Journal of Medicinal Chemistry

Perspective

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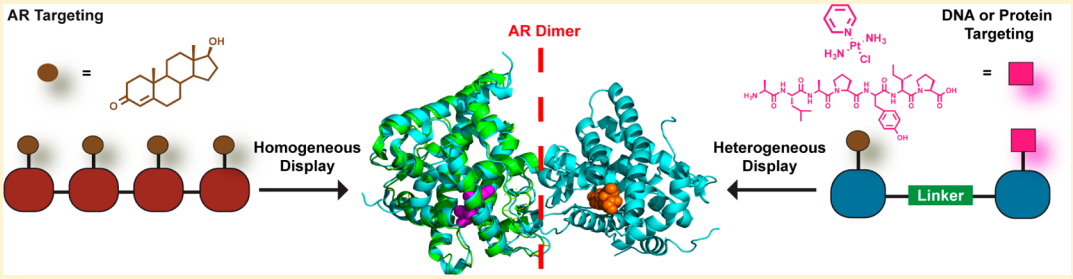
Targeting the Androgen Receptor with Steroid Conjugates

Miniperspective

Paul M. Levine,[†] Michael J. Garabedian,[‡,§] and Kent Kirshenbaum*[,†]

[†]Department of Chemistry, New York University, New York, New York 10003, United States

[‡]Department of Biochemistry and Molecular Pharmacology and [§]Department of Urology, NYU Langone School of Medicine, New York, New York 10016, United States



ABSTRACT: The androgen receptor (AR) is a major therapeutic target in prostate cancer pharmacology. Progression of prostate cancer has been linked to elevated expression of AR in malignant tissue, suggesting that AR plays a central role in prostate cancer cell biology. Potent therapeutic agents can be precisely crafted to specifically target AR, potentially averting systemic toxicities associated with nonspecific chemotherapies. In this review, we describe various strategies to generate steroid conjugates that can selectively engage AR with high potency. Analogies to recent developments in nonsteroidal conjugates targeting AR are also evaluated. Particular focus is placed on potential applications in AR pharmacology. The review culminates with a description of future prospects for targeting AR.

■ INTRODUCTION

There is a critical need to develop potent and selective therapeutic agents capable of targeting malignant tissue without compromising normal cell viability. While chemotherapeutic agents (e.g., doxorubicin and docetaxel) remain widely used in the clinic, they lack inherent selectivity desired to limit toxicity to normal cells.[1] In addition, administration of chemotherapeutic agents can induce drug resistance, resulting in disease progression.[2] Thus, the development of more targeted therapies could circumvent nonspecific interactions and potentially overcome drug resistance in cancer therapy.

Intriguing studies are currently exploring new methods to engage biomolecular targets with high affinity and specificity, including the generation of multivalent and heterobifunctional constructs. Advances in chemical synthesis techniques, such as cross-coupling and conjugation strategies, have enabled chemists to decorate a plethora of molecular species with targeting moieties, providing access to elaborate molecular architectures that can be tailored to occupy distinct binding sites within one or multiple biomacromolecules. Although these types of compounds fall outside the molecular weight range of typical drug compounds (500−3000 Da), increasing interest in developing new chemical entities that can modulate biomolecular targets in novel ways and address selectivity requirements are emerging.

To date, there have been only limited examples evaluating the potential for targeting the androgen receptor (AR) with steroidal conjugates. The AR is an important drug target for treatment of prostate cancer and has been the subject of research for several decades. A large number of bioactive compounds targeting AR have been identified via screening efforts.[3] In this review, we begin by providing a rationale for continued studies in prostate cancer pharmacology targeting the AR. Particular focus is placed on examining current approaches to specifically engage and modulate AR activity with steroid conjugates utilizing rational design principles. Lastly, future prospects for identifying novel AR modulators will be explored.

■ PROSTATE CANCER: A GLOBAL CONCERN

Androgens are a class of steroid hormones that consist of 19-carbon derivatives of cholesterol and are synthesized by the testis and adrenal glands.[4] They are also precursors for estrogens, the female sex hormones, produced by hydroxylation, elimination, and aromatization of androgens through the enzyme aromatase. Functioning primarily through the AR, which is a ligand-dependent transcription factor, androgens play a fundamental role in the development and survival of male reproductive tissues, such as the prostate, by influencing gene expression levels.[5]

Received: January 17, 2014
Published: June 17, 2014

   dx.doi.org/10.1021/jm500101h | J. Med. Chem. 2014, 57, 8224−8237

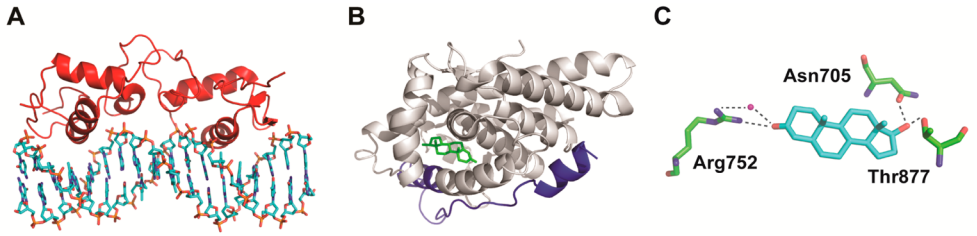


Figure 1. X-ray crystal structure of (A) androgen receptor (AR) DNA binding domain (ribbon, red) in complex with the androgen response elements (sticks, PDB code 1R4I) and (B) AR ligand binding domain (ribbon, gray) and portion of hinge region (ribbon, blue) in complex with native ligand (sticks, green, PDB code 1I37). (C) Amino acids residues that establish high affinity binding with native ligand DHT (PDB code 2AMA).

The body maintains control of testosterone (the most abundant androgen in men) levels within a normal reference range of ∼240−800 ng/dL.[6] Health problems are associated with deviations outside this range.[7] Low levels of testosterone resulting from zinc deficiency or aging can lead to fatigue and erectile dysfunction.[8] By contrast, high levels of testosterone have been linked to a variety of diseases, including prostate cancer.[9]

Prostate cancer remains the most common cancer among men and is globally estimated to affect 900 000 patients every year.[10] As the second leading cause of cancer-related deaths in men (258 000/year), approximately one out of every six men will be diagnosed with prostate cancer in the U.S. If detected early, an arsenal of therapeutic options currently provide a promising chance for long-term survival. However, ∼40% of patients will develop castration-resistant prostate cancer (CRPC), arising from drug resistance (vida infra), which is associated with poor survival rates.[11]

■ ANDROGEN RECEPTOR: STRUCTURE AND FUNCTION

The AR is a 110 kDa protein that shares sequence homology with other nuclear hormone receptors in the superfamily, including the progesterone receptor (PR), glucocorticoid receptor (GR), and estrogen receptor (ER).[12] The AR consists of four basic elements: N-terminal domain, DNA binding domain, hinge region, and the ligand binding domain (LBD).[13] The first domain is the 559 amino acid long intrinsically disordered N-terminal domain, which contains the ligand-independent activation function 1 (AF-1). Activation function sites encode signature motifs containing LxxLL or FxxLF sequences to recruit co-regulatory proteins that are essential for transcription. The most highly conserved region within all nuclear hormone receptors, including AR, is the centrally located DNA binding domain, consisting of two zinc finger domains that recognize specific DNA consensus sequences known as the androgen response elements (Figure 1A). The third domain, dubbed the hinge region, connects the DNA binding domain to the ligand-binding domain (Figure 1B). The ligand-binding domain (LBD) contains ligand-dependent activation function 2 (AF-2), forms the ligand-binding pocket, and mediates interactions between the AR and heat shock proteins (Figure 1B).[13] Importantly, AF-2 can interact with an FxxLF binding motif located within the N-terminal domain, a feature unique to AR.[13]

