmol/L niclosamide or 20 μmol/L enzalutamide overnight in media containing 10% FBS or 10% CS-FBS and whole cell lysates were subjected to luciferase assay. B, LNCaP cells were cotransfected with PSA luciferase promoter and AR V7 in CS-FBS condition for 24 hours, followed by treatment with 1.0 μmol/L niclosamide or 20 μmol/L 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 μmol/L niclosamide or 20 μmol/L 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 lysates were subjected to ChIP assay (left). C4-2 AR-V7 cells were treated with 0.5 μmol/L, 1.0 μmol/L niclosamide, or 20 μmol/L 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.
AR-V7 than LNCaP and C4-2 cells. The results were also confirmed by Western blot analysis, 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). 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 (Fig. 1C, right). To examine the effect of AR-V7 on cell growth in prostate cancer cells, CWR22Rv1 cells were transiently transfected with AR exon 7 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), knocked down full-length AR had moderate growth inhibition on CWR22Rv1 cells, whereas 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 analysis (Fig. 1D, right). 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 1,120 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 that lacks the AR. The HEK293 cells were stably cotransfected 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 hours and luciferase activities were measured. Niclosamide, an FDA-approved antihelminthic 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-6.0 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 hours and luciferase activities were measured. Niclosamide, an FDA-approved antihelminthic 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.

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Niclosamide inhibited AR-V7 transcriptional activity

To 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 and 1 µmol/L) or 20 µmol/L 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 whereas 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. A total of 0.5 µmol/L niclosamide significantly inhibited AR-V7 expression but had little effect on full-length AR (AR FL) expression, which suggested that niclosamide is more effective at inhibiting AR-V7 expression.
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 cells were treated with 20 µmol/L 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 analysis (inside). 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). 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 hours. D, C4-2B MR cells were treated with DMSO, 10 or 20 µmol/L enzalutamide, 0.5 or 1.0 µmol/L 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 µmol/L niclosamide with or without 20 µmol/L 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. (Continued on the following page.)
Niclosamide Overcomes Resistance to Enzalutamide

potent in inhibition of the truncated AR. Niclosamide inhibited AR-V7 protein expression starting at 4 hours 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 (CHX). The protein synthesis inhibitor CHX (50 μg/mL) was added with or without 2 μmol/L niclosamide at time 0 hour. At specified time points, cells were harvested, and the levels of AR-V7 protein were measured by Western blot analysis using antibodies specific against AR-V7. As shown in Fig. 3C, niclosamide increased AR-V7 protein degradation compared with the untreated control cells. To examine whether niclosamide induced AR-V7 protein degradation via the ubiquitin–proteasome system, the 26S proteasome inhibitor MG132 (5 μmol/L) was added to the cells treated with niclosamide. MG132 was able to reduce the 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 μmol/L niclosamide for 48 hours and cell numbers were determined. As shown in Fig. 4A, 0.5 μmol/L niclosamide significantly inhibited cell growth in prostate cancer cells, with little effect on PZ-HPV-7 normal prostate epithelial cells. To further examine the anticancer effects of niclosamide, cell death ELISA was performed. As shown in Fig. 4B, 0.5 μmol/L 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 D, 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), 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). Because 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), single-agent treatments with low dose of niclosamide or enzalutamide had moderate effects on CWR22Rv1 cells whereas 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 with single-agent treatments (Fig. 6A, right). 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 Materials and 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 whereas 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 whereas 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 antiandrogen therapies for advanced prostate cancer. In this 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, patients with advanced prostate cancer, especially those resistant to enzalutamide.

The potential molecular mechanisms underlying the development of resistance to enzalutamide are under intensive investigation. A 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 CRPC, 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 was also shown to be able to down regulate both full-length AR and AR variants (34). In this 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 5,000 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 antitumor activity in several cancers such as colorectal cancer (37–39), ovarian cancer (40), acute myeloid leukemia (AML; ref. 41), breast cancer, and prostate cancer (20). Niclosamide has been shown to promote Fzd1 endocytosis, downregulate 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 that inhibits prostate cancer cell growth and induces apoptosis. Niclosamide can inhibit enzalutamide-resistant prostate cancer cell growth and tumor growth. Furthermore, niclosamide exhibits a 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 the treatment of advanced metastatic prostate cancer.

Disclosure of Potential Conflicts of Interest

C. Liu, W. Lou, and A.C. Gao are co-inventors of a patent application covering the use of niclosamide. C.P. Evans is a consultant/advisory board member for and reports receiving speakers bureau honoraria from Astellas/Medivation and Janssen; a commercial research grant from Astellas/Medivation; and has ownership interest (including patents) in...
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Liu, W. Lou, A.C. Gao

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

Study supervision: C. Liu, W. Lou, A.C. Gao

Authors' Contributions

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

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References


Correction: Niclosamide Inhibits Androgen Receptor Variants Expression and Overcomes Enzalutamide Resistance in Castration-Resistant Prostate Cancer

In this article (Clin Cancer Res 2014;20:3198–210), which was published in the June 15, 2014, issue of Clinical Cancer Research (1), the AR-V7 sequence, which was listed on page 2 under the Methods section subtitle "Cell transfection and luciferase assay," was published incorrectly as "(GUAGUUGUAAGUAUCAUGA)." It should read as follows: "(GUAGUUGUGAGUAUCAUGA)." The authors regret this error.

Reference

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