Recent Progress in the Development of Protein-Protein Interaction Inhibitors Targeting Androgen Receptor-Coactivator Binding in Prostate Cancer

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# ABSTRACT

The androgen receptor (AR) is a key regulator for the growth, differentiation and survival of prostate cancer cells. Identified as a primary target for the treatment of prostate cancer, many therapeutic strategies have been developed to attenuate AR signaling in prostate cancer cells. While frontline androgen-deprivation therapies targeting either the production or action of androgens usually yield favourable responses in prostate cancer patients, a significant number acquire treatment resistance. Known as the castration-resistant prostate cancer (CRPC), the treatment options are limited for this advanced stage. It has been shown that AR signaling is restored in CRPC due to many aberrant mechanisms such as AR mutations, amplification or expression of constitutively active splice-variants. Coregulator recruitment is a crucial regulatory step in AR signaling and the direct blockade of coactivator binding to AR offers the opportunity to develop therapeutic agents that would remain effective in prostate cancer cells resistant to conventional endocrine therapies. Structural analyses of the AR have identified key surfaces involved in protein-protein interaction with coregulators that have been recently used to design and develop promising AR-coactivator binding inhibitors. In this review we will discuss the design and development of small-molecule inhibitors targeting the AR-coactivator interactions for the treatment of prostate cancer.

**KEYWORDS**

Prostate cancer; androgen receptor; coregulator recruitment; protein-protein interactions; coactivator binding inhibition; peptidomimetics.

**ABBREVIATIONS**

ABS, androgen-binding site; AF, activation function; AR, androgen receptor; ARA, androgen receptor-associated protein; ARE, androgen-responsive elements; BF3, binding function 3; CBI, coactivator binding inhibitor; CBP, CREB-binding protein; CPRC, castration-resistant prostate cancer; CYP, cytochrome P450; DBD, DNA-binding domain; DHT, 5α-dihydrotestosterone; ER, estrogen receptor; FP, fluorescence polarization; GRIP1, glutamate receptor interacting protein 1; LBD, ligand-binding domain; NLS, nuclear localization signal; NR, nuclear receptor; NTD, N-terminal domain; PCa, prostate cancer; PELP1, proline-, glutamic acid- and leucine rich protein 1; PPI, protein-protein interaction; PSA, prostate specific antigen; SAR, structure-activity relationship; SPR, surface plasmon resonance; SRC, steroid receptor coactivator; TF, transcription factor; TR-FRET, time-resolved fluorescence resonance energy transfer;

# 1. Introduction

The androgen receptor (AR) is a member of the nuclear receptor (NR) superfamily of ligand regulated transcription factors that plays an integral role in primary and secondary male sexual development. As a key regulator for the growth, terminal differentiation and function of the prostate gland, excessive stimulation of the AR can result in prostate cancer (PCa) and the related but benign prostatic hyperplasia [[1-5](#_ENREF_1)]. PCa is the most common solid tumor and the second leading cause of cancer death among men worldwide with ~20% of patients developing metastatic castration-resistant prostate cancer (CRPC) [[6](#_ENREF_6)]. Playing a critical role in PCa development and progression, the AR has emerged as a primary therapeutic target. Different strategies have been developed to block the production or action of androgens that provide growth and survival signals to prostate cells [[7-14](#_ENREF_7)]. The currently used AR antagonists, such as flutamide, bicalutamide, nilutamide and enzalutamide (MDV3100), act by binding to the androgen binding site (ABS) of the AR, resulting in conformational changes that prevent its activation [[7](#_ENREF_7), [11](#_ENREF_11), [14](#_ENREF_14)]. Although initial responses to AR antagonists are usually favorable and suppression of prostate tumor growth is observed, with time, the diseases transforms and progresses to metastatic CRPC where patients develop resistance to antiandrogen drugs [[8](#_ENREF_8), [15-19](#_ENREF_15)]. The therapeutic options are very limited for this disease stage and include the addition of cytotoxic agents such as taxanes, the selective CYP17 inhibitor abiraterone acetate, radium-223 (for men with bone metastases) or the vaccine sipuleucel-T to the androgen deprivation therapy [[17](#_ENREF_17), [20-22](#_ENREF_20)]. Many studies on CRPC have shown that functional AR signaling is inappropriately restored in castrate or androgen-depleted environment [[23](#_ENREF_23), [24](#_ENREF_24)]. A wide variety of cellular modifications have been proposed for the emergence of resistance in CRPC including AR mutation (*e.g.* in the ABS), overexpression of the AR and/or its coactivators, constitutively active splice variants, intracrine androgen production and alternative AR activation [[25-35](#_ENREF_25)]. Taken together, these mechanisms underscore the addiction of CRPC to AR signaling [[17](#_ENREF_17), [24](#_ENREF_24)]. As a result, new chemical approaches with innovative mode of action are urgently needed to overcome resistance to antiandrogens and successfully inhibit AR signaling in advanced PCa. In this review we will discuss the recent progress in the design and development of promising small-molecule inhibitors targeting AR-coactivator protein-protein interactions for the treatment of prostate cancer.