The crystal structure of the AR LBD bound to native ligand (DHT) reveals the amino acid residues critical for maintaining high binding affinity (Figure 1C).[14] Although van der Waals forces contribute to binding affinity, hydrogen bonds establish stronger interactions with the native ligand.[14] Arg752 forms a hydrogen bond with the O3 atom (ketone) of the steroid ligand. Mutagenesis of Arg752 has been shown to compromise binding affinity, suggesting the importance of this interaction for achieving high affinity.[15] In addition, Asn705 and Thr877 form hydrogen bonds with the 17-β hydroxyl group of the steroid ligand. Mutagenesis of Asn705 and Thr877 have also resulted in reduced binding affinity and specificity, establishing their importance to maintaining high affinity.[16,17] It is important to note that modifications to the 17-β hydroxyl group can result in diminished binding affinity, while even large substituent modifications at the 17-α position often retain strong binding interactions.[13,18]

The AR is a ligand-dependent transcription factor that is stabilized in the cytoplasm by chaperone proteins (Figure 2).[19] Competitive displacement of the chaperones by dihydrotestosterone (DHT), an androgen biosynthesized from testosterone through the enzyme 5α-reductase, activates the AR.[20] Upon activation, a conformational change brings the N- and C-termini into proximity and facilitates AR dimerization.[21,22] Upon translocation into the nucleus, AR binds to palindromic 5′-TGTTCT-3′ consensus sequences (androgen response elements) in the promoter regions of target genes.[23,24] This event stimulates the recruitment of necessary cofactors, including LxxLL or FxxLF motif-containing proteins, and other components of the transcriptional machinery to regulate gene expression.[25]

■ ANDROGEN-DEPENDENT AND -INDEPENDENT PROSTATE CANCER

The AR mediates a variety of androgen-dependent diseases including benign prostatic hypertrophy (BPH), prostatic intraepithelial neoplasia (PIN), and prostate cancer.[26] It has been proposed that prostate cancer often originates from high-grade prostatic intraepithelial neoplasia (HGPIN), a process in which subtle alterations in the shape and size of prostate cells occur. More importantly, progression of prostate cancer has been linked to elevated expression of AR in malignant tissue, suggesting that AR plays a central role in prostate cancer cell biology.[27] Although many hypotheses regarding the involvement of AR in prostate cancer progression have been postulated, the precise molecular mechanisms are not fully understood.

Patients diagnosed with localized or metastatic prostate cancer usually undergo androgen deprivation therapy (reduction of circulating androgen levels), through chemical castration (gonadotropin-releasing hormone agonists) or surgical castration.[28] Unfortunately, these methods do not completely eliminate circulating levels of androgens, as the tumor itself is

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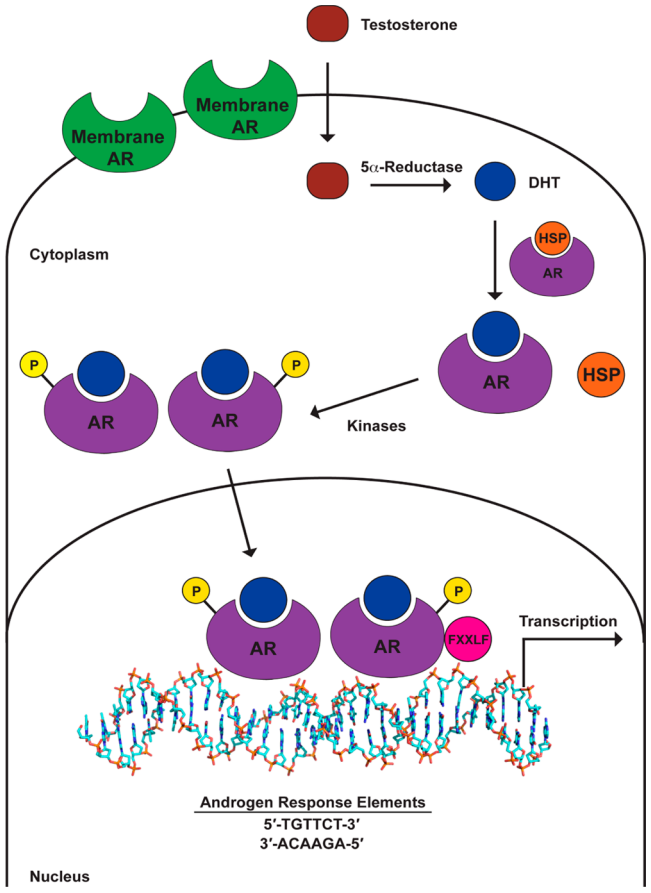

Figure 2. Schematic diagram depicting the mechanism of AR activation. Abbreviations: DHT, dihydrotestosterone; HSP, heat shock protein; P, phosphorylation site; FxxLF, coactivator protein. Figure is adapted from ref 31.

capable of local androgen synthesis, due to the expression of androgen biosynthetic enzymes.[29] This has led to numerous research efforts focusing on the development of inhibitors that interfere with key enzymes, such as cytochrome P450 17A1 (CYP17A1), in androgen biosynthesis as exemplified by the recent FDA approval of abiraterone (Zytiga).[30]

The standard treatment approach for prostate cancer involves androgen deprivation therapy in conjunction with small molecule anti-androgens that block AR signaling (Figure 3A).[31] Anti-androgens compete with DHT for binding to AR, thus inhibiting AR transactivation through a variety of mechanisms, including disruption of nuclear localization, interruption of DNA binding, and interference with coactivator recruitment.[32,33] Unfortunately, most patients receiving anti-androgen therapy eventually develop drug resistance as indicated by rising levels of serum prostate-specific antigen (PSA), a gene regulated by AR, leading to the lethal disease state termed castration-resistant prostate cancer or CRPC.[34]

Current mechanisms proposed for advancement to CRPC include the following:[35,36]

(1) alterations in AR co-regulatory protein balance;
(2) somatic gain of function mutations within AR, with the majority in the LBD, resulting in activation by other steroid hormones and anti-androgens;
(3) generation of new fusion gene products;
(4) AR "ligand-independent" activation via cross-talk with other signaling pathways.

These mechanisms have garnered significant attention because of their ability to "reactivate" AR and disease progression, and provide a conceptual underpinning to guide development of new therapeutic interventions. Nevertheless, currently CRPC is primarily treated with chemotherapeutic agents, immunotherapy, or abiraterone (vida supra).[37]

Recently, a number of potential therapeutic agents targeting "reactivated" AR have been identified via chemical screening efforts and include compounds that act on the AF-2 (Figure 3B) or BF3 site (Figure 3C) on AR to regulate its activity.[38−43] The BF3 site is a hydrophobic binding pocket located adjacent to AF-2 on the surface of AR that can allosterically regulate binding interactions between AR and coactivator proteins. The development of noncompetitive modulators (that do not compete against DHT for ligand binding) could circumvent drug resistance in AR pharmacology. While promising, these noncompetitive approaches have yet to yield candidates for clinical implementation, likely because of the high concentrations required to suppress AR activity.[44] In the future, it may be important to utilize structure-based design to generate more potent AF-2 or BF3 inhibitors.

