JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Communication

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Multivalent Peptidomimetic Conjugates: A Versatile Platform for Modulating Androgen Receptor Activity

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Supporting Information

ABSTRACT: We introduce a family of multivalent peptidomimetic conjugates that modulate the activity of the androgen receptor (AR). Bioactive ethisterone ligands were conjugated to a set of sequence-specific peptoid oligomers. Certain multivalent peptoid conjugates enhance AR-mediated transcriptional activation. We identify a linear and a cyclic conjugate that exhibit potent antiproliferative activity in LNCaP-abl cells, a model of therapy-resistant prostate cancer. The linear conjugate blocks AR action by competing for ligand binding. In contrast, the cyclic conjugate is active despite its inability to compete against endogenous ligand for binding to AR *in vitro*, suggesting a non-competitive mode of action. These results establish a versatile platform to design competitive and non-competitive AR modulators with potential therapeutic significance.

There is a critical need to develop potent and selective compounds that can modulate androgen receptor (AR) activity to provide a therapeutic modality for prostate cancer. Androgens are steroid hormones that can interact with the AR to play an important role in human endocrinology and disease.[1] AR is a ligand-dependent transcription factor capable of binding the native androgen dihydrotestosterone (DHT).[2] The classical mechanism of AR activation involves DHT displacing a chaperone protein, thus inducing a conformational change that promotes receptor dimerization.[3] Upon phosphorylation and translocation into the nucleus, AR binds to specific DNA sequences and recruits necessary transcriptional co-factors to regulate gene expression.[4,5]

Androgens functioning through the AR can also promote prostate cancer development, growth and progression.[6] The advancement of prostate cancer from an androgen-dependent disease state to one that is androgen-independent represents the disease's lethal transformation, as limited therapeutic options exist for patients with advanced disease.[7] The standard approach for treating androgen-dependent prostate cancer is androgen ablation by suppression of testosterone production. This treatment option is typically accompanied by competitive DHT antagonists, such as bicalutamide, to block AR signaling.[8] While initially effective at suppressing tumor growth, these therapies often evoke castrate-resistant (or androgen-independent) prostate cancer progression.[9]

