Synthesis and biological evaluation of niclosamide PROTACs

Erick Munoz\textsuperscript{a}, Guanglin Chen\textsuperscript{a}, Ahamed Hossain\textsuperscript{b}, Sitong Wu\textsuperscript{a}, Esveidy Oceguera Nava\textsuperscript{a}, Jasmine Hang\textsuperscript{a}, Tong Lee\textsuperscript{a}, Qiang Zhang\textsuperscript{b}, Guangdi Wang\textsuperscript{b, \textdagger}, Qiao-Hong Chen\textsuperscript{a, \textdagger}

\textsuperscript{a}Department of Chemistry, California State University, Fresno, 2555 E. San Ramon Avenue, M/S SB70, Fresno, CA 93740, USA

\textsuperscript{b}Department of Chemistry and RCMI Cancer Research Center, Xavier University of Louisiana, 1 Drexel Drive, New Orleans, LA 70125, USA

Abstract

Roughly 268,000 new cases of prostate cancer and 34,000 deaths from prostate cancer are projected by the American Cancer Society to occur in the United States in 2022. Androgen receptor is a key protein in the proliferation and survival of prostate cancer cells and has been revealed to be overexpressed in 30\% to 50\% of castration-resistant prostate cancer patients. One promising approach to reducing the level of this protein is Proteolysis Targeting Chimeras (PROTACs) that is an emerging drug discovery technology. PROTACs are hetero-bifunctional molecules where one end binds to a protein of interest and the other to an E3 ligase ligand, initiating the Ubiquitin-Proteasome Pathway for protein degradation. Two PROTACs with niclosamide as androgen receptor ligand and VHL-032 as the E3 ligase ligand have been designed and synthesized for suppressing proliferation of androgen receptor-positive prostate cancer cells via degrading androgen receptor. The \textit{in vitro} antiproliferative assessment suggested that they can selectively suppress PC-3, LNCaP, and 22Rv1 prostate cancer cell proliferation, but cannot inhibit DU145 cell proliferation. However, the mechanism of both compounds in suppressing prostate cancer cell proliferation is not through the AR PROTAC mechanism because they did not degrade AR in our Western Blotting assay up to 1 μM.

Prostate cancer remains to be one of main health concerns, as evidenced by the estimated 268,000 new cases and about 34,000 deaths in the United States in 2022.\textsuperscript{1} Specifically, about 1 in 8 men will be diagnosed with prostate cancer at some point in their lifetime, with the probability increasing to 6 out of every 10 men when they are older than 65. Although the early-stage prostate cancer is not life-threatening, no curative therapeutics available for patients with aggressive and lethal castration resistant prostate cancer (CRPC) allows prostate cancer to be the second leading cause of cancer death in American men.\textsuperscript{1} The androgen receptor (AR) signaling pathway remains active in patients with CRPC.

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\textsuperscript{\textdagger}Corresponding authors: gwang@xula.edu (G. Wang), qchen@csufresno.edu (Q.-H. Chen).

Declaration of Competing Interest

The authors declare that they have no known competing financial interest or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2022.128870.
The AR belongs to the steroid hormone group of nuclear receptors and is made up of three main functional domains: the N-terminal transcriptional regulation domain (NTD), the DNA binding domain (DBD), and the C-terminal ligand binding domain (LBD). Dihydrotestosterone (DHT) binds to AR with high affinity and displaces heat-shock proteins from the AR to drive the interaction between the N and C termini of the AR and bind importin-α to translocate the AR into the nucleus. Once in the nucleus, receptor dimers bind to the androgen response element and ultimately promotes transcription, which leads to responses such as cell proliferation and survival.\textsuperscript{2-3} Initiated by Huggins and Hodges,\textsuperscript{4} androgen deprivation therapy has been used to slow prostate cancer progression through lowering the androgen concentration in the bloodstream by medical or surgical castration. Some patients inevitably progress to CRPC, where prostate cancer can still progress even with near castrate levels of androgen. The most recent FDA-approved hormonal therapeutics to combat CRPC is to interrupt the AR signal pathway through AR antagonists that compete with androgens for binding with ARs.\textsuperscript{5-7} Unfortunately, these therapeutics only allow for the prolongation of the patient’s survival time by a few months,\textsuperscript{8} as exemplified by enzalutamide (Xtandi) with a survival time prolongation of five months.\textsuperscript{9} The intrinsic and acquired drug resistance pose the major problem for the AR antagonists.\textsuperscript{8} The mode of action of these AR antagonists is occupancy-driven pharmacology via the LBD, but since cancer cells divide rapidly, there is a chance that compensatory secondary mutation(s) at the LBD of AR trigger the drug resistance. Additionally, androgen receptor splice variant 7 (AR-V7) lacks the LBD,\textsuperscript{10} which is the main target of androgens and the FDA-approved AR antagonists, but still retains its transcription factor activity in a ligand-independent manner through the cross talk of two signal transduction pathways.\textsuperscript{11}