In contrast, continuing interest in anti-androgen drug development has led to the FDA approval of enzalutamide, which targets the AR ligand binding domain for the treatment of CRPC (Figure 3A).[45] Unfortunately, recent evidence suggests that drug resistance to enzalutamide can emerge from point mutations within the AR LBD, such as F876L.[46] Additionally, drug resistance has been proposed to arise from constitutively active AR splice variants lacking the AR ligand binding domain.[47] This has led researchers to focus on innovative ways to antagonize AR splice variants and the development of N-terminal domain inhibitors (Figure 3D).[48−50] It is important to note, however, that no structural information exists for the AR N-terminal domain, complicating the design of N-terminal domain antagonists.[51]

Although it is tempting to speculate that AR splice variants are mainly responsible for drug resistance to enzalutamide, the precise molecular mechanisms remain unknown. Evidence suggests that full-length AR is required for signaling, although different sets of studies demonstrate that ER splice variants can be constitutively active in the absence of ligand.[52] Also, an intriguing report has similarly suggested that the GR can become constitutively active in the absence of its LBD.[53] Future research may illuminate whether other nuclear hormone receptors can exhibit similar modes of action.

■ TARGETING AR WITH STEROID CONJUGATES

Bioactive "hit" compounds, typically identified from screening efforts, often lack the potency and selectivity required for translation to a clinical setting. For this reason, most "hit" compounds must be optimized into "lead" compounds through iterative rounds of synthesis and rigorous bioassays. While this strategy remains widely utilized in both academic and industrial research programs, rational design of therapeutic agents aims to streamline these issues by initially identifying more potent and selective compounds. Below, we describe different strategies that have been used to target AR with steroid conjugates, along with preliminary evaluation of their potential applications in AR pharmacology.

PROTACS. Protein synthesis and degradation is an essential component of cellular homeostasis.[54] The ATP-dependent ubiquitin-proteasome pathway is a quality control mechanism that conducts the programmed metabolic degradation of

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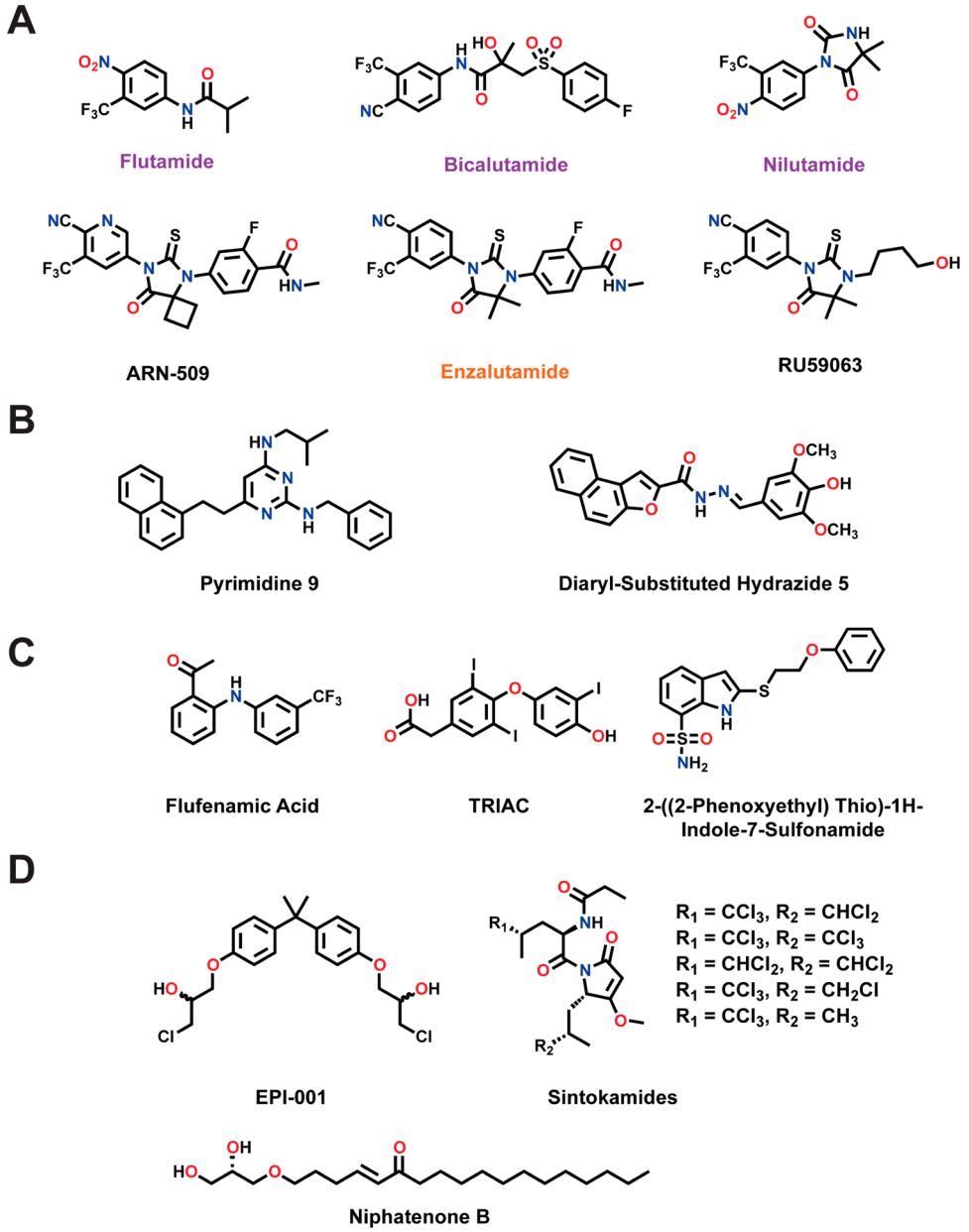

Figure 3. Small molecule inhibitors targeting the AR: (A) anti-androgens; (B) activation function 2 inhibitors; (C) allosteric (BF3 site) regulators; (D) N-terminal domain inhibitors. Purple denotes approved therapies for androgen-dependent prostate cancer, and orange represents approved therapies for castration-resistant prostate cancer.

proteins.[55] Ubiquitin-protein ligase (E3) associates with ubiquitin-conjugating enzyme (E2), providing subsequent tagging of ubiquitin chains to protein substrates that results in degradation by the proteasome.[56−58] Rational design strategies aimed toward selectively targeting proteins for degradation through E3 could establish an approach to diminish the levels of aberrantly functioning proteins.

The Crews lab has pioneered a general strategy to modulate levels of selective proteins by engagement of the ubiquitin system.[59] By use of conjugates dubbed proteolysis targeting chimeric molecules (PROTACS), the first steroid conjugate to selectively induce AR degradation was developed.[60] PROTACS consist of three components: a targeting moiety (DHT), a linker, and a recognition element for E3. The modular synthesis of PROTACS establishes a significant pharmacological advantage because PROTACS are particularly amenable to chemical modification, permitting control over the physicochemical features of the products.