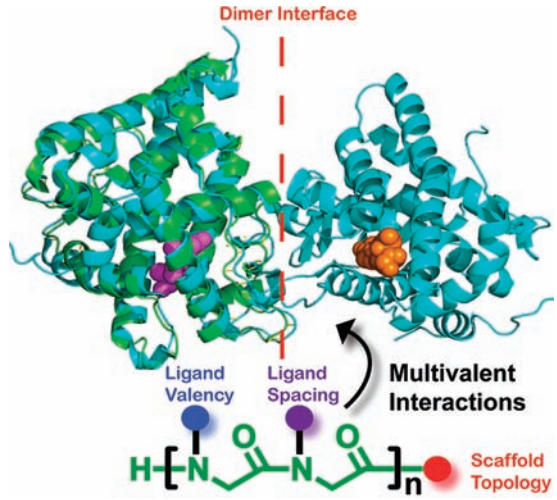


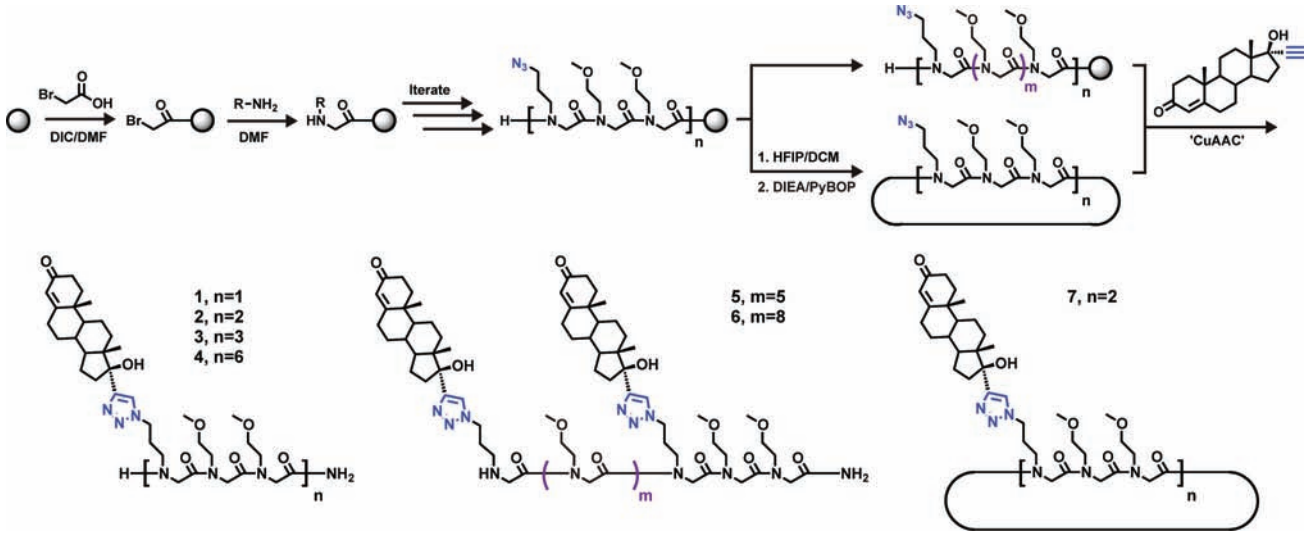
Figure 1. Peptoid scaffolds permit design of oligomers to modulate androgen receptor activity through multivalent interactions. Colored circles represent sites amenable to chemical modification and ligand display. Homology model of the AR ligand binding domain dimer (green ribbon, PDB 1I37) bound to native ligand (DHT, magenta).[14,15]

AR is expressed in both androgen-dependent and androgen-independent prostate cancer cells.[10] Two cell model systems, LNCaP (androgen-dependent) and LNCaP-abl (androgen-independent) have been established to study AR function in these different disease states. AR-based drug discovery typically focuses on the development of competitive DHT antagonists that bind in the ligand-binding domain (LBD) of the AR.[11] Drug resistance can therefore arise through mutations within the AR-LBD.[12] Recent evidence suggests that allosteric binding sites on AR can also regulate receptor activity through non-competitive mechanisms, providing additional targets for pharmacology.[13] Thus, the development of non-competitive modulators that act independently or synergistically with competitive antagonists could shift the paradigm for prostate cancer therapy. The approach described herein introduces a versatile multivalent scaffold to design competitive or non-competitive AR modulators with potential therapeutic significance (Figure 1).

Received: January 16, 2012
Published: April 17, 2012

dx.doi.org/10.1021/ja300170n | *J. Am. Chem. Soc.* 2012, 134, 6912–6915

Scheme 1. General Synthesis of Linear and Cyclic Multivalent Peptoid Conjugates[a]



[a]Rink Amide and 2-chlorotrityl chloride resin were used to generate linear and cyclic peptoid oligomers, respectively. Rink Amide resin was cleaved with 95% trifluoroacetic acid following conjugation reactions.

Multivalent pharmacological strategies have several advantages over traditional small molecule approaches. Multivalent constructs have been shown to enhance binding to target receptors through avidity effects.[16] The assembly of a multivalent display on a modular oligomer framework can also enable control over important physico-chemical features of the products.[17] In particular, control over size, charge and branching of the scaffold can influence solubility, cellular uptake and other desirable pharmacological characteristics.

We previously introduced the use of a *N*-substituted glycine oligomer scaffold, termed a "peptoid", to establish multivalent displays of diverse bioactive ligands.[18] Peptoids are a class of peptidomimetics in which the side chains are appended to the amide nitrogen atoms, engendering proteolytic stability and enhanced cellular permeability.[19,20] The ability to incorporate highly diverse side chain groups facilitates the discovery of peptoid sequences that exhibit a wide variety of sophisticated functions ranging from enantioselective catalysis to modulation of protein−protein interactions.[21−23] Peptoids can be synthesized on solid support to allow generation of monodisperse products, offering distinct advantages over other multivalent display approaches (e.g., random copolymers or dendrimers).[24] The sequence-specific assembly of peptoids enables precise tuning of ligand valency and spacing to potentially enhance affinity and specificity for corresponding biomolecular targets.[17] In addition, the peptoid scaffold can be used as a versatile platform for optimizing other properties critical for biological activity, such as the ability to alter the topology of the scaffold to achieve optimal ligand−receptor interactions.[25]