# 2. THE ANDROGEN RECEPTOR

In the absence of ligands, the AR predominantly resides in the cytoplasm where it is associated with heat shock proteins in a transcriptionally inactive form until it is activated by testosterone or the more potent metabolite, 5α-dihydrotestosterone (DHT) [[36](#_ENREF_36), [37](#_ENREF_37)]. Upon ligand binding, the AR undergoes a substantial conformational change leading to dissociation from repressor proteins, dimerization, translocation to the nucleus and association to androgen-responsive elements (ARE) in the regulatory regions of target genes [[38](#_ENREF_38)]. Through this pathway, the AR regulates the expression of more than a thousand genes including the prostate specific antigen (PSA), an important biomarker for PCa. The DNA-bound receptor can then exert a positive or negative effect on gene transcription by recruiting either coactivators or corepressors and their respective multi-protein machinery [[35](#_ENREF_35), [39-41](#_ENREF_39)]. More than 300 proteins that exhibit AR coactivator or corepressor properties have been identified [[42](#_ENREF_42), [43](#_ENREF_43)]. Coregulator recruitment is a crucial regulatory step in AR signaling and the direct blockade of coactivator binding to AR offers the opportunity to develop therapeutic agents that would remain effective even in instances where currently used AR antagonists fail [[17](#_ENREF_17), [44-47](#_ENREF_44)].

**<INSERT FIGURE 1 (single column)>**

Like other NRs, the AR displays a modular structure composed of an N-terminal domain (NTD) bearing the activation function (AF) 1, a DNA-binding domain (DBD), a connecting hinge region containing a nuclear localization signal (NLS) and a C-terminal ligand-binding domain (LBD) (Fig. 1). The LBD is composed of 12 anti-parallel α-helices with the ABS buried in the interior that undergo significant rearrangement upon agonist binding. The helix 12 (H12) is the most flexible part of the LBD and its repositioning after androgen binding completes the AF-2 binding surface by creating a shallow hydrophobic groove that allows the docking of leucine-rich, LXXLL motif-containing coregulatory proteins, where X is any amino acid [[48-53](#_ENREF_48)]. In addition, the intrinsic dipole moment of the coactivator α-helix is matched on the AF-2 by a negatively charged glutamic acid residue (E897 on H12) at the N-terminus and a positively charged lysine residue (K720 on H3) at the C-terminus, which form a charge clamp (Fig. 2) [[50](#_ENREF_50), [54](#_ENREF_54), [55](#_ENREF_55)]. The LXXLL motif, also known as the NR box, is present in several coactivators such as members of the p160 coactivator family [[56-58](#_ENREF_56)]. Mainly formed by residues from helices 3, 4, 5 and 12, the AF-2 surface on the AR-LBD is unique among NRs in preferring to interact with the more bulky hydrophobic motifs F/WXXLF over the LXXLL [[53](#_ENREF_53), [59-63](#_ENREF_59)]. Interestingly, this motif can be found within AR cognate coactivators including AR-associated protein (ARA) 54, ARA55 or ARA70 but also on the AR-NTD (23FQNLF27 and 433WHTLF437) (Fig. 1) [[50](#_ENREF_50), [64](#_ENREF_64)]. These intramolecular interdomain N/C interactions play an important role in selective AR-dependent gene regulation and are however disrupted by DNA binding, which in turn would expose the NTD and AF-2 surfaces for interactions with coregulators [[65](#_ENREF_65)]. Residues flanking the LXXLL or FXXLF motifs are the major contributors for coactivators selectivity and affinity [[54](#_ENREF_54), [55](#_ENREF_55), [57](#_ENREF_57)]. Also known as the coactivator binding pocket, the AF-2 is the major protein-protein interaction (PPI) surface used by NRs for coactivator recruitment. While the AF-2 site certainly represents a target of choice to develop AR-coactivator interaction inhibitors, other binding sites have been recently exploited and showed very promising results.