The limitations of AR antagonists could be mitigated through PROTACs (Proteolysis Targeting Chimeras) due to their catalytic nature. This study thus aims to develop PROTACs which consist of niclosamide (1, Fig. 1) attached to VH-032 (2) via different linkers (Fig. 2). Niclosamide (1) is an FDA approved anthelminthic drug which has also been demonstrated to inhibit AR-V7 protein expression and AR-V7 transcriptional activity in CWR22Rv1 cells.\textsuperscript{12} Niclosamide (1) is envisioned as an appropriate candidate to incorporate into a PROTAC due to it being a potent AR-V7 inhibitor but also having unacceptable bioavailability as reported in its Phase I clinical trials.\textsuperscript{13-14} VH-032 (2, Fig. 1) was planned to be used in our target PROTACs (3, Fig. 2) to recruit von Hippel-Lindau (VHL) proteins\textsuperscript{15} because recruitment of these proteins for degradation is so far the most explored among all the E3 ligases. A VHL ligand was initially used in peptide based PROTACs,\textsuperscript{16} and then a series of optimized VHL ligands with nanomolar binding affinities were established. Among them, VH-032 (2, Fig. 1) was demonstrated to be the most potent VHL ligand and is now mainly used in other PROTACs with minor replacements.\textsuperscript{17-18} The niclosamide-PROTAC 4 (Scheme 1) was designed to possess a linear linker with a 7-atom chain to allow sufficient flexibility for ternary complex formation. Niclo-Click PROTAC 5 (Scheme 2) was designed to increase rigidity of the linker by incorporating a triazole moiety in hope to enhance the molecular recognition and stability of the ternary complex. The 7-atom chain and 12-atom chain were selected for PROTAC 4 and 5, respectively, on the grounds of the empirical linker length structure–activity relationship studies\textsuperscript{19} and the successful pioneering AR PROTACs.\textsuperscript{18}
As illustrated in Scheme 1, the synthesis of Niclosamide-PROTAC 4 started with the linker preparation due to the materials being more affordable. Niclosamide (1) and VH-032 (2) were then sequentially appended to two ends of the linker via an S_N2 reaction followed by a peptide coupling reaction. The linker intermediate 8 was prepared through a Williamson ether synthesis from diethylene glycol (6) and tert-butyl bromoacetate (7) mediated by sodium hydride. Running the reaction overnight was identified as the most efficient procedure as the starting materials and product are invisible in TLC under ultraviolet light and other visualization methods. The difficulty in monitoring the reaction as well as purification via PTLC was overcome by observing a “shadow” on the TLC and PTLC through overloading. After tosylation, the linker intermediate 9 was reacted with niclosamide (1) in DMF mediated with potassium carbonate followed by the tert-butyl ester deprotection via trifluoroacetic acid in DCM to afford carboxylic acid 10. The product from the reaction between niclosamide (1) and linker intermediate 9 was hard to be separated from the excess niclosamide which was getting dragged across on the TLC plate. We therefore decided to directly use the crude product without purification for the next step tert-butyl ester deprotection reaction in hope that the polar carboxylic acid group would help separate the compound 10 from niclosamide (1). Surprisingly, the results are even better than we expected because compound 10 was readily purified through recrystallization with acetone. The desired Niclosamide-PROTAC 4 was then obtained through the peptide coupling reaction of carboxylic acid 10 and amine VH-032 (2).

Niclo-Click PROTAC 5 was synthesized following the sequence described in Scheme 2. The synthesis began with preparation of the alkyne-azide Huisgen cycloaddition reaction precursors, alkyne 12 and azide 13. Alkyne 12 was synthesized through the Williamson ether thesis from niclosamide (1) and tosylate 11 that was obtained from commercially available 3-butyn-1-ol. Azide 13 was prepared from azidation of tosylate 9. The advanced carboxylic acid intermediate 15 was achieved by alkyne-azide Huisgen cycloaddition reaction of alkyne 12 and azide 13 catalyzed by copper iodide followed by tert-butyl ester deprotection. The desired Niclo-Click PROTAC 5 was synthesized by the amide coupling reaction of carboxylic acid 15 and amine VH-032 (2).