Initial ex vivo studies aimed toward degrading AR yielded PROTAC-5 (Figure 4A).[60] PROTAC-5 was outfitted with a peptide sequence (ALAPYIP) as an E3 recognition domain and to induce ubiquitination upon hydroxylation of the central proline residue.[61] To assess biological activity, PROTAC-5 was administered to human embryonic kidney cells (HEK293) that stably expressed an AR fluorescent fusion protein. Protein degradation was quantified by a reduction in the fluorescence signal. At a concentration of 25 μM, PROTAC-5 successfully degraded AR without compromising normal cell viability. In control studies, vehicle treated cells maintain fluorescence, suggesting that PROTAC-5 engages AR in the cell and induces degradation. To confirm these results, cells were treated with PROTAC-5 and immunoblotted for AR. A significant decrease

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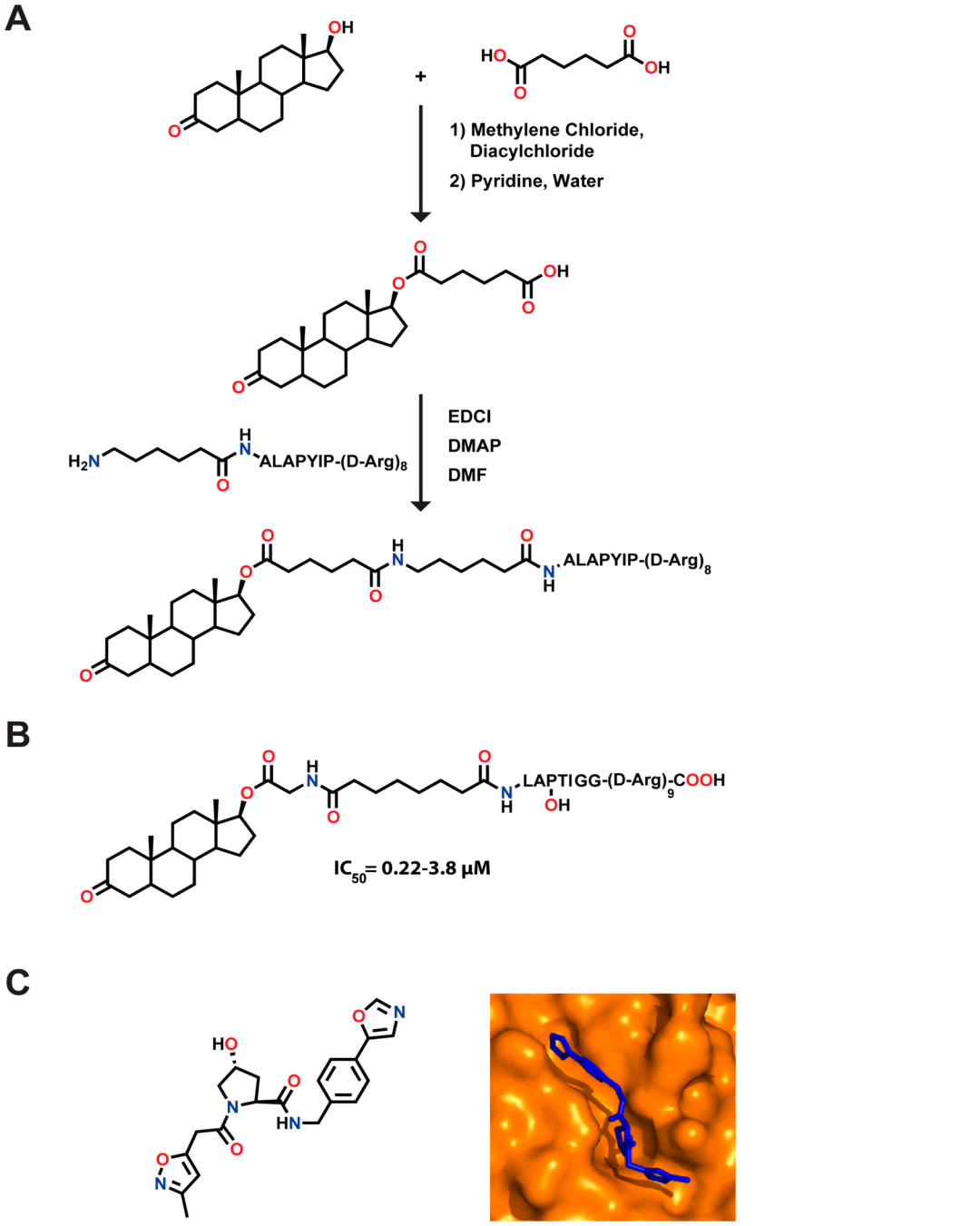

Figure 4. Proteolysis targeting chimeric molecules (PROTACS) for AR: (A) synthesis of PROTAC-5; (B) chemical structure of PROTAC-AA; (C) chemical structure of small molecule E3 recognition element (left) and cocrystal structure of small molecule E3 recognition element (blue sticks) and E3 (orange surface rendered, right; PDB code 3ZRC). Figure is adapted from refs 53, 55, and 56.

in AR protein level was detected, confirming that PROTAC-5 targets and degrades AR.

More recently, a derivative of PROTAC-5, dubbed PROTAC-AA (Figure 4B), was administered to an AR-expressing prostate cancer cell line (LNCaP) to evaluate effects on cell proliferation.[62] PROTAC-AA contains a shorter hydroxylated recognition element for E3 and a slightly modified arginine tail to enhance cell permeability. The arginine tail enhances cell permeability through an uptake mechanism mimicking the Antennapedia and HIV Tat proteins.[63,64] PROTAC-AA inhibited cell growth with an inhibitory concentration (IC_{50}) value of 3.8 μM at 72 h and 0.217 μM at 144 h. A control PROTAC lacking the arginine tail displayed IC_{50} values 12.5 μM at 72 h and 1.5 μM at 144 h. Western blot analysis was performed to establish that AR protein levels were reduced. Taken together, these results suggest that the arginine tail enhances biological activity while maintaining specificity. Importantly, in prostate cancer cell lines that do not express AR (PC-3 and DU-145 cells), PROTAC-AA had no significant effect on cell viability, establishing selective activity.

While PROTACS remain promising candidates for applications in AR pharmacology, difficulties in large-scale production may impede rapid translation into the clinic. Current efforts have focused on developing more "druglike" PROTAC

dx.doi.org/10.1021/jm500101h | J. Med. Chem. 2014, 57, 8224−8237

molecules and the recent discovery of the first small molecule targeting E3 (Figure 4C) with an IC_{50} value of 4.1 μM.[65] Competitive fluorescence polarization data indicated that the small molecule binds to E3, which was confirmed by a cocrystal structure. Subsequent optimization led to the first submicromolar small molecule targeting E3 (IC_{50} = 0.90 μM).[66] In the future, we may begin to see small molecule PROTACS targeting AR, which may include, for example, enzalutamide tethered to similar small molecules that are capable of recruiting E3.

SNIPERs. Apoptosis, or programmed cell death, is a physiological cell suicide mechanism critical to cellular homeostasis.[67] Inadequate activation of the apoptotic pathway can play a role in the development of cancer and autoimmune diseases.[68] Inhibitors of apoptosis proteins (IAPs) play a fundamental role in regulating apoptosis and other cellular processes. IAPs contain a RING domain that possesses E3 activity, establishing the ability to induce proteasomal degradation by tagging proteins with ubiquitin chains.[69]

The Hashimoto lab has developed specific and nongenetic IAPs-dependent protein erasers (SNIPERs).[70] Relative to PROTACS, SNIPERs consist of a targeting moiety (DHT), linker, and a recognition element for IAPs. The targeted ubiquitination of proteins by SNIPERs relies on small molecule IAP recognition elements (Figure 5). The biological activity of

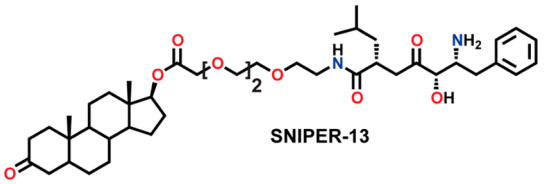

Figure 5. Chemical structure of specific and nongenetic IAPs dependent protein eraser 13 (SNIPER-13). Figure is adapted from ref 61.

an AR targeting compound, SNIPER-13, was evaluated by Western blot. In human mammary tumor (MCF-7) cells that express AR, SNIPER-13 decreased AR protein levels at a concentration of 30 μM. The high concentration required to induce degradation may be attributed to the hydrolytically unstable ester and oxime linkages.[71] These results suggest that SNIPERs can be utilized to modulate AR activity.