# 3. Targeting the activation function 2 site

The manipulation of PPIs with chemical compounds is very attractive and has provided great potential for the discovery of new drugs [[66](#_ENREF_66), [67](#_ENREF_67)]. However, the development of low molecular weight PPI inhibitors is beset by a number of challenges such as the large size of the surface area and the often lack of distinctive structural features that can be used as a basis for inhibitor design [[68-71](#_ENREF_68)]. Considering the nature of the interaction surface, the ability to mimic protein secondary structure is crucial to efficiently bind a protein interaction domain and disrupt target PPI with a synthetic molecule. Alternatively to large library screenings, one strategy is to identify the binding motif of partner proteins at the interaction site and determine its structure to design and develop molecules mimicking this motif [[72-74](#_ENREF_72)].

**<INSERT FIGURE 2 (single column)>**

Phage display technology and structural studies of NR box peptides bound to AR showed that the LXXLL and FXXLF motifs adopt an α–helical conformation for optimal binding and that side chains of the leucine and/or phenylalanine at the *i*, *i*+3 and *i*+4 positions interact with hydrophobic pockets in the AF-2 domain (Fig. 2) [[46](#_ENREF_46), [50](#_ENREF_50), [53](#_ENREF_53), [59-62](#_ENREF_59), [75-80](#_ENREF_75)]. Most importantly, many of these studies have also demonstrated *in vitro* that peptides derived from these motifs were able to block interactions between the AR and coactivators bearing L/FXXLL/F motifs [[46](#_ENREF_46), [53](#_ENREF_53), [77-80](#_ENREF_77)]. Peptides are very useful tools in drug design and development but their use as therapeutic agents is unfortunately limited by a rapid metabolic degradation, low bioavailability and poor cell permeability. To overcome these drawbacks, the transfer of the essential functional and conformational requirements for AF-2 binding into small organic molecules able to mimic peptide structures is a very interesting strategy. Such molecules, called peptidomimetics, do not possess a peptide backbone structure but are able to mimic protein secondary structures and arrange essential functional groups into the required three-dimensional pattern to interact with the target protein [[72](#_ENREF_72), [81](#_ENREF_81), [82](#_ENREF_82)]. Based on L/FXXLL/F motifs structural behavior (Fig 3, **1**), α-helix mimetics have been elegantly used as scaffolds by different research groups to design and develop coactivator binding inhibitors (CBI).

**<INSERT FIGURE 3 (double column)>**

Building on successful results obtained in the design of estrogen receptor (ER) CBIs [[83-85](#_ENREF_83)] and considering the AR-LBD preference for motifs bearing bulkier aromatic residues, Gunther et al. used a 2,4,6-trisubstituted pyrimidine scaffold to mimic the *i*, *i* + 3 and *i* + 4 arrangement of interacting hydrophobic side chains in the AF-2 groove (Fig. 3, **2**) [[86](#_ENREF_86)]. This scaffold was previously designed by an ‘’outside-in’’ approach from structural data of the ER-bound LXXLL motif and substituted with alkyl groups to mimic the leucine side chains orientation in this short α-helix conformation [[85](#_ENREF_85)]. To target the AR AF-2 site, a series of peptidomimetic compounds bearing larger benzyl and naphtyl moieties on the pyrimidine core was prepared to mimic the phenylalanine and tryptophan residues found in AR-NTD and a great number of AR-coactivators. As a consequence of solubility problems with compounds bearing larger aromatic substituents, the library could not be entirely evaluated in TR-FRET assay for direct AR-LBD binding and a luciferase reporter gene assay was used. The tested compounds did not show cellular toxicity and their ability to cause inhibition by binding to the AR surface and not by displacing DHT from the ABS was confirmed. As expected, AR selectivity over ER could be achieved with compounds bearing at least two aromatic substituents with IC50 values ranging from 1.5 to 6.6 µM. Most importantly, some of the reported compounds retain their activity in the reporter gene assay with full-length AR containing the LNCaP mutation (T877A), frequently found in patients with metastatic CRPC [[87](#_ENREF_87)]. These results were promising and further optimizations are needed to improve the solubility of these compounds in order to facilitate their *in vitro* evaluation for accurate structure-activity relationship (SAR) analysis and affinity improvement and ultimately allow their evaluation in animal models.