The in vitro antiproliferative potency of two PROTACs 4 and 5 together with the intermediate compounds (10, 12, 13, 14, and 15) towards two AR-negative (PC-3 and DU145) and two AR-positive (LNCaP and 22Rv1) prostate cancer cell lines were assessed using WST-1 cell proliferation assays, according to the methods described in the Experimental section (Supporting Information). DU-145 and PC-3 are both AR-negative prostate cancer cell lines where LNCaP and 22Rv1 are AR-positive prostate cancer cell lines with one key difference. LNCaP includes full length AR that is androgen responsive, but 22Rv1 includes both full length and LBD (ligand binding domain)-truncated AR-V7. Niclosamide was tested as a positive control, and all IC_{50} values were summarized in Table 1. Niclosamide has anti-proliferative potency across all four prostate cancer cell lines, possibly due to the multiple pathways it can inhibit cancer cell proliferation. Most Niclosamide-PROTAC precursor compounds had an IC_{50} (half-maximal inhibitory concentration) of >10 μM except for compound 14, which curiously had an IC_{50} of 3.08 μM against LNCaP cells. The linker portion may not be the cause since the other
compounds tested did not exhibit inhibition under 10 μM. A possible reason could be due to the triazole ring in compound 14, with the carboxylic acid in compound 15 preventing inhibition. Further testing would be required to determine which portion is contributing to cell inhibition. As for the two Niclosamide-PROTACs (4 and 5), both are selective against PC-3, LNCaP, and 22Rv1 prostate cancer cell lines with some decrease in potency as compared to niclosamide (1). Niclosamide-PROTAC 4 has less potency towards LNCaP compared to Niclo-click PROTAC 5, but for 22Rv1 the opposite is true. Either case, the results show that these compounds are effective against prostate cancer cell lines. The AR degradation assay of the two niclosamide PROTACs (4 and 5) has been evaluated through Western Blotting assay, using the known AR degrader ARV110 as a positive control. As shown in Fig. 3, however, two niclosamide PROTACs have no effect on lowering the AR protein level up to 1 μM, suggesting that the mechanism of both compounds 4 and 5 in suppressing prostate cancer cell proliferation is not through the AR PROTAC mechanism. This is because compounds 4 and 5 did not degrade AR in our Western Blotting assay up to 1 μM (Fig. 3). The designed PROTACs 4 and 5 with niclosamide warhead did not degrade the AR directly but can suppress the prostate cancer cell proliferation. The data imply that these two compounds are inactive against AR degradation and their antiproliferative activity towards PC-3, LNCaP, and 22Rv1 are associated with other mechanisms. For example, niclosamide has been reported not only as an AR-V7 inhibitor but also a suppressor of STAT3 activation. The ligand-independent transcription factor activity of AR-V7 is through STAT3 activation in the IL6/JAK2/STAT3 pathway. STAT3 signaling has been demonstrated to induce cell proliferation while also preventing cell apoptosis through the upregulation of c-MYC and PIM1 and downregulation of p53. Equally important, STAT3 signaling can also interact with the AR N-terminal domain to facilitate AR transactivation.

In conclusion, two niclosamide-based PROTACs were designed, synthesized, and evaluated. The designed PROTACs with niclosamide warhead did not degrade the receptor directly but can suppress the prostate cancer cell proliferation. The data imply that the anti-proliferative activity of these two compounds towards PC-3, LNCaP, and 22Rv1 are directly associated with other protein targets.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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**Data availability**

Data will be made available on request.
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Fig. 1.
Niclosamide and VH-032.
Fig. 2.
Generalized Structure for target niclosamide PROTACs.
Fig. 3.
Western blot assay of AR with the synthesized PROTACs in different doses from 1 nM to 1000 nM.
Scheme 1.
Synthesis of Niclosamide PROTAC 4.
Scheme 2.
Synthesis of Niclo-Click PROTAC 5.
Table 1

Anti-Proliferative Activities of PROTACs and Intermediates Against Prostate Cancer Cell Lines.

| Compound                  | IC₅₀ (μM)ᵃ | DU145ᵇ | PC-3ᵇ | LNCaPᶜ | 22Rv1ᶜ |
|---------------------------|------------|--------|-------|--------|--------|
| Niclosamide (1)           | 3.65 ± 0.34| 1.92 ± 0.53| 0.80 ± 0.27| 1.61 ± 0.87 |
| 10                        | >10        | >10    | >10   | >10    |
| 12                        | >10        | >10    | >10   | >10    |
| 13                        | >10        | >10    | >10   | >10    |
| 14                        | >10        | >10    | 3.08 ± 0.89| >10    |
| 15                        | >10        | >10    | >10   | >10    |
| Niclosamide PROTAC 4      | >10        | 2.34 ± 0.48| 2.37 ± 0.96| 4.94 ± 0.66 |
| Niclo-Click PROTAC 5      | >10        | 2.79 ± 1.08| 1.04 ± 0.18| 6.88 ± 0.38 |

ᵃIC₅₀ is the half-maximal inhibitory concentration measured via WST-1 cell proliferation assay. Each IC₅₀ value was tested in triplicates. The data were presented as mean ± standard deviation.

ᵇHuman AR-negative prostate cancer cell line.

ᶜHuman AR-positive prostate cancer cell line.