The modular assembly of SNIPERs allows for the incorporation of virtually any "targeting moiety". This characteristic, and the ability to recruit E3 with a small molecule, establishes a versatile molecular platform to address many protein targets. In the future, research efforts may focus on generating stable linkages between the targeting moiety and the IAP recognition element or altering linker lengths to optimize activity of SNIPER conjugates against various protein targets.

Metallo-Conjugates. Metallo-based cytotoxic agents, such as cisplatin, remain a viable option for the treatment of cancer.[72] From a mechanistic standpoint, these compounds exert their biological activities by binding to nucleobases in DNA and inducing damage to DNA that ultimately triggers apoptosis.[73] Although widely used in the clinic, these agents are generally nonspecific and exhibit shortcomings that include severe side effects resulting from compromised normal cell viability and drug resistance. This has led to the exploration of metallo-based chemotherapeutic agents that target specific organs or tumors to minimize adverse side effects.

Conjugation of a "targeting moiety" to metallo-based cytotoxic agents could potentially circumvent nonspecific interactions by selectivity targeting cells that overexpress particular proteins, establishing a delivery vector to localize the effects of new therapeutic agents.[74] Recent studies from the Hannon group have discovered the first metallo-based chemotherapeutic conjugates to target AR.[75] The authors developed an efficient protocol to readily synthesize an array of steroid conjugates to act as delivery vehicles. Ethisterone, the 17α-ethynyl homologue of testosterone, was conjugated to pyridines, quinolines, and isoquinolines utilizing Sonogashira cross-coupling conditions. Subsequent coordination to platinum(II) complexes yielded metallo-based bifunctional agents (Figure 6).

Initial evaluation of the cytotoxic effects for the two most promising metallo-based bifunctional agents in cell lines that express AR (T47D cells) revealed promising biological activity for a cis conjugate (IC_{50} = 15.9 μM) but not the trans conjugate (IC_{50} = 63 μM), suggesting that the geometry of the platinum(II) complexes plays a critical role. Additionally, the cis conjugate exhibited greater potency than cisplatin itself (IC_{50} = 32 μM). Cell uptake studies reveal that the "targeting moiety" enhances drug delivery, suggesting that the hydrophobic character of ethisterone facilitates molecular transport across the cellular membrane. Importantly, the presence of the "targeting moiety" in the cis conjugate (relative to control compounds lacking steroid moieties) led to significant structural effects on DNA.[76] The distortion of DNA upon

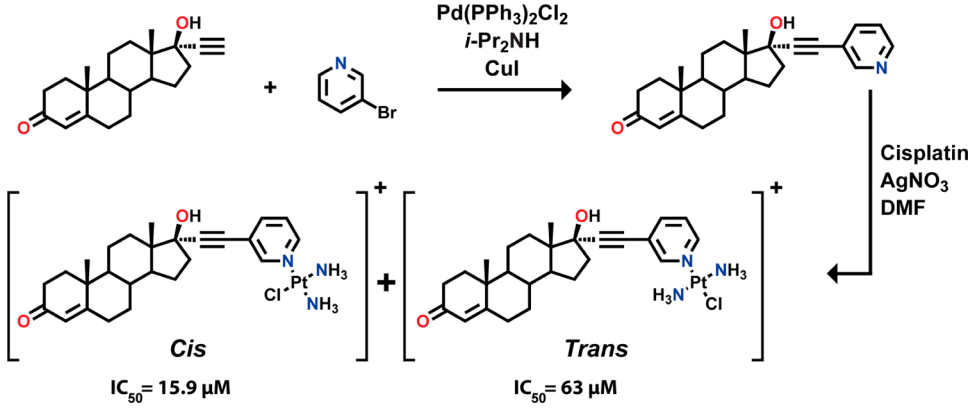

Figure 6. Synthesis of platinum(II) steroid conjugates for targeted drug delivery. Figure is adapted from ref 65.

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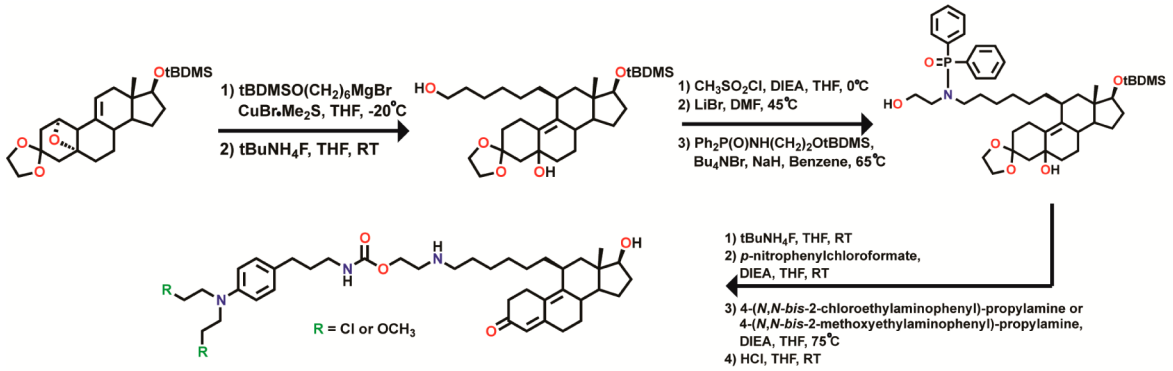

Figure 7. Synthesis of alkylating agents for targeted DNA damage. Figure is adapted from ref 71.

binding the cis conjugate was greater than that observed for cisplatin, suggesting that the steric bulk of ethisterone promotes greater unwinding of DNA to accommodate binding of the complex. These results demonstrate the utility of targeting strategies for drug delivery.

Metallo-based conjugates have significant utility as a platform for targeted drug delivery. The work outlined above suggests that metallo-based conjugates can be crafted to exert toxic effects preferentially on cell types that overexpress AR. Additional studies would be valuable for elucidating mechanistic features. For example, demonstration that coadministration of a competitive ligand, such as DHT, abrogates the activity of the metallo-conjugate would further support the hypothesis that targeting is mediated specifically through binding to AR. Ultimately, similar strategies could potentially be elaborated for targeting additional metallo-conjugates to a range of malignant cell types, while mitigating cytotoxic effects on other tissues.

Alkylating Conjugates. Alkylating agents act through DNA damaging mechanisms and are commonly used in cancer therapy.[77−80] These agents primarily alkylate guanine bases in DNA, inducing cellular apoptosis. Crafting alkylating therapies to specifically target malignant cells could minimize cytotoxic effects to normal cells and lead to the development of potent anticancer agents.

In an effort to block DNA repair enzymes, the Essigmann group has developed heterobifunctional DNA-damaging agents to specifically target prostate cancer cells (Figure 7).[81] The alkylating agent *N*,*N*-bis-2-chloroethylaniline was linked to a steroid hormone that targets AR, allowing the conjugate to simultaneously bind AR and DNA. This strategy results in the blockade of DNA repair enzymes in prostate cancer cells that overexpress AR, subsequently leading to the disruption of AR-mediated transcription and signaling. Using radiometric competitive binding assays, the relative binding affinity of *N*,*N*-bis-2-chloroethylaniline was determined to be ∼20% for AR and 4.2% for PR. This result establishes that the conjugate is more selective for AR than PR. In addition, only *N*,*N*-bis-2-chloroethylaniline, and not the negative control (*N*,*N*-bis-2-methoxyethylaniline), covalently modified DNA. Administration of the alkylating agent at a concentration of 10 μM induced apoptosis, as determined by flow cytometry and cleavage of poly ADP-ribose polymerase (PARP) in Western blot analysis. As expected, the negative control did not induce apoptosis at an equivalent concentration. More importantly, xenograft studies in immunocompromised mice revealed 90% inhibition of tumor growth through intraperitoneal injection (daily dose of 30 mg/kg). These results demonstrate the effectiveness of using targeted alkylating agents to selectively suppress prostate cancer tumor growth.

Peptoid Conjugates. An emerging avenue in molecular pharmacology is the development of multivalent therapeutic agents. Multivalency can be used to establish enhanced binding affinity, termed avidity, and specificity for corresponding biomolecular targets through multisite binding contacts.[82] Displaying ligands or "targeting moieties" on modular oligomer frameworks allows chemists to precisely craft architectures capable of inhibiting highly specific protein−protein or protein−nucleic acid interactions. In addition, the ability to create monodisperse molecular scaffolds enables control over important physicochemical features of the products, including solubility and cellular uptake.[83]

Peptoids are a class of peptidomimetics composed of N-substituted glycine units joined through tertiary amide linkages. Peptoids have recently been exploited as multivalent platforms to design conjugates capable of targeting different nucleic acids and protein receptors.[84−86] Peptoids are stable against proteases and display enhanced cell permeability profiles.[87,88] The incorporation of over 200 different peptoid side chains has enabled numerous applications in chemistry and biology, including enantioselective catalysis, molecular recognition, antimicrobial activity, intracellular delivery, and antitumor activity in vivo.[89−96] Peptoids are compatible with solid-phase synthesis techniques and can be assembled in a sequence-specific manner to afford monodisperse products.[97] Additionally, the conformation of peptoid oligomers can be controlled though macrocyclic constraints and side chain interactions.[98,99]

Utilizing peptoids as a versatile chemical platform, the Kirshenbaum lab designed multivalent ethisterone conjugates to specifically target the AR LDB and modulate AR activity via different mechanisms of action.[100] Ethisterone was conjugated at the 17-α position to the peptoid scaffold via highly stable triazole linakges. Initial efforts evaluated effects of valency, spacing, and conformational ordering on AR activity (Figure 8). Previous studies had demonstrated the cell permeability of similar steroidal peptidomimetic conjugates.[84] Fluorescence polarization assays were conducted to determine if the conjugates compete against DHT for binding to the AR ligand binding domain. Results from these studies revealed that hexavalent (**4**) and spaced divalent conjugates (**5** and **6**) compete for binding. Mono-, di-, and trivalent conjugates (**1**−**3**) and a cyclic divalent conjugate (**7**) did not compete for AR binding. A control peptoid conjugate outfitted with PR ligands did not activate AR in a luciferase reporter assay, suggesting the

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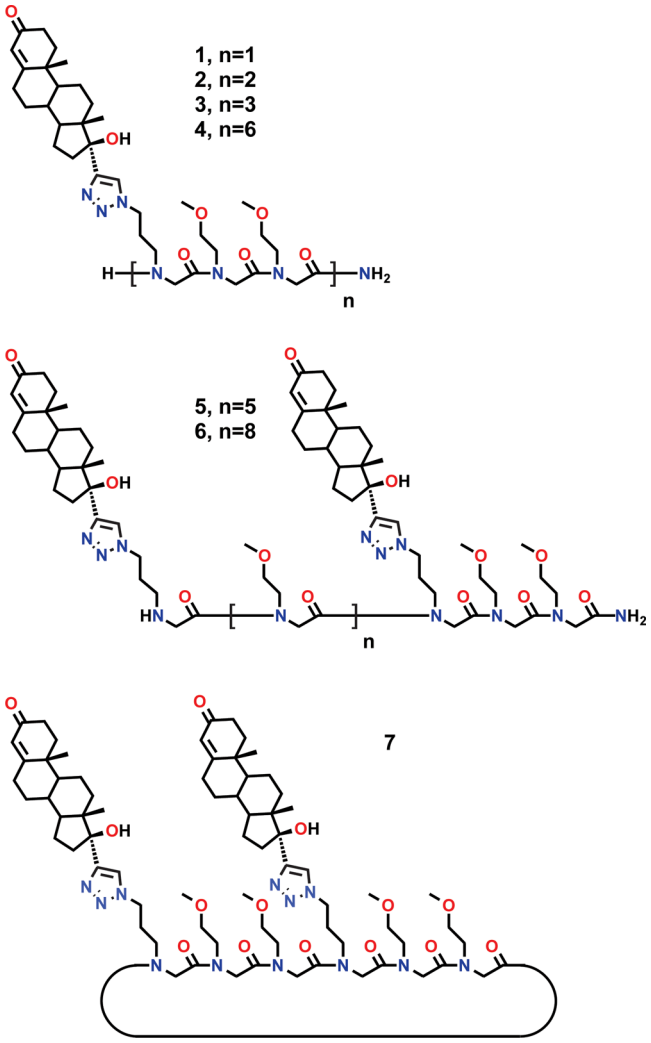

Figure 8. Chemical structures of ethisterone peptidomimetic conjugates. Figure is adapted from ref 90.

ethisterone conjugates are selective for AR. In cell proliferation studies that model castration-resistant prostate cancer (LNCaP-abl cells), conjugates **6** and **7** exhibited potent antiproliferative properties. As expected, the anti-androgen bicalutamide (vide supra) failed to suppress proliferation in this resistant cell line. Importantly, cytotoxic effects of conjugates **6** and **7** were not observed in cell lines that do not express AR (PC-3 and HEK293 cells), establishing that conjugates selectively target AR.

In a follow-up investigation, the authors used confocal microscopy, time-resolved fluorescence resonance energy transfer, chromatin immunoprecipitation, flow cytometry, and microarray analysis to gain insight into the mechanism of action for conjugates **6** and **7**.[101] Upon administration of conjugates **6** and **7** to HEK293 cells transfected with an AR fluorescent fusion protein, conjugate **6** did not promote AR nuclear localization while conjugate **7** did, suggesting competitive and noncompetitive mechanisms of action, respectively. AR coactivator recruitment assays revealed that conjugate **6** did not promote binding between AR and coactivator proteins while conjugate **7** partially recruited coactivator proteins. In DNA binding experiments, both conjugates **6** and **7** reduced the occupancy of AR to the PSA enhancer (vida supra). Conjugate **7**, but not conjugate **6**, induced arrest in the G_0/G_1 phase of the cell-cycle and displayed contrasting patterns in global gene expression. Intriguingly, conjugate **6** and **7** share extensive chemical similarities, indicating that the disposition of the ligand presentation on the scaffolds can exert a significant influence on the mechanism of action. Conjugate **6** did not promote AR nuclear localization or coactivator binding and inhibited DNA binding. In contrast, conjugate **7** promoted AR nuclear localization and induced cell-cycle arrest through a noncompetitive mode of action.