Caboni et al. combined a structure-based drug design approach and virtual screening to identify potent CBIs [[88](#_ENREF_88)]. The use of virtual screening to identify inhibitors of the AR-AF-2 has been previously reported [[89](#_ENREF_89)] but in this case, their strategy was to use available structural data for AR-bound coactivator peptides to build a 3D pharmacophore model based on coactivators common features and aromatic moieties of the FXXLF motif. The resultant model was then used to virtually screen commercially available databases and discover potential AR CBIs. After the selection of a first series of hit compounds and docking studies followed by molecular similarity search, a series of diarylhydrazide derivatives were identified (Fig 3, **3**). It was proposed that this novel class of diarylhydrazide, composed of two aromatic moieties on each side of a rigid linker, is able to mimic the *i* and *i* + 4 phenylalanine side chains in the FXXLF motif. Selected diarylhydrazide derivatives were found to inhibit AR coactivator recruitment by TR-FRET assay with IC50 values between 13 and 26 µM using both wild type and mutant T877A AR-LBDs. With the exception of progesterone receptor (IC50 ~22-28 µM), the compounds showed selectivity for the AR over glucocorticoid receptor (GR), ERα and ERβ. Finally, diarylhydrazide **3** exhibited low toxicity in cell viability assays on different prostate cell lines and antiproliferative activity and inhibition of DHT-stimulated PSA expression in LNCaP cells at 10 and 20 µM. This study supports the applicability of virtual screening to identify new scaffolds, discover lead compounds and develop potent AR modulators targeting PPI after optimization.

In another approach, Weiser et al. used biaryl scaffolds to replicate the α-helical rotation of the LXXLL motif backbone and display the *i* and *i* + 4 leucine side chains in the appropriate arrangement [[90](#_ENREF_90)]. Supported by theoretical data and promising results in the design of ER CBIs using biaryl scaffolds, they designed 3,3′-disubstituted bis-4,4′oxybiphenyls to mimic the hydrophobic side chains arrangement and also the electronic interactions with the charge clamp to lock the inhibitors in place (Fig 3. **4a-d**) [[90-94](#_ENREF_90)]. A series 3,3′-disubstituted biphenyls including 4,4′-asymmetrical phenolic-ester, amino-ester and amino-acid derivatives was prepared and their ability to disrupt ERα- and AR-coactivator binding evaluated in respective cell-based transactivation assays. While 4-phenolic-4′-ester and 4-amino-4′-acid derivatives were able to block the ERα-GRIP1 interaction, the 4-amino-4′-esters bearing larger substituents **4a-d** were significantly less active against ERα signaling and the only ones to exhibit inhibitory activity for AR signaling. In some cases cellular toxicity was observed at higher doses. The reported results confirmed the ability of biaryl scaffolds to mimic short α-helical structures and disrupt NR-coactivators interactions. They also showed the existence of subtle variations between the ERα and AR AF-2 sites and that, additionally to side chains hydrophobicity and positioning, the charge clamp can also play an important role in NR selectivity. The described approach is very interesting and further optimization should lead to compounds with increased affinity and selectivity for the AR.

Oligobenzamides are also well recognized for their ability to mimic α-helical structures [[72](#_ENREF_72), [95-97](#_ENREF_95)]. Ahn et al. used bis- and tris-benzamide scaffolds to rationally design peptidomimetics of the LXXLL motif in the PELP1 coregulatory protein [[98](#_ENREF_98), [99](#_ENREF_99)]. Considerably involved in AR signaling in PCa both in presence and absence of androgens, the AR-PELP1 interaction represent a very attractive target [[100](#_ENREF_100), [101](#_ENREF_101)]. Bis- and tris-benzamide derivatives have been prepared to display the *i* and *i* + 4 (*i* + 7 for tris-benzamides) hydrophobic side chains of the helix (Fig 3, **5**). Co-immunoprecipitation experiments showed that the bis-benzamide **5** was able to block DHT-induced AR-PELP1 interaction in LNCaP cells via the LBD and unable to inhibit the interaction between ERβ and PELP1, confirming its specificity for the AR. It was also demonstrated by different experimental approaches that bis-benzamide **5** was able to block AR signaling by suppressing expression of DHT-induced genes and also to prevent AR nuclear translocation. Furthermore, compound **5** was found to inhibit cell proliferation in a variety of AR-positive PCa cell lines but not AR-negative PCa cell line PC3. The results also showed that the antiproliferative activity was dose-dependent with an IC50 value of ~40 nM and that the effect could be rescued by overexpression of PELP1. Finally, bis-benzamide **5** showed antitumor activity in xenogratfs following direct intratumoral injections and inhibition of AR expression in human tumour explants. No toxicity was observed for the tested bis-benzamide derivatives in cell viability assays. Subtle side chains variation on bis-benzamide **5** yielded compounds with improved antiproliferative activity having IC50 value as low as 20.2 nM in PCa cells lines. Currently in preclinical development, these promising compounds are presently the most characterized and advanced among peptidomimetic CBIs targeting the AR-LBD AF-2 site.

# 4. targeting the binding function 3 site

A second surface on the AR-LBD amenable to pharmacological manipulation has been identified by Estébanez-Perpiñá et al. and named binding function 3 (BF-3) (Fig 1c) [[49](#_ENREF_49)]. Adjacent to and as large as AF-2, this hydrophobic binding site is near the junction of H1, the H3-H5 loop, and H9. Recent studies have demonstrated the important role of BF-3 in AR signaling regulation through the recruitment of AR coregulators such as FKBP52 [[102](#_ENREF_102)] and Bag-1L [[103](#_ENREF_103)] and crosstalk with the adjacent AF-2 [[49](#_ENREF_49), [104](#_ENREF_104)]. Using a crystallographic screening approach, Estébanez-Perpiñá et al. were the first to report compounds that allosterically inhibit the interaction of AR with coactivators by binding to the BF-3 site [[49](#_ENREF_49)]. In this study, compounds such as flufenamic acid (FLUF) **6**, triiodothyronine (T3) **7** and triiodothyroacetic acid (TRIAC) **8** were found to bind the BF-3 site and inhibit AF-2 interactions interfering with AR activity (Fig. 4a). While it was proposed that their binding to the BF-3 site induces a conformation change in both the BF-3 and AF-2 regions that disfavors coactivator binding to the AF-2, these molecules were found at both AF-2 and BF-3 sites in crystallized structures [[49](#_ENREF_49)]. Therefore they can be referred as mixed AF-2/BF-3 inhibitors. Although these compounds had low potency (IC50 > 50µM) in FP assays and multiple off-target effects, they established the BF-3 site as a novel binding surface and attractive therapeutic target to develop alternative approaches for the treatment of PCa.

**<INSERT FIGURE 4 (single column)>**

To identify selective AR BF-3 inhibitors, Cherkasov et al. used a combination of virtual screening and experimental evaluations followed by SAR studies on selected lead compounds for optimization [[105](#_ENREF_105), [106](#_ENREF_106)]. In an initial study, a virtual screening campaign was performed on the Zinc database and the 213 retrieved compounds were evaluated for their ability to inhibit AR transcriptional activity in cell-based assays. Several compounds showed activity in the submicromolar range and the four most potent molecules with no obvious cytotoxicity were used for further studies. Binding of the selected compounds to the AR was confirmed by surface plasmon resonance (SPR) and their interaction with the BF-3 site elucidated by X-ray crystallographic studies (PDB: 2YLP, 2YLQ, 2YLO and 3ZQT). Surprisingly, unlike previously reported BF-3 binders such as FLUF **6**, T3 **7** and TRIAC **8**, the compounds did not induce coactivator displacement from the AF-2 site [[105](#_ENREF_105)]. Therefore they were proposed to be specific BF-3 inhibitors and represent proper lead candidates for further optimization. Based on the benzimidazole derivative **9 (**Fig. 4b**)**, SAR studies were performed to improve affinity to the BF-3 site and increase activity. Different sites on compound **9** were rationally explored by chemical modifications and a first series of potent and selective BF-3 inhibitors was designed (Fig 4, **10**-**12**) [[106](#_ENREF_106)]. In cellular assays, compounds **11** and **12** were able to reduce the expression of PSA in LNCaP and enzalutamide-resistant MR49F PCa cells with IC50 values ranging from 1.6 to 6.8 µM and to inhibit their growth at 6 µM. The AR-specificity of the compounds was demonstrated by the absence of antiproliferative activity on the AR-negative PCa cell line PC3 and their inability to inhibit ERα transcriptional activity in luciferase reported gene assay.