We designed a series of peptoid-based multivalent conjugates to evaluate the influence of valency, spacing and conformational ordering on AR activity. We used Cu-catalyzed azide−alkyne [3+2] cycloaddition (CuAAC) "click" reactions to conjugate ethisterone, a 17α-ethynyl homologue of DHT, to peptoid side chains, thus generating a family of multivalent conjugates.[26] Ethisterone was chosen as a ligand because it is known to compete for AR binding and suppresses levels of AR transcriptional activation relative to DHT.[27,28] Additionally, the ethynyl moiety provides accessibility to CuAAC reactions, a powerful synthetic tool due to its bioorthogonality, high yields and mild reaction conditions.[26]

Using modified solid-phase peptoid synthesis protocols, we synthesized linear and cyclic peptoid oligomers containing azido-alkyl functionalized side chains at specific positions in the oligomer sequence.[18] Following oligomerization (and cyclization as required), the peptoid scaffolds were used as substrates for CuAAC-mediated conjugation of ethisterone ligands (Scheme 1). In order to alleviate steric congestion, the ethisterone moieties were conjugated at least three residues apart (*i*, *i*+3) in the peptoid oligomer sequence. It is important to note that the distance between the hormone binding pockets of dimerized AR is approximately 26 Å (Figure S6). Increasing the spacing between the steroid ligands could potentially span the AR dimer and allow simultaneous binding to the two pockets. To enhance overall water solubility, all other intervening positions in the peptoid sequence included the hydrophilic monomer *N*-(methoxyethyl)glycine.

We synthesized a set of linear peptoid conjugates with one, two, three, or six ethisterone ligands (conjugates **1**−**4**) displayed along the peptoid backbone. Additional divalent peptoid conjugates containing ethisterone ligands separated by five (conjugate **5**) or eight (conjugate **6**) intervening monomer units were synthesized (Scheme 1). We also generated a cyclic divalent construct (conjugate **7**) to constrain the spatial disposition of the ethisterone moieties.

An *in vitro* ligand-binding assay was used to evaluate binding of the multivalent peptoid conjugates to the AR. In this assay, competitive binding was detected by a change in fluorescence polarization that occurs upon displacement of a fluorescently labeled hormone ligand (Fluormone) from AR by a competitive ligand.[29] Conjugates **1**−**3** and **7** do not compete against Fluormone for binding at concentrations up to 10 μM (Figure 2A and Figure S7). Conjugates **4**−**6** compete for binding, indicated by a decrease in fluorescence polarization relative to vehicle treatment. To differentiate effects due to multivalency and not hydrophobicity, a control peptoid conjugate was synthesized (**8**, see SI). Conjugate **8**, a divalent dodecamer containing one inactive hydrophobic moiety and one bioactive ethisterone ligand, did not compete for ligand