The modularity of peptoid synthesis establishes a versatile chemical platform to generate an array of three-dimensional architectures to target and modulate the activity of different biomolecular targets. Generation of peptidomimetic conjugates capable of antagonizing AR via distinct mechanisms of action could circumvent drug resistance in AR pharmacology. Peptoids offer a chemical platform that can be utilized to optimize biological activity and hold significant promise as next generation therapeutics for prostate cancer.

TOWARD NOVEL AR ANTAGONISTS BY MOLECULAR DESIGN: TAKING INSPIRATION FROM THE ESTROGEN RECEPTOR

The estrogen receptor (ER) has a well-characterized mechanism of action. It is known that native ligand (estradiol) binding to the ER ligand binding domain induces a conformational rearrangement that promotes dimerization, as determined by site-specific mutational analysis.[102] Additionally, X-ray crystal structures of ER dimers in the presence of ligand and other ER modulators have been reported, establishing a template for molecular design by elucidating the structural parameters of the ER dimer complex.[103,104]

Pioneering work from the Katzenellenbogen lab has probed ER function with various bivalent conjugates tethered by different linkers.[105] Using high-resolution structural information, the first steroidal constructs aimed toward targeting the ER dimer have been synthesized (Figure 9). Initial studies

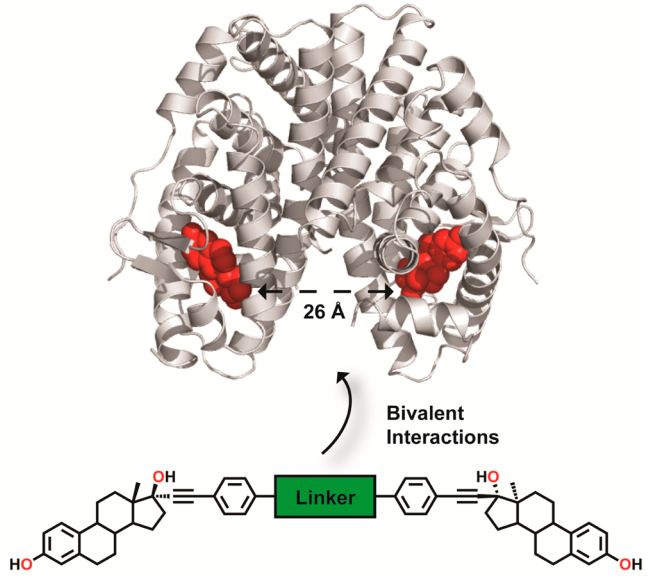

Figure 9. Steroidal bivalent conjugates modulate estrogen receptor (ER) activity through bivalent binding interactions. Crystal structure of the ER ligand binding domain (gray ribbon, PDB code 1ERE) is bound to native ligand (estradiol, red spheres). Figure is adapted from ref 95.

dx.doi.org/10.1021/jm500101h | J. Med. Chem. 2014, 57, 8224−8237

focused on developing a correlation between linker length and binding affinity. The authors concluded that bivalent conjugates incorporating a ~35 Å linker were most suitable for enhancing ER binding affinity.

In more recent studies, nonsteroidal bivalent conjugates that induce agonistic and antagonistic ER conformations (Figure 10) were designed and synthesized in order to distinguish intra-

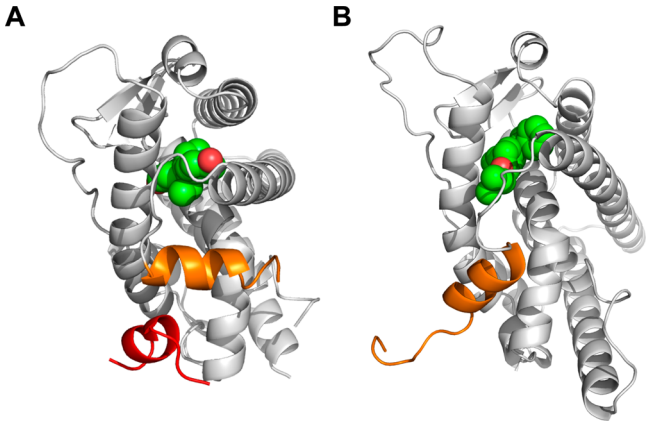

Figure 10. ER conformation is dependent upon ligand binding: (A) ER bound in an agonist conformation (gray ribbon; diethylstilbestrol, colored spheres; helix-12 in orange; coactivator peptide in red; PDB code 3ERD); (B) ER bound in an antagonist conformation (gray ribbon; hydroxytamoxifen, colored spheres; helix-12 in orange; PDB code 3ERT).

from intermolecular binding events.[106,107] Bivalent agonist conjugates displayed weak binding affinity, presumably due to burial of the hydrophilic linker within the protein interior. In contrast, it was determined that antagonist conjugates incorporating a 14.4 Å linker induced an intramolecular binding event (i.e., one targeting moiety optimized for competitive binding and the other for binding to additional hydrophobic pockets such as activation function 2, Figure 11).

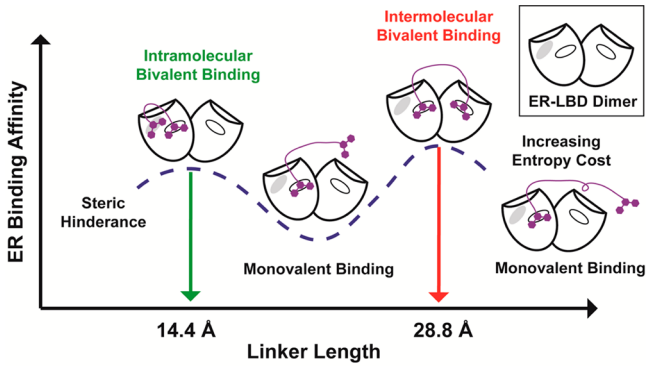

Figure 11. Diagram depicting intra- and intermolecular ER binding events that are dependent on linker length. Figure is obtained from ref 96.

Additionally, a 29 Å linker was found to induce an intermolecular binding event. Increases in linker length above 29 Å resulted in reducing binding affinities, presumably due to unfavorable entropic effects. Importantly, most antagonistic nonsteroidal bivalent conjugates were more potent at inhibiting cell proliferation in breast cancer cells (MCF7) than a monovalent pharmacophore control.

A critical objective from a molecular design approach is the ability to induce different ER conformations that are dependent upon ligand binding. As discussed above, the conformation induced upon agonist or antagonist ligand binding to ER (Figure 10) plays a critical role in the biological outcome. In an antagonist conformation, an intra- or intermolecular binding event can occur between two distinct "targeting moieties". An open question is whether structure-based design can be utilized to generate heterobifunctional conjugates that target two distinct binding sites on AR (i.e., one targeting moiety optimized for competitive binding at the ligand binding domain and the other for binding to an additional hydrophobic pocket, such as AF-2 or BF3).

TARGETING AR WITH NONSTEROIDAL CONJUGATES

Over the past decade, targeting canonical or membrane-associated AR with heterobifunctional or multivalent constructs displaying anti-androgen drug ligands has emerged as a potential family of therapeutics. These compound classes hold great promise as effective therapeutic agents due to their ability to modulate AR activity through unique mechanisms of action. Because of the large number of reports, we highlight only a few representative examples of promising strategies that have been used to target AR with nonsteroidal conjugates.

Recently, the Oyelere lab reported a nonsteroidal heterobifunctional conjugate outfitted with histone deacetylase inhibitors (Figure 12A).[108] Histone deacetylase inhibitors

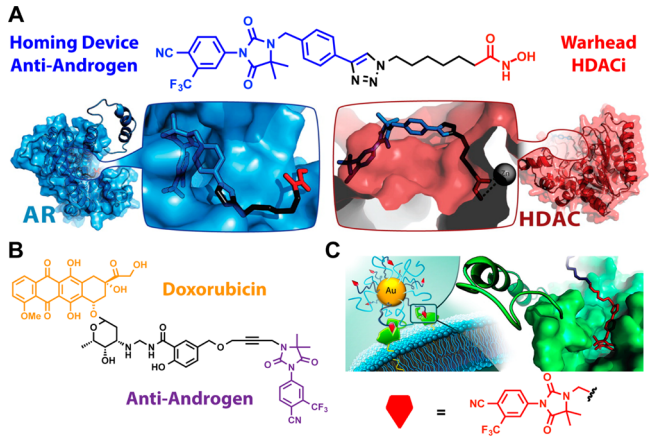

Figure 12. Nonsteroidal conjugates targeting AR: (A) chemical structure of heterobifunctional conjugate displaying histone deacetylase inhibitor linked to a nonsteroidal antiandrogen ligand; (B) chemical structure of heterobifunctional conjugate displaying doxorubicin linked to a nonsteroidal antiandrogen ligand; (C) schematic depiction of a multivalent gold nanoparticle displaying nonsteroidal antiandrogen ligands that target membrane-associated AR. Figure is obtained from refs 98, 99, and 101.

show great promise in preclinical cancer models, but their inability to selectively target malignant tissue has restricted therapeutic development. By conjugating histone deacetylase inhibitors to nonsteroidal anti-androgen ligands, selective modulation of AR activity at concentrations lower than clinical anti-androgens was achieved. These results introduce a novel method to antagonize the AR and pave the way for next generation therapeutics.

In similar studies, the Koch lab reported a nonsteroidal heterobifunctional conjugate containing doxorubicin, a non-

dx.doi.org/10.1021/jm500101h | J. Med. Chem. 2014, 57, 8224−8237

selective cytotoxic therapeutic DNA intercalator used in the clinic (Figure 12B).[109,110] To enhance selectivity, doxorubicin was conjugated to a nonsteroidal anti-androgen ligand through a salicylamide linker that can be hydrolyzed ($t_{1/2}$ = 57 min under physiological conditions) to yield a doxorubicin–formaldehyde Schiff base. The anti-androgen conjugate successfully delivered the doxorubicin-formaldehyde Schiff base to cells overexpressing AR, highlighting the ability of this approach to enhance selectivity by releasing the pharmacophore in prostate cancer cells.

Lastly, the El-Sayed lab introduced the first nonsteroidal multivalent conjugates that selectively target membrane-associated AR (Figure 12C).[111] Bicalutamide was conjugated to gold nanoparticles, generating architectures that display approximately $(2.25 \pm 0.02) \times 10^3$ ligands/particle. The multivalent compounds enhanced potency by 1 order of magnitude, in comparison to the monovalent ligand, in prostate cancer cells. These results establish that conjugation of numerous copies of a known pharmacophore to a molecular scaffold can significantly increase antiproliferative effects and may overcome resistance that arises from monovalent treatment.

CONCLUSION

There is a growing appreciation for the design of potent and selective therapeutic agents targeting the AR for prostate cancer patients. Targeted drug therapy is beginning to play a pivitol role in new drug discovery efforts. Classically, small molecules identified via chemical screening efforts have been considered to offer a relatively straightforward path for clinical implementation. In certain cases, extensive high-resolution structural information enables structure–activity relationship profiles that can be utilized for optimization, facilitating translation into the clinic. Unfortunately, their therapeutic responses can be short-lived because of acquired resistance.

The studies highlighted in this review indicate how new chemical entities are being designed to engage AR with high potency. Many of these compounds feature novel steroidal conjugates. Additional preclinical studies will be required to validate their potential for clinical translation. In many cases, it will be necessary to evaluate critical parameters such as selectivity, in vivo potency, and binding affinity. As discussed, chemical modifications at certain positions on the steroid core can result in diminished binding affinities, potentially limiting their utility in AR pharmacology.

These molecular architectures have been demonstrated to elicit potent biological responses and more importantly target the AR in novel ways. In the future, we may begin to see examples of monodisperse homo- and heterogeneous bivalent or multivalent displays in which high-resolution structural data enable evaluation of structure–activity relationships that have propelled many small molecule drug discovery efforts. More importantly, heterobifunctional displays will likely be designed to target two distinct binding sites on AR, enhancing potency and establishing new modes of AR antagonism. These constructs could potentially address the challenge of overcoming resistance in prostate cancer patients.

AUTHOR INFORMATION

Corresponding Author

*E-mail: kent@nyu.edu. Phone: 212-998-8486.

Notes

The authors declare no competing financial interest.

Biographies

Paul M. Levine received his Ph.D. from New York University (NYU) in 2014 under the mentorship of Professor Kent Kirshenbaum. At NYU, he designed a chemical platform to generate multivalent steroid-peptidomimetic conjugates as potent modulators of androgen receptor signaling. Paul is currently pursuing his postdoctoral studies in the laboratory of Professor Rebecca Scheck at Tufts University, MA. His current research involves developing short, structured peptide catalysts as encodable motifs that will report on protein conformation and association in living cells.

Michael J. Garabedian is a Professor of Microbiology and Urology at the NYU Langone School of Medicine. His research group studies how nuclear hormone receptors regulate cell physiology and pathophysiology in areas such as prostate cancer, cardiovascular disease, and neuroendocrine function. In addition to extensive publications, work done by his group has led to several patents in nuclear receptor function and activity. Dr. Garabedian serves on the editorial boards of several biomedical journals and grant review panels. He is also actively involved in graduate and medical student education. Dr. Garabedian earned undergraduate degrees in chemistry and biology from the University of California, Irvine. He completed a Ph.D. in Biochemistry at Brandeis University, MA.

Kent Kirshenbaum obtained his B.A. in Chemistry at Reed College in Portland, OR. He then conducted Ph.D. research in Pharmaceutical Chemistry at the University of California, San Francisco, where he worked with Prof. Ken Dill toward the discovery of new folding codes in biomimetic polymer systems. He went on to pursue postdoctoral studies in protein chemistry at California Institute of Technology, where his research with Prof. David Tirell involved manipulation of the protein biosynthetic machinery to create proteins incorporating non-natural amino acids with useful abiotic functional groups. He is currently an Associate Professor in the Department of Chemistry at NYU, where his research focuses on investigating sequence–structure–function relationships in peptoid oligomers.

ACKNOWLEDGMENTS

This work was supported by the NSF (Award CHE-1152317 to K.K.) and was also supported in part by Grant UL1 TR000038 from the National Center for Advancing Translational Sciences, National Institutes of Health. P.M.L. thanks New York University for a Dean's Dissertation Fellowship.

ABBREVIATIONS USED

AR, androgen receptor; LBD, ligand-binding domain; DHT, dihydrotestosterone; PSA, prostate specific antigen; CRPC, castration-resistant prostate cancer; ER, estrogen receptor

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