In another study, to avoid possible tautomerization of the imidazole N atoms and based on promising results obtained with compound **12** and an indole derivative of **11,** Cherkasov et al. used a focused library of indole-containing compounds to perform a combination of virtual screening and experimental evaluations [[107](#_ENREF_107)]. A series of 1*H*-indole-2-carboxamides was selected for further optimization by rational design (Fig. 4b). The resulting compound **13** was found to reduce PSA expression in LNCaP and enzalutamide-resistant MR49F cells and to potently inhibit their growth with IC50 values of 0.55 and 1.31 µM, respectively. While no antiproliferative activity has been observed for AR-independent PC3 cells, no DHT or AF-2 peptide displacement was detected, confirming AR specificity and activity through interactions with the BF-3 site.

In a more recent study, compound **10** was used as template in a shape-based similarity search in the ZINC database to identify new BF-3 inhibitors with increased activity [[108](#_ENREF_108)]. Selected compounds were subjected to molecular docking in the AR BF-3 site and compounds respecting the established constraints tested in a cell-based AR transcriptional activity assay. As the most promising inhibitor of this series, the ability of the indole-based compound **14** (Fig. 4) to inhibit the production of PSA and growth of PCa cells was evaluated. As expected, it induced a significant decrease in PSA expression in LNCaP and MR49F PCa cells and inhibited their growth with IC50 values of 0.71 and 2.01 µM, respectively. As described above, the AR specificity of compound **14** was confirmed by its inactivity on PC3 cells and its decreased inhibitory activity against GR, ERα and to a lesser extent PR. Finally, *in vivo* studies on LNCaP and MR49F tumor xenografts showed that compound **14** was able to reach serum levels above its IC50 and significantly inhibit tumor growth and reduce serum PSA levels.

Recently, Jehle et al. demonstrated that a duplicated GARRPR motif found at the N-terminal region of the cochaperonne Bag-1L interacts with the AR via the BF-3 site [[103](#_ENREF_103)]. This interaction plays an important role in the modulation of AR activity [[109](#_ENREF_109), [110](#_ENREF_110)]. Different binding studies showed that compounds **11** and **14** blocked the interaction of Bag-1L motif peptides with the AR, implying that these compounds binds directly to the AR BF-3 site [[103](#_ENREF_103), [108](#_ENREF_108)]. Taken together, these results show the great potential of the BF-3 site as a therapeutic target for the treatment of CRPC. The identified selective BF-3 inhibitors exhibited very promising activities and provide strong lead candidates to generate a new drug class for clinical applications.

# 5. targeting the n-terminal domain

The AR-NTD plays an essential role in ligand-dependent AR transcriptional activity but in addition possesses a strong ligand-independent transactivation function [[111-114](#_ENREF_111)]. Indeed C-terminal truncated ARs lacking the LBD retain a constitutive activity and such splice variants can be observed in CRPC [[115-120](#_ENREF_115)]. The AF-1 region located in the NTD contributes significantly to these functions by interacting with other proteins such as components of transcription factors (TF) IIF and IIH and coactivators SRC1-3 and CREB-binding protein (CBP) [[121-123](#_ENREF_121)]. Using the intact NTD domain (AR1-558) as a decoy, Sadar et al. were able to inhibit both ligand-dependent and independent transcriptional activities by sequestering coregulatory proteins [[124](#_ENREF_124)]. The NTD is the least conserved domain across steroid receptors with less than 15% homology and very few disease-associated mutations of the AR are been reported in this domain [[111](#_ENREF_111), [112](#_ENREF_112)]. For such reasons, the AR-NTD represents a very attractive target to develop inhibitors that could block ligand-dependent and ligand-independent AR transactivation, and hence be active in hormone-sensitive and castration-resistant PCas.

**<INSERT FIGURE 5 (single column)>**

The design of inhibitors targeting the AF-1 site was initially impeded by the lack of structural information for the NTD, which is highly flexible and intrinsically disordered. In an effort to discover AR-NTD inhibitors, Sadar et al. used a transactivation assay based on the AR-NTD to screen a library of marine sponge extracts [[125](#_ENREF_125)]. Several small chlorinated peptides from the sponge *Dysidea* sp. named Sintokamides were identified and no cytotoxicity was observed in a cell viability assay (Fig. 5, **15**). Sintokamide A **15** was found to significantly reduce PSA expression and block AR-NTD transactivation at 5 µg/mL in luciferase reporter gene assays and to inhibit proliferation of AR-positive LNCaP cells but not AR-negative PC3 cells. Another screen was more recently performed on extracts of the marine spronge *Niphates digitalis* leading to the discovery of niphatenones, a group of glycerol ether lipids, as AR-NTD inhibitors (Fig. 5, **16**) [[126](#_ENREF_126)]. Niphatenone B **16** reduced the production of PSA and AR-NTD transactivation and inhibited growth of LNCaP cells at 14 µM but had no effect on PC3 cells [[126](#_ENREF_126), [127](#_ENREF_127)]. Moreover, Meimetis et al. showed that compound **16** blocks N/C interaction in the AR and binds to AF-1 by using Click chemistry. Unfortunately, because of observed decrease in GR activity, binding to the GR AF-1 and reactivity with glutathione, the niphatenone scaffold became less attractive for further drug development [[127](#_ENREF_127)].

Another screening campaign of library containing marine sponge extracts using an AR-NTD based transactivation assay led to the discovery of EPI-001 **17**, a very potent bisphenol A diglycidic ether analog (BADGE) from the marine sponge *Geodia lingreni* (Fig 5) [[111](#_ENREF_111), [128](#_ENREF_128), [129](#_ENREF_129)]. Andersen et al. demonstrated that compound **17** specifically and covalently binds to the AF-1 region and block interactions with CBP and the RAP74 subunit of TFIIF as well as AR N/C interaction. The proposed mechanism for covalent bond formation involves the nucleophilic attack of an AF-1 residue on an intermediate epoxide formed in the binding site from an internal nucleophilic substitution of the chlorohydrin function, which is required for activity. It was also demonstrated with denatured AF-1 and alkylation experiments with glutathione that EPI-001 binds selectively to intrinsically disordered AR-NTD and is not a random alkylator [[129](#_ENREF_129)]. EPI-001 **17** is a mixture of 4 stereoisomers and it was reported that, while no stereospecificity for covalent binding to AR was observed, the stereoisomer EPI-002 (2R, 20S) **18** had improved antitumoral properties both *in vitro* and *in vivo*. Compounds **17** and **18** were found to block the expression of both androgen-dependent and androgen-independent AR regulated genes and to selectively inhibit AR-dependant proliferation in a variety of AR-positive PCa cell lines including 22RV1 cells that express both full-length AR and constitutively active splice variant lacking the LBD. *In vivo* studies with EPI-001 and EPI-002 in castrated mice bearing respectively LNCaP and VCaP CRPC subcutaneous xenografts showed low toxicity and strong inhibition of tumor growth while no tumor reduction was observed in PC3 xenograft models. Before treatment with EPI-002, VCaP cells were found to express both full-length and splice variant ARs. Moreover, the compound **17** did not inhibit transcriptional activities of other steroid receptors and did not interfere with ligand-binding at the ABS, suggesting high binding site specificity. These compounds were the first identified selective protein-protein interaction inhibitors for the AR AF-1 site and the first reported inhibitors of constitutively active AR splice variants lacking the LBD. Their excellent pharmacokinetic properties and their ability to inhibit AR signaling in androgen-dependent PCa and CRPC have prompted their development for clinical applications.

# 6. Conclusion and future perspectives

There has been considerable progress over recent years in our understanding of the AR and its action in different PCa cell lines and disease stages. Important advances in the structure-function relationships of the AR, structural behaviour of AR domains, identification of binding partners, role of androgen regulated genes and functions of AR splice variants in PCa evolution have improved our knowledge of the AR axis in PCa. The limited treatment options for CRPC patients and the increasing appreciation of the role of AR in advanced PCa have prompted the development of new approaches to target AR signaling. Different strategies to destabilize the AR, prevent its nuclear translocation, inhibit its binding to DNA or block coactivator recruitment have been explored. The latter, mediated by protein-protein interactions, is a very attractive target and offers the opportunity to develop selective non-competitive AR antagonists that would overcome resistance to traditional antiandrogens and remain effective in advanced PCa. Several binding domains outside the ABS have been explored to block AR-coactivator interactions and inhibit AR signaling. Among AR domains, the AF-1, AF-2 and BH-3 sites have yielded the most promising compounds. These PPI inhibitors have been discovered and developed using a wide variety of approaches including structure-based drug design, screening of natural compound libraries, ligand-based peptidomimetics and different combinations of virtual library screening and experimental evaluation. While many compounds described herein need more comprehensive SAR studies and pharmacological optimization to progress into clinical evaluation, some like EPI-001 and oligobenzamides have already showed very encouraging results in *in vivo* studies. Overall the future of CBIs is very promising and the development of these non-competitive AR antagonists will help fulfill the high medical needs for compounds that can overcome resistance to androgen-deprivation therapies and most importantly provide more treatment options for CPRC patients to obtain maximal therapeutic benefit.

# Acknowledgments

This research was supported by the Fonds de recherche du Québec – Santé (FRQS) and the National Sciences and Engineering Research Council of Canada (NSERC). François Bédard thanks the Fonds de recherche du Québec – Nature et Technologie (FRQNT), the Fondation de l’Université Laval and the Centre de recherche en endocrinologie moléculaire et oncologique et génomique humaine (CREMOGH) for postgraduate scholarships.

# Conflict of interest

None declared.

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**Figure legends**

**Figure 1**. Structure of the AR and its LBD. (a) Functional domains of the AR. (b) Crystal structure of the AR-LBD bound to testosterone (balls and sticks; cyan, carbon; red, oxygen) with key helices 3, 4, 5 and 12 (red ribbon) forming the coactivator binding site (PDB: 2Q7I) [[63](#_ENREF_63)]. (c) Space-filling model of the AR-LBD showing the BF-3 (yellow) and AF-2 (red) sites.

**Figure 2**. Close-up view of the AR-NTD peptide 20GAFQNLFQSV30 containing the FXXLF motif (green) bound to the coactivator binding groove (red) of AR-LBD with the charge clamp from Glu897 (yellow) and Lys720 (blue) functional groups. (Figure prepared using PDB: 2Q7I [63[63](#_ENREF_63)].)

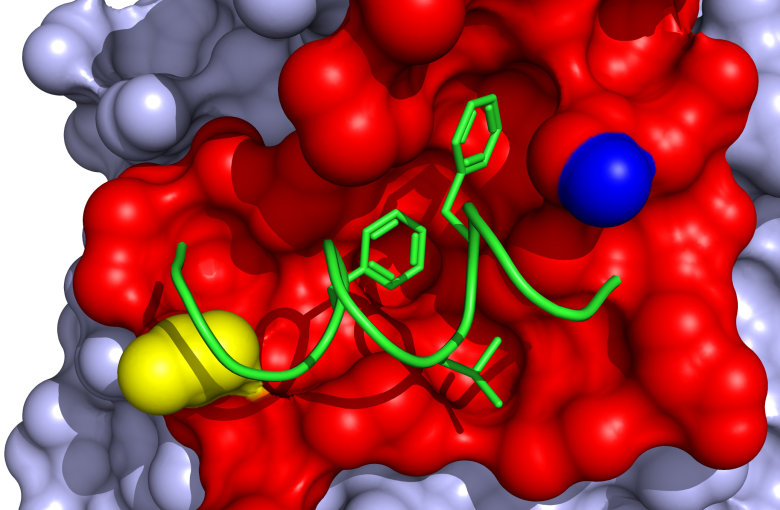
**Figure 3.** Representation of the FXXLF binding motif **1** (side and top views) and structures of coactivator binding inhibitors blocking the AR-LBD AF-2 site.

**Figure 4**. Structures of compounds targeting the AR-LBD BF-3 site. (a) Mixed AF-2/BF-3 inhibitors. (b) Selective BF-3 inhibitors.

**Figure 5**. Structures of AR-NTD AF-1 directed inhibitors.



**Figure 1**



**Figure 2**



**Figure 3**



**Figure 4**



**Figure 5**