dx.doi.org/10.1021/ja300170n | *J. Am. Chem. Soc.* 2012, 134, 6912−6915

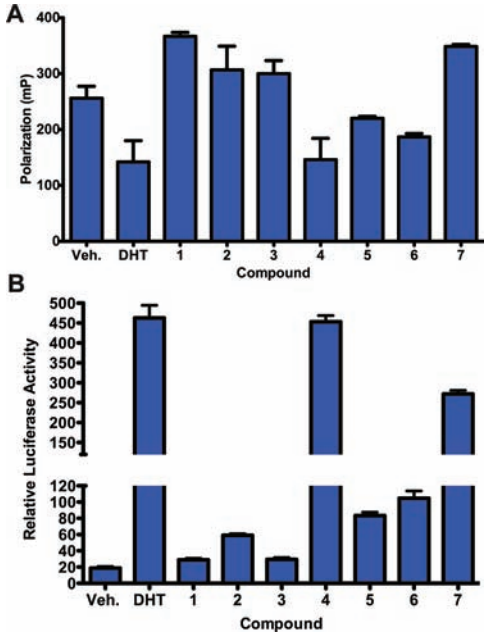


Figure 2. (A) Multivalent peptoid conjugates can compete with fluorescetly labeled hormone ligand and bind to AR (Veh., fluorescence polarization in absence of any competitor; DHT, 1 μM; conjugates **1**–**3** and **5**–**7**, 10 μM; conjugate **4**, 100 nM). (B) Transcriptional activation by AR in the presence of multivalent peptoid conjugates quantified by luciferase activity in LB1 cells (Veh., EtOH treated cells; DHT, 10 nM; conjugates **1**–**7**, 1 μM). All data presented as mean + SD of triplicates.

binding (Figure S8). Increasing the valency and spacing of the ethisterone moieties along the peptoid backbone enhances AR binding, consistent with multivalent interactions.[30]

To determine if the conjugates function as AR agonists, we evaluated the ability of the conjugates to induce AR-mediated transcriptional activation in LNCaP cells. For this study, we used a LNCaP cell line that stably expresses the AR-responsive luciferase reporter gene under the probasin promoter.[31] These cells, termed LB1, were treated with conjugates **1**–**7** at a concentration of 1 μM for 24 h, and AR-mediated transcriptional activation was measured (Figure 2B). As a positive control for full AR-mediated transcriptional activation, cells were also treated with DHT. Compared to DHT, which resulted in a 23.5-fold induction of the reporter gene over baseline, conjugates **1**–**3** were weak activators of AR, displaying only a 1.5- to 3-fold induction of the reporter gene. In contrast, hexavalent conjugate **4** produced a robust induction of the reporter gene to levels similar to DHT. For the linear divalent conjugates, dodecamer conjugate **6** showed greater reporter gene induction as compared to nonamer conjugate **5** (6- vs 4.5-fold). This is consistent with the competitive AR binding data observed for conjugates **1**–**6**. Cyclic conjugate **7** displayed a strong 11-fold induction of reporter gene activity despite its inability to compete with DHT for binding to the AR. These results indicate that different multivalent conjugates can operate through either competitive or non-competitive mechanisms to regulate AR-mediated transcriptional activation.

We next evaluated if conjugates **1**–**7** were able to suppress the proliferation of LNCaP-abl cells. These cells are models of advanced disease that express AR, and proliferate in the absence of hormone. LNCaP-abl cells were treated with conjugates **1**–**7** for 72 h, and cellular proliferation was measured utilizing the CyQUANT assay (Figure S9).[32] Conjugates **1** and **2**, which fail to competitively bind AR or activate AR gene expression, had little impact on the proliferation of the LNCaP-abl cells. In contrast, conjugate **3**, which elicited only a 3-fold induction of AR reporter gene activity, suppressed the proliferation of the LNCaP-abl cells. An agonist-induced transcription assay confirms conjugate **3** is functioning as an AR antagonist (Figure S10). Conjugate **4**, which induced a potent AR transcriptional response, inhibited cell proliferation to the same extent as conjugate **3**. Conjugates **6** and **7** displayed the greatest levels of inhibition of cell proliferation. Although conjugates **3**–**7** suppress the proliferation of LNCaP-abl cells, they do so through different mechanisms, given the distinctive profiles for competitive binding elicited by the conjugates.

Conjugates **6** and **7** were further evaluated for their ability to inhibit LNCaP-abl cell proliferation. At concentrations of 1 μM or 10 nM, conjugates **6** and **7** evoke a potent reduction in cell proliferation, relative to vehicle treatment (Figure 3A and Figure S11). To demonstrate that suppression of proliferation can be specifically attributed to the presence of the steroid

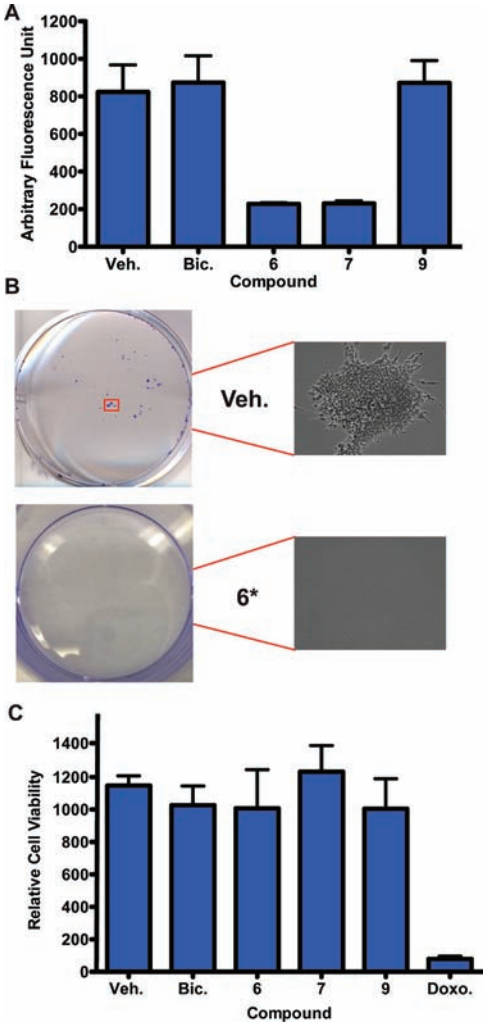


Figure 3. Effect of multivalent peptoid conjugates on cell proliferation in LNCaP-abl (A,B) and HEK293 (C) cells (Veh., EtOH treated cells; Bic., Bicalutamide, 1 μM; conjugates **6**–**8**, 1 μM; control peptoid conjugate **9**, 1 μM; Doxo., positive cytotoxic control Doxorubicin, 1 μM.[33] All data presented as mean + SD of triplicates. *Similar results observed for conjugate **7** (Figure S13).

dx.doi.org/10.1021/ja300170n | J. Am. Chem. Soc. 2012, 134, 6912–6915

ligands, two additional control compounds (conjugates **9** and **10**, see SI) were synthesized. Conjugate **9**, lacking the ethisterone moieties, and conjugate **10**, containing bioactive progesterone receptor ligands, had no effect on cell proliferation (Figure 3A and Figure S12). The standard AR monotherapy bicalutamide also had no effect on cell proliferation. In addition, LNCaP-abl cells were treated with conjugates **6** and **7** at a concentration of 1 μM for 14 days, and stained with crystal violet to detect differences on long-term proliferation and colony-formation. Conjugates **6** and **7** strongly diminished the number and size of colonies formed relative to vehicle treatment (Figure 3B).

To evaluate the general cell toxicity of conjugates **6** and **7**, a cell viability assay was utilized. Conjugates **6** and **7** did not exhibit cytotoxic effects in human embryonic kidney (HEK293) cells or AR-deficient prostate cancer (PC3) cells (Figures 3C and S14). These results suggest that conjugates **6** and **7** are selectively active and exert anti-proliferative activity in LNCaP-abl cells. Our findings highlight the potential biomedical significance of multivalent peptoid conjugates for advanced prostate cancer.

The peptoid-based conjugates represent the first multivalent constructs designed to specifically target the AR. The oligomeric scaffold provides a versatile platform that can be utilized to modulate AR activity. This study shows that multivalent ethisterone conjugates can compete for AR binding and modulate AR-mediated transcription. A linear and a cyclic conjugate exhibit potent anti-proliferative activity in therapy-resistant prostate cancer cells through competitive and non-competitive mechanisms, respectively. Cytotoxicity was not observed in non-AR expressing HEK293 or PC3 cells. Future studies will evaluate the mechanism of action of these compounds, and explore their potential applications in AR pharmacology and chemical biology.

■ ASSOCIATED CONTENT

Supporting Information

Complete ref 11; detailed peptoid and submonomer synthesis; mass spectra analysis; and assay methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was financially supported by the NSF (CAREER Award CHE-0645361 to K.K.). We thank Justin Holub and Bishwajit Paul for their helpful discussions and suggestions